

A Study of Allelic Imbalance in Human Breast Cancer

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at the University of Leicester

by

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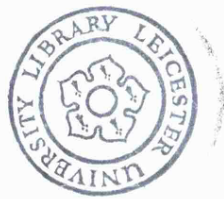
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Abstract

The analysis of loss of heterozygosity (LOH) in tumours can be used to map candidate sites of tumour-suppressor genes in the human genome. A panel of breast cancer patients was assembled as pairs of tumour and lymphocyte DNA samples and LOH studies carried out by Southern hybridization with polymorphic loci mapping to chromosomes 1 and X. Loci on other chromosomes were also studied as controls. Sequences on chromosome 1 were found to be subject to a variety of alterations creating very complex patterns of rearrangements, including LOH. Deletion mapping identified five independent regions of loss, three of which may correspond to regions identified in previous reports, although individually none occurred with high frequency to support the targeting of a specific locus. The short arm of chromosome 1 primarily underwent interstitial deletions whereas the long arm was subject to both whole arm events (gains and losses) and more localized events. Small deletions on chromosome 1 were found exclusively in patients with poorly-differentiated tumours (i.e. tumours of high grade, $p < 0.05$). A similar study of the X chromosome revealed a high frequency of small regionalized deletions, primarily defining three small independent regions, one located in the distal portion of the pseudoautosomal region of Xp, another spanning the pseudoautosomal boundary into the sex-specific sequences of Xp and a possible third on distal Xq. No gains in copy number were identified at any locus on the X chromosome, in distinct contrast to chromosome 1. Small-scale deletions occurring on the X chromosome were found in patients with no spread of cancer to the lymph nodes, thus correlating with a favourable prognosis ($p < 0.005$). Fluorescence *in situ* hybridization to formalin-fixed paraffin-embedded tumour sections defined the actual copy number of chromosome 1 for nine tumours. The results obtained confirmed the changes established by Southern hybridization.

"Put it before them briefly so they will read it,
clearly so they will appreciate it,
picturesquely so they will remember it
and, above all, accurately
so they will be guided by its light."

Joseph Pulitzer

(or not)

Contents

Chapter 1: Introduction	1
1.1 Chromosome 1 and cancer	3
1.1.1 Somatic cell hybrids	4
1.1.2 Cell lines	6
1.1.3 Loss of heterozygosity (allelic loss)	7
1.1.3.1 Loss of heterozygosity on chromosome 1 in breast tumours	11
1.1.3.2 Loss of heterozygosity on chromosome 1 in other tumours	13
1.1.4 Cytogenetic studies of tumours	15
1.1.4.1 Breast tumours	17
1.1.4.2 Fluorescence <i>in situ</i> hybridization	21
1.2 The X chromosome and cancer	24
1.3 Aims of the project	30
1.4 Strategy	31
 Chapter 2: Materials and methods	 34
2.1 Materials	34
2.1.1 Chemicals	34
2.1.2 Enzymes, proteins and antibodies	38
2.1.3 Bacterial strains and culture conditions	38
2.1.4 Tissue samples	38
2.1.5 Deoxyribonucleic acids	39
2.2 Methods of sterilization	39
2.3 Experimental protocols	40
2.3.1 Preparation of genomic DNA	40
Purification of genomic DNA from human tissues	40
Standard procedures	40
Qiagen protocols	41
Preparation of sheared salmon testes DNA	41

2.3.2	Preparation of plasmid DNA	42
	Transformation of competent <i>E. coli</i> cells	42
	Large scale preparation of plasmid DNA	42
	Small scale purification of plasmid DNA	43
2.3.3	Polymerase chain reaction (PCR) methods	43
2.3.4	Gel electrophoresis	44
	Purification of DNA fragments from agarose gels	44
2.3.5	Restriction enzyme digestions	44
2.3.6	Southern hybridization	45
	Southern (DNA) blotting	45
	Preparation of ³² P radiolabelled probes	45
	Hybridization of membrane-immobilized deoxyribonucleic acids	45
	Removal of probes and re-use of DNA blots	46
2.3.7	Laser scanning densitometry	46
2.3.8	Fluorescence <i>in situ</i> hybridization	46
	Preparation of lymphocyte splashes	46
	Preparation of tissue sections	47
	Preparation of probes	48
	Hybridization	48
	Washing protocol	49
	Detection protocols for a single biotinylated probe	49
	Detection protocols for a single digoxigenylated probe	49
	Dual-detection protocol for biotinylated and digoxigenylated probes	50
	Mounting of hybridized slides	50
	Fluorescence microscopy	50
2.3.9	Statistical analyses	51

Chapter 3: Allelic imbalance studies of other chromosomes 52

3.1 Introduction 52

3.2 Results 53

3.2.1	Construction of the panel of patients and probe bank	53
3.2.2	Chromosome 2	54
3.2.3	Chromosomes 7 and 20	55
3.2.4	Scoring the autoradiographs for allelic imbalance	56
3.2.5	Chromosomes 13 and 17	58

3.3 Discussion	59
3.3.1 Allelic imbalance detection	59
3.3.2 Chromosomes 2, 7 and 20	59
3.3.3 Chromosomes 13 and 17	61
3.3.4 Future work	61
 Chapter 4: Allelic imbalance studies of the X chromosome	 63
4.1 Introduction	63
4.2 Results	63
4.2.1 Panel of breast cancer patients and the probe bank	63
4.2.2 Loss of heterozygosity in the pseudoautosomal region: evidence for two discrete regions of loss	64
4.2.3 Loss of heterozygosity in the sex-specific region of the X chromosome: additional small discrete regions of loss identified	66
4.2.4 Loss of heterozygosity on the X chromosome: gross deletions	67
4.2.5 Concomitant gain and loss of alleles on the X chromosome: two patients identified	68
4.3 Discussion	68
4.3.1 Loss of heterozygosity on the X chromosome	68
4.3.2 Concomitant gain and loss of alleles on the X chromosome	72
4.3.3 Future work	73
 Chapter 5: Allelic imbalance studies of chromosome 1	 74
5.1 Introduction	74
5.2 Results	75
5.2.1 Panel of breast cancer patients and the probe bank	75
5.2.2 Loss of heterozygosity on chromosome 1: identification of five discrete regions	76
5.2.3 Loss of heterozygosity on chromosome 1: gross deletions	78
5.2.4 Concomitant gain and loss of alleles on chromosome 1: a second mechanism of tumour-suppressor inactivation	78
5.2.5 Gain in copy number of loci on chromosome 1	79

5.2.6 Somatic mutations: generation of new alleles	80
5.3 Discussion	80
5.3.1 The significance of loss of heterozygosity on chromosome 1	80
5.3.2 Concomitant gain and loss of alleles on chromosome 1	83
5.3.3 Gain in copy number of regions of chromosome 1	84
5.3.4 Future work	85
Chapter 6: Fluorescence <i>in situ</i> hybridization to tissue sections	86
6.1 Introduction	86
6.2 Results	87
6.2.1 Developing the technique of FISH to tissue sections	87
6.2.2 Normal breast tissue	90
6.2.3 Tumour RW 628	91
6.2.4 Tumour RW 864	92
6.2.5 Tumour RW 866	93
6.2.6 Tumour RW 867	94
6.2.7 Tumour RW 870	95
6.2.8 Tumour RW 873	95
6.2.9 Tumour RW 874	97
6.2.10 Tumour RW 904	98
6.2.11 Tumour RW 921	99
6.3 Discussion	99
6.3.1 Copy number of D1Z1 in tissue sections	99
6.3.2 Future work	102
Chapter 7: Correlation of Southern data with clinico-pathological variables	104
7.1 Introduction	104
7.2 Results	104
7.2.1 Datasets for statistical analysis and contingency tables	104
7.2.2 The chi-square distribution and test of association	105
7.2.3 Yates' correction for the chi-square distribution	106
7.2.4 The Fisher-Irwin test for exact probability	106

7.2.5 Deviation of allelic loss at RB1 and D17S5 from the literature	109
7.3 Discussion	109
Chapter 8: Conclusion	112
Appendix I: Other chromosomes	116
Appendix II: The X chromosome	117
Appendix III: Chromosome 1	119
Appendix IV: Raw data from densitometry	121
Appendix V: Clinical data	131
Appendix VI: Statistics	132
VI.1 Chi-square test	132
VI.2 Yates' correction	134
VI.3 Fisher-Irwin exact test of probability	135
Appendix VII: Percentage points of the chi-square distribution	138
Bibliography	139

Figures and Tables

Chapter 1: Introduction

Figures

1.1 Possible mechanisms of inactivation of a tumour-suppressor gene	7
1.2 Diagram to show Southern blot analysis of a patient heterozygous for locus A	7
1.3 Alleles created by an RFLP system	8
1.4 Alleles created by a VNTR system	8
1.5 Summary of allelic imbalance (AI) for polymorphic markers mapping to chromosome 1	11
1.6 Summary of the patterns of homologies between the human X and Y chromosomes	25

Chapter 2: Materials and methods

Tables

2.1 Details of the probes used for Southern analysis	45
i Probes detecting loci on chromosome 1	45
ii Probes detecting loci on the X chromosome	45
iii Probes detecting loci on other chromosomes	45
2.2 Details of the probes used for FISH	48

Chapter 3: Allelic imbalance studies of other chromosomes

Figures

3.1 Autoradiographs of the VNTR from intron 16 of the RB1 gene on chromosome 13q14	54
3.2 Autoradiographs of D17S5 on chromosome 13q14	54
3.3 Autoradiographs of the three controls, D2S44, D7S21 and D20S26	54

Tables

3.1 Summary of probes and data on allelic imbalance on other chromosomes	54
3.2 Summary of results for all scanned loci	56

Chapter 4: Allelic imbalance studies of the X chromosome

Figures

4.1	Autoradiographs of four loci on the X chromosome from PB 121	64
4.2	Autoradiographs of five loci on the X chromosome from PB 124	64
4.3	Autoradiographs of five loci on the X chromosome from RW 647	64
4.4	Autoradiographs of five loci on the X chromosome from RW 659	64
4.5	Autoradiographs of five loci on the X chromosome from RW 663	64
4.6	Autoradiographs of five loci on the X chromosome from RW 926	64
4.7	Autoradiographs of the DXS278 locus on the X chromosome from three patients	64
4.8	Loci studied on the X chromosome and a summary of the allelic imbalances observed	64
4.9	Tumours with small independent deletions in the pseudoautosomal region of Xp	64
4.10	Tumours for which the order of DXYS17 and DXYS89 is important	65
4.11	Tumours with larger deletions in the pseudoautosomal region of the X chromosome	65
4.12	Small deletions on the short arm of the X chromosome in the sex-specific sequences	66
4.13	Small areas of LOH on the long arm of the X chromosome	67
4.14	Tumours most affected by change in the order of DXS52 and DXS438	67
4.15	Tumours with large-scale deletions of the short arm of the X chromosome	67
4.16	A single tumour with a possible large-scale deletion involving the long arm of the X chromosome	67
4.17	Tumours with highly-fragmented X chromosomes	67
4.18	A single tumour with the whole loss of one copy of the X chromosome	68
4.19	Concomitant gain and loss of alleles for loci on the X chromosome	68

Table

4.1	Summary of probes used in the study and data on allelic imbalance on the X chromosome	64
-----	---	----

Chapter 5: Allelic imbalance studies of chromosome 1

Figures

5.1	Autoradiographs of three loci on chromosome 1 from PB 5	75
5.2	Autoradiographs of five loci on chromosome 1 from PB 134	75

5.3	Autoradiographs of four loci on chromosome 1 from RW 690	75
5.4	Autoradiographs of five loci on chromosome 1 from RW 864	75
5.5	Autoradiographs of six loci on chromosome 1 from RW 867	75
5.6	Autoradiographs of five loci on chromosome 1 from RW 869	75
5.7	Autoradiographs of five loci on chromosome 1 from RW 942	75
5.8	Autoradiographs of the MUC1 locus on chromosome 1 from PB 40	75
5.9	Loci studied on chromosome 1 and a summary of the allelic imbalances observed	75
5.10	Patients defining a discrete region of LOH in the distal region of chromosome 1p	76
5.11	Patients defining a discrete region of LOH in the mid-region of chromosome 1p	76
5.12	Patients defining a discrete region of LOH in the proximal region of chromosome 1p	76
5.13	Patients defining a discrete region of LOH in the mid-region of chromosome 1q	77
5.14	Patients defining a discrete region of LOH in the distal region of chromosome 1q	77
5.15	Tumours with LOH possibly involving the whole of the short arm of chromosome 1	78
5.16	Tumours with LOH involving the whole or most of the long arm of chromosome 1	78
5.17	Tumours with loss of the whole or most of one copy of chromosome 1	78
5.18	Patients with GAL at loci along the length of chromosome 1	78
5.19	Patients with an interstitial GAL event on chromosome 1	79
5.20	Patients with small regional gains in copy number on chromosome 1	79
5.21	Patients with gain in copy number of the whole of the long arm of chromosome 1	79
5.22	A single patient with gain in copy number involving the whole of chromosome 1	79
5.23	A single patient with LOH involving the whole of the short arm and GCN involving the whole of the long arm of chromosome 1	79

Table

5.1	Summary of probes used in the study and data on allelic imbalance (AI) on chromosome 1	75
-----	--	----

Chapter 6: Fluorescence *in situ* hybridization to tissue sections

Figures

6.1 D1Z1 and D2Z1 co-hybridized to normal human lymphocyte splash preparations	87
6.2 Single hybridization of D1Z1 to normal human lymphocyte splash preparations	88
6.3 Summary of Southern hybridization data for chromosome 1 for the tumours studied by FISH also	89
6.4 D1Z1 and D2Z1 co-hybridized to normal breast tissue sections	90
6.5 D1Z1 and D2Z1 co-hybridized to tumour RW 628	91
6.6 Single hybridization to detect D1Z1 in tumour RW 864	92
6.7 Sections of tumour RW 866	93
6.8 Co-hybridization of D1Z1 and D2Z1 to tumour RW 867	94
6.9 Co-hybridization of D1Z1 and D2Z1 to tumour RW 870	95
6.10 Sections of tumour RW 873	95
6.11 Single hybridization to detect D1Z1 in sections of tumour RW 874	97
6.12 Single hybridization to detect D1Z1 in sections of tumour RW 904	98
6.13 Single hybridization to detect D1Z1 in sections of tumour RW 921	99

Tables

6.1 Copy number determination for D1Z1 on chromosome 1q12 in each of the breast tissue sections used	89
6.2 A summary of the primary ranges of copy number counts for D1Z1 from FISH and the data obtained by Southern analysis	89

Chapter 7: Correlation of Southern data with clinico-pathological variables

Tables

7.1 Rearrangements observed in tumours by Southern hybridization and subjected to tests of association	104
7.2 Summary of the pairs of data showing possible association	106








Abbreviations

+ve	positive
-ve	negative
AG	allelic gain
AI	allelic imbalance
AL	allelic loss
BHK	baby hamster kidney
bp	basepair(s)
BSA	bovine serum albumin
°C	degrees Centigrade
CEPH	Centre d'Etude du Polymorphisme Humain, 27, rue Juliette Dodu, 75010 Paris, France
CI	chromosome index
cM	centiMorgan(s)
cm	centimetre(s)
CsCl	caesium chloride
DAPI	4',6-diamino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DM(s)	double minute(s)
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DOP-PCR	degenerate oligonucleotide primed-PCR
dTTP	deoxythymidine triphosphate
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	diaminoethanetetra-acetic acid, disodium salt
EGFR	epidermal growth factor receptor
ER	oestrogen receptor
ERE(s)	oestrogen responsive element(s)
EtBr	ethidium bromide
FAP	familial adenomatous polyposis
FCM	flow cytometry
FISH	fluorescence <i>in situ</i> hybridization
FITC	fluorescein isothiocyanate
g	gramme(s)
GAL	concomitant gain and loss of alleles

GCN	gain in copy number of one allele
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HNPCC	hereditary non-polyposis colorectal cancer
hr	hour(s)
HPRT	hypoxanthine phosphoribosyltransferase
HSR(s)	homogeneously staining region(s)
i	informative (Southern analysis)
IMS	industrial methylated spirits
ISH	<i>in situ</i> hybridization
kb	kilobasepair(s)
kg	kilogramme(s)
l	litre(s)
LOH	loss of heterozygosity
M	molar
Mb	megabasepair(s)
mg	milligramme(s)
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
mRNA	messenger ribonucleic acid
μl	microlitre(s)
μm	micron(s), micrometre(s)
μM	micromolar
n	number (Southern analysis, number studied)
n/a	not applicable
NA	nucleic acid
NCI	normalized chromosome index
NEO	neomycin (resistance gene)
nm	nanometre(s)
no.	number
nuc	nucleotide(s)
PAR	pseudoautosomal region
PBS	phosphate-buffered saline
PCI	phenol/chloroform/isoamyl alcohol
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	propidium bromide
PR	progesterone receptor
Q	Millipore Super-Q system treated water

RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SCB	sex chromatin body
SDS	sodium dodecyl sulphate
SE	saline/EDTA buffer
SRO	smallest region of overlap
SSC	standard saline citrate
SSCP	single-strand conformation polymorphism
TACF	telomere-associated chromosome fragmentation
TAE	Tris/acetate/EDTA buffer
TE	Tris/EDTA buffer
TK	thymidine kinase
TNB	Tris/NaCl/Blocking reagent buffer
TNE	Tris/NaCl/EDTA buffer
TNT	Tris/NaCl/Tween buffer
TRITC	tetramethyl rhodamine isothiocyanate
u	uninformative
U	unit(s), especially of enzyme activity
V	volt(s)
VNTR	variable number of tandem repeats
vs.	versus
(v/v)	(volume/volume)
(w/v)	(weight/volume)
YAC	yeast artificial chromosome

Key to Figures in Chapters 4, 5 and 6 and Appendices II and III

	-, not tested
	u, uninformative
	i, informative and unaffected by imbalance
	AI, allelic imbalance, indeterminate as LOH, GAL or GCN
	LOH, loss of heterozygosity
	GAL, concomitant gain and loss of alleles
	GCN, gain in copy number of alleles

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CHAPTER 1

Introduction

In North America and Western Europe breast carcinoma is the most common malignancy in women affecting one woman in 12, amounting to ~25,000 new cases every year in the UK alone (Cancer Statistics Registration: England and Wales, 1985; Cancer Research Campaign Annual Report, 1989). Contributing factors are thought to include genetic, hormonal and dietary elements as well as social class, body mass and age at menarche. Despite advances in treatment, only modest increases in survival have been achieved. This has stimulated the introduction of screening programmes for the earlier detection of breast cancer on the basis that small invasive cancers are less likely to have metastasized and so survival should be better.

Carcinomas form only a subset of all neoplastic changes affecting the breast. Benign breast disorders also occur and can be broadly categorized into two groups: localized specific lesions, including fibrosarcoma, and 'fibrocystic change', which includes cysts, stromal fibrosis and epithelial proliferative lesions. Malignant tumours differ from benign changes in that they have the ability to invade local tissue and metastasize to distant sites. Breast carcinomas actually originate in the epithelial cells of the ductules or acini (saccular evaginations situated at the ends of the ductules within the lobules of the breast), usually spreading via the lymphatic system to other sites.

Breast carcinomas form a highly heterogeneous group, broadly divided into infiltrating or non-infiltrating tumours, depending on whether the integrity of the basement membrane has been compromised. The non-infiltrating (non-invasive) class, where the malignant cells are enclosed by the basement membrane, are either ductal carcinoma *in situ* (DCIS, also known as intraductal carcinoma) or lobular carcinoma *in situ* (LCIS, also known as intralobular carcinoma). Although DCIS represents 50-80% of all the non-invasive carcinomas, it represents only ~1-5% of all breast carcinomas in general. DCIS is rarely evident on gross examination but its detection increases in populations subject to mammographic screening (Harris *et al*, 1987). These tumours have

various growth patterns, such as comedo, cribriform, solid and papillary. The majority of carcinomas are infiltrating, and appear with various pathologies and clinical properties, the most common of which is infiltrating ductal (ID) representing 70-80% of all invasive breast carcinomas. Other growth patterns have been characterized as infiltrating lobular (IL), tubular, mucinous and medullary. Invasive carcinomas may contain non-invasive elements and be adjacent to or mixed in with normal or benign compartments.

Clinical staging of patients is done on the basis of tumour size, degree of lymph node development and presence or absence of distant metastases. Breast cancer generally manifests itself as a systemic disease and 20-30 year follow-up studies have revealed that eventually 75-85% of patients die with some evidence of metastatic disease (American Joint Committee for Cancer Staging and End-Results Reporting, 1978).

One of the major problems in trying to reduce the morbidity and mortality from breast cancer is the lack of a clear understanding of its natural history (i.e. of the development and progression). Recent developments regarding colorectal carcinoma (Vogelstein *et al*, 1988; Fearon, 1992) indicate that molecular analysis may be the way forward.

Cancer cells contain many genetic alterations that accumulate gradually during the process of tumourigenesis. Although the nature and order of appearance is not fully understood for any type of neoplasia, their cumulative number is considered crucial for the expression of the transformed phenotype (Fearon and Vogelstein, 1990; Bishop, 1991; Marshall, 1991; Weinberg, 1991). The transformed phenotype abounds in structural and functional alterations that are thought to be acquired independently during the development of the tumour. Finally, there is a breakdown in the regulation of cell proliferation, cells multiply without restraint, cell-cell interactions become modified, and the neoplastic cells invade the surrounding tissue and metastasize.

No coherent picture of cancer genetics can be formed without knowledge of the triggering cause and the mechanism(s) responsible for the accumulation of multiple genetic lesions in the neoplastic cell, the physiological function of the various 'cancer genes', and the contribution each and every genetic aberration makes to the transformed phenotype of each single tumour. It is not clear whether the accumulation of multiple genetic events in the developing tumour cells is merely the consequence of random genetic changes coupled with intense selection, or whether enhanced genomic instability in the neoplastic cells favours,

or is required for, the onset of these changes (Bishop, 1991). Numerous hereditary syndromes predispose to cancer, and *in vitro* studies on non-neoplastic somatic cells from such patients have shown that they are, indeed, genetically unstable (Tlsty *et al*, 1989, 1992; Tlsty, 1990). Genomic instability, therefore, may be an inheritable genetic trait associated with a predisposition to cancer, and similar genetic defects may be operative even in spontaneous tumours (Ponder, 1990).

Many experimental data have accumulated in the last 20 years implicating loci physiologically involved in regulation of cell proliferation as being the genomic defects of cancer cells. On the basis of their biochemical activity, where known, and the type of genetic alteration that affects the neoplastic cell, 'cancer genes' fall into two classes, the proto-oncogenes and the tumour-suppressor genes. In general, the difference between them is that proto-oncogenes undergo gain-of-function events by point mutation, chromosomal translocation and/or gene amplification while tumour-suppressor genes undergo loss-of-function events by allelic loss and inactivating mutation. Alterations to these genes have been found to occur in a wide variety of unrelated tumours, although clearly only a sub-fraction of all cancer-related genes contained in the human genome have been identified to date.

There is evidence to suggest that genes, specifically tumour-suppressor genes, encoded on chromosome 1 are of fundamental importance in many of the major human malignancies. Although a number of candidates have been isolated, they have not been found to account for the rearrangements to chromosome 1 observed and further candidates must be identified. Loss of heterozygosity studies are one approach to identifying genes associated with sporadic tumours in particular, since it can uncover regions of the genome specifically targeted for deletion when the same area is repeatedly deleted in a panel of tumours. Thus, loss of heterozygosity could identify candidates for further study, although it by no means provides proof of a functional tumour-suppressor gene.

1.1 Chromosome 1 and Cancer

The evidence for the presence of one or more tumour-suppressor genes on chromosome 1 derives from four types of study. Somatic cell hybrids provided the first evidence for the existence of tumour-suppressor genes within the human genome. Chromosome 1 can suppress a number of tumourigenic

characteristics in both intraspecies and interspecies hybrids. Related to these studies, analyses of cell lines established in cell culture have yielded interesting results involving chromosome 1 and the maintenance of a tumourigenic phenotype. Loss of heterozygosity (LOH), also known as allelic loss or reduction to homozygosity, involving chromosome 1 has been determined in several studies using genetic markers mapping along the length of the chromosome for breast tumours and other tumours. Cytogenetic analysis of tumours has provided information on more than 14,000 neoplasms with an abnormal karyotype, from which over thirty chromosome 1 abnormalities are recurrently found in specific types of benign and malignant neoplastic disorders (Mitelman *et al.*, 1991). Specifically, six cytogenetic bands on chromosome 1 are consistently involved in non-random rearrangements in breast tumours (Mitchell and Santibanez-Koref, 1990).

1.1.1 Somatic Cell Hybrids

In 1969, the phenomenon of tumour-suppression was established through the study of the fusion products of malignant (tumourigenic) mouse cells and normal mouse cells (Harris *et al.*, 1969). Some of these hybrids, referred to as either MxN (malignant x normal) or TxN (tumourigenic x normal) hybrids, failed to form tumours. However, re-expression of tumourigenicity was observed in part of the hybrid cell population which correlated with the loss of specific chromosomes from the hybrids. The use of intraspecific human cell hybrids, which are more stable, later confirmed this phenomenon (Stanbridge, 1976).

The study of cell-cell fusions, one form of somatic cell hybrid, may be of limited value, since usually more than one chromosome is lost from the tumourigenic segregants within the hybrid cell population. Therefore, panels of different segregants which have lost differing combinations of chromosomes are usually required in order to determine which of the lost chromosomes was causing suppression of the tumourigenic phenotype. Despite this disadvantage, two independent studies have successfully established that chromosome 1 has a suppression effect on some cell hybrids. Benedict *et al.* (1984) found that intraspecies fusions between HT1080 cells (a highly tumourigenic human fibrosarcoma cell line) and normal human fibroblast cell lines must lose one or more copies of chromosome 1 to regain the tumourigenic phenotype. Interspecies hybrids have also indicated that chromosome 1 is involved in tumourigenic suppression since fusions between anchorage-independent

transformed BHK (baby hamster kidney) fibrosarcoma cells and normal human fibroblasts regained the anchorage-independent phenotype upon the loss of human chromosome 1 (Stoler and Bouck, 1985). Further work with these fusions between BHK cells and normal human fibroblasts has shown that not only is the anchorage-independent phenotype regained with the loss of human chromosome 1, but also the actin cytoskeleton resembled that of the BHK cells and the cells were potently angiogenic (Bouck *et al.*, 1986). A study of tumourigenic suppression in mouse cell lines has revealed that there is an operative tumour-suppression locus in the A4-C3 region on mouse chromosome 4 (Jonasson *et al.*, 1977; Islam *et al.*, 1989). A gene causing suppression of anchorage-independence has also been localized to rat chromosome 5q22-q23 (Islam *et al.*, 1989). Both the A4-C3 region of mouse chromosome 4 and rat chromosome 5q22-q23 are homologous to human chromosome 1p in terms of linked markers, so that these findings greatly enhance the strength of conclusions drawn from the human tumour cell studies (Harris, 1990).

The use of microcell transfer to form somatic cell hybrids has enabled specific mapping of putative tumour-suppressor loci to human chromosome 1. Microcells are cell fragments containing a single chromosome or translocation product, tagged with a selectable marker such as an HPRT (hypoxanthine phosphoribosyltransferase) gene, neomycin resistance (NEO) gene, or thymidine kinase (TK) gene. Two groups have carried out extensive work on chromosome 1 in microcell transfer protocols.

Using a variety of tumour cell lines for microcell transfer of single chromosomes, Oshimura's group confirmed the tumour-suppressing properties of chromosome 1. Transfer of human chromosome 1 into the human neuroblastoma cell line SK-N-MC (Oshimura *et al.*, 1990), the human fibrosarcoma cell line HT1080 (Kugoh *et al.*, 1990), the human uterine endometrial carcinoma cell line HHUA (Yamada *et al.*, 1990b) and the murine Kirsten sarcoma virus-transformed NIH 3T3 (DT) cells (Yamada *et al.*, 1990a), brought about suppression of tumourigenicity in nude mice. Also, the introduction of chromosome 1 into the HT1080, HHUA and DT cells generated alterations in cellular morphology and *in vitro* transformed properties (Kugoh *et al.*, 1990; Yamada *et al.*, 1990a, 1990b). The lack of a suppressive effect of chromosome 1 on YCR cells (Shimizu *et al.*, 1990) indicates that the locus/loci responsible for the effect in other cell lines are not expressed in all tissue types.

Barrett's group found that introduction of human chromosome 1 into BHK cells had a generalized effect on cell growth and could suppress anchorage-

independent growth (Annab *et al.*, 1992); selection against cells with an intact chromosome 1 was also observed. In another protocol, this group also established that sequences on chromosome 1 are involved in cellular senescence (Sugawara *et al.*, 1990); when human chromosome 1 was reintroduced into human-Syrian hamster hybrids which had lost both copies of the chromosome and had failed to senesce, the cells subsequently aged and died normally. Further use of this cell line and microcells containing a translocation X;1q chromosome, localized the putative tumour-suppressor locus to the long arm of chromosome 1 and, more recently, refined to the region 1q23-q31 (Barrett unpublished, cited in Cheng and Diaz, 1991). Thus it is possible that there are two tumour-suppressor loci on chromosome 1, one associated with negative growth control and the other, localized to 1q23-q31, involved in cellular senescence. While both of these functions may be under the control of a single locus on 1q, transfer of an X;1q derivative chromosome had no effect on a neuroblastoma cell line NGP.1A.TR1 whereas an X;1p derivative induced neuronal differentiation and most of those cells died (Bader *et al.*, 1991); cell lines that grew out of the survivors lacked the t(X;1p) chromosome and were still tumourigenic. Introduction of a normal copy of chromosome 1 was also found to be capable of suppressing tumourigenicity of a colon carcinoma cell line, COKFu (Tanaka *et al.*, 1993), and the 1p36-p34 portion was identified in all clones undergoing suppression that did not retain an intact normal chromosome 1. The essential region of chromosome 1 was refined to 1p36 as revertants possessing the introduced copy were isolated and found to have lost this region of the chromosome.

1.1.2 Cell Lines

Analysis of the establishment of human cell lines in culture has demonstrated that changes which are visible cytogenetically do occur within the cells. One study conducted over a period of four years has shown the importance of chromosome 1 rearrangements to the evolution of the PC/AA cell line. PC/AA, a non-tumourigenic colorectal diploid adenoma cell line, had become immortalized *in vitro* concomitant with the development of an isochromosome 1q (Paraskeva *et al.*, 1988). A 3T3 feeder cell-independent variant, PC/AA/FI, was found to carry two isochromosomes of 1q derived from the two original chromosome 1 homologues present (Paraskeva *et al.*, 1989). More recently this cell line has been shown to be converted along two different pathways into either a mucinous carcinoma or an adenocarcinoma (Paraskeva *et al.*, 1992); both pathways to tumourigenicity include abnormalities of chromosome 1 and provide further evidence for the adenoma-carcinoma evolutionary sequence

proposed by Fearon and Vogelstein (1990). Cytogenetic analyses of human colorectal cancer cells have shown a high frequency of chromosome 1p deletions as well as a number of other chromosomal abnormalities (Leister *et al.*, 1990). However, it is not known if these chromosome alterations correlate with each other.

Investigations of an Epstein-Barr virus-transformed human chronic lymphocytic leukaemic B-cell line identified a clone with a trisomy of 1q11-q32 (D10-1) which was more tumourigenic in nude mice, grew faster and produced more metastases than other clones in the study (Ghose *et al.*, 1990). It is therefore possible that the increased copy number of 1q11-q32 in clone D10-1 confers advantages in proliferation and metastasis formation.

1.1.3 Loss of Heterozygosity (Allelic Loss)

Tumour-suppressor genes appear to act recessively within the cell. Therefore, in order to obtain loss-of-function of a tumour-suppressor gene, both copies of the gene must be inactivated (Ponder, 1988). The loss/inactivation of the first copy is usually achieved by a small deletion, point mutation or even possibly by genetic imprinting and so is rarely seen cytogenetically. It may be a constitutional alteration (i.e. it can be found in all cells throughout the body), observed mostly in heritable tumours, or it may occur in the tumour cell, which is a more frequent occurrence. The loss of the second copy of the gene within a tumour cell often does involve the loss of all or part of the chromosome, or an unbalanced translocation (figure 1.1). This second loss is very important as heterozygous markers mapping to this deleted region of the chromosome will show either a complete loss or partial loss of one allele in Southern blot analysis (figures 1.1.c and 1.2). The reduction of intensity of one allele is more commonly observed in breast tumours than the complete loss of the band since breast tumours are generally heterogeneous in their cellular content; they usually consist of several tumour cell sub-populations, which exhibit differing genotypes, as well as normal cells, such as stromal cells and capillary cells, which have the constitutional genotype of the patient. Southern blot analysis of a panel of patients with several markers can then determine the extent of LOH on the chromosome.

Most probes used in analysis of LOH by Southern blot analysis detect either restriction fragment length polymorphisms (RFLPs) or minisatellites. Primarily, RFLPs have been used in the detection of LOH in human tumours, since until recently they were the predominant form of polymorphism known to the

Legend to figure 1.1: Possible mechanisms of inactivation of a tumour-suppressor gene: specific chromosomal mechanisms which would allow phenotypic expression of a mutant allele of the tumour-suppressor gene, after Cavenee *et al.* (1983).

The first inactivating mutation may be inherited or may occur within the tumour (a). Within the tumour cell (b), the inactivation event involving the second (wild type) allele may occur by a number of mechanisms: mitotic nondisjunction and loss of the chromosome carrying the wild-type tumour-suppressor allele (i, ii) results in hemizyosity (i) or homozygosity when reduplication of the remaining homologue occurs (ii); mitotic recombination with the other homologue to give homozygosity (iii) or another chromosome to produce an unbalanced translocation giving rise to hemizyosity at the tumour-suppressor locus (iv); several regionalized events must also be considered, such as deletion (v) or point mutation (vi) which produces loss-of-function of the second allele without perturbation of flanking sequences.

The consequences that the rearrangements in (b) have for a polymorphic locus mapping close to the tumour-suppressor gene on the chromosome can be observed by Southern analysis when a patient is constitutionally informative (c). Loss of an allele is presented as a complete loss of one band in the tumour track; duplicated alleles are presented as extra-thickened lines.

- t1, t2 = inactivated tumour-suppressor alleles
- + = wild-type tumour-suppressor allele
- = absence of tumour-suppressor allele

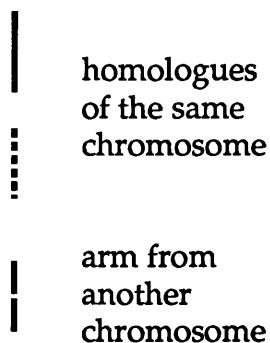


Figure 1.1

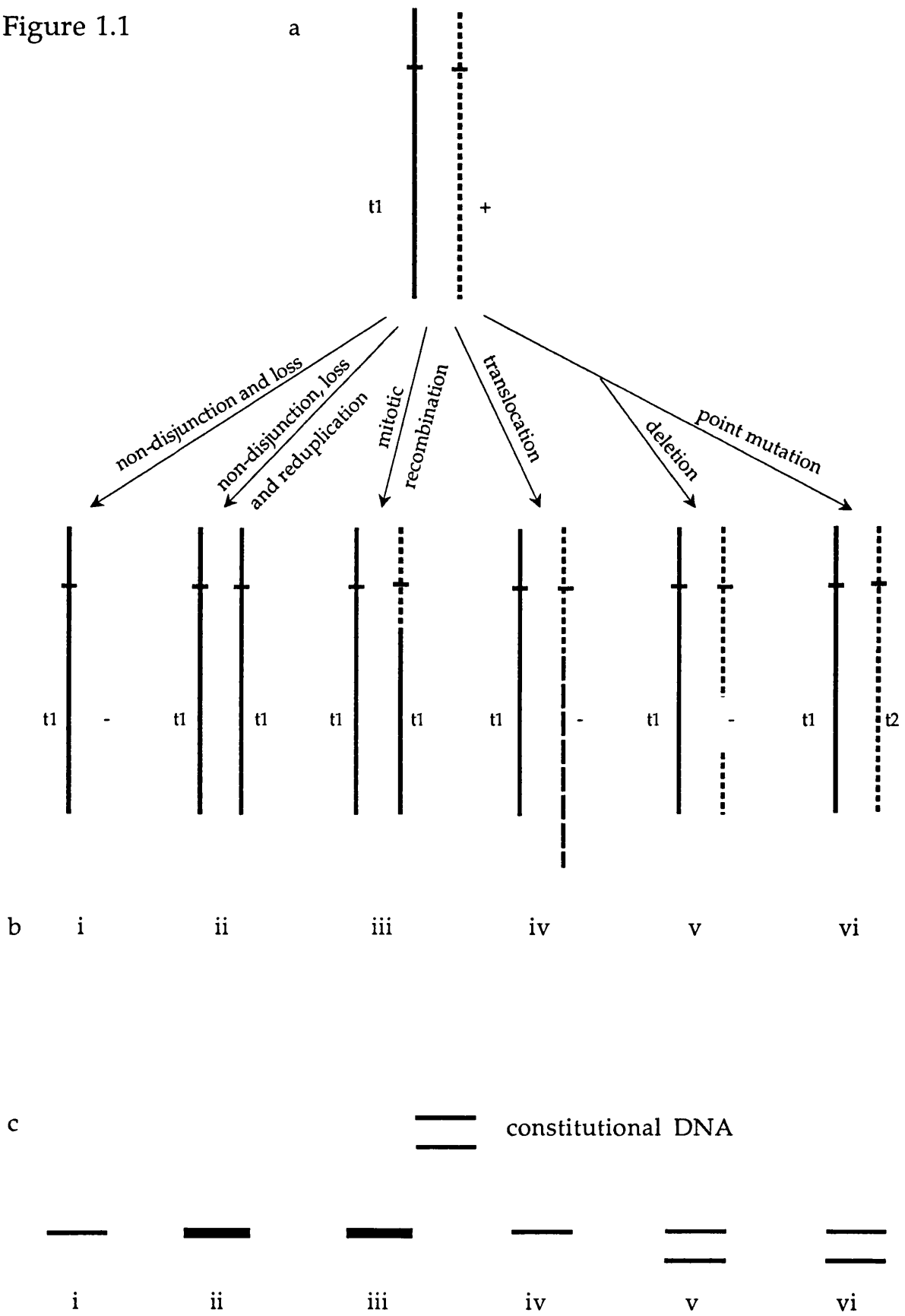
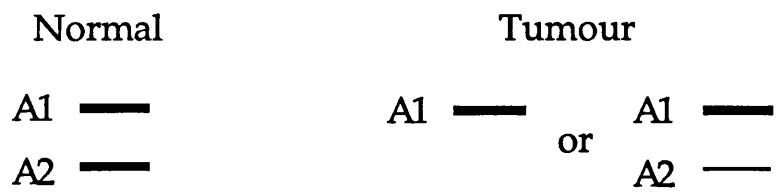


Figure 1.2: Diagram to show Southern blot analysis of a patient heterozygous for a polymorphic locus *A*. The tumour genotype must be compared to the normal constitutional genotype of the patient in order to determine if LOH is occurring. This is essential since two patterns are possible in the tumour DNA. Where there is complete loss of allele *A2* within the tumour, it must be established that the patient is constitutionally heterozygous. Where there is partial loss of *A2* (shown by a reduction of intensity of that band), it must be established that this is not due to partial degradation of one of the alleles.

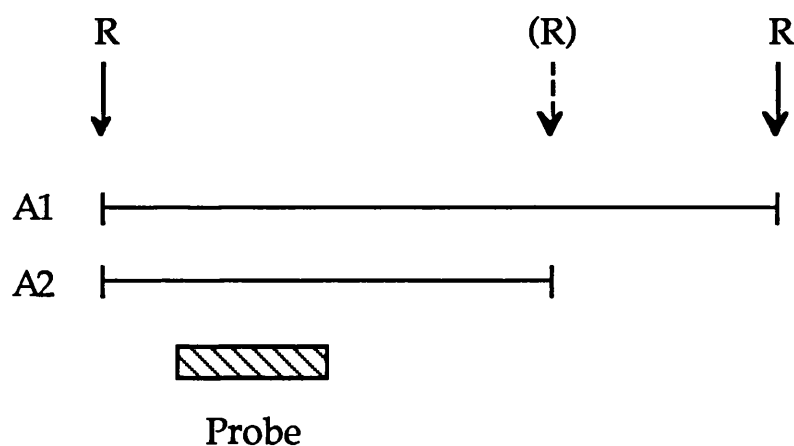


human genome. RFLPs are biallelic systems which exist due to the occasional presence of a particular restriction enzyme recognition site (figure 1.3). However, RFLPs are of limited value since the recognition site in question is either present or absent in an individual so that the frequency of heterozygotes (informative patients) is at most 50% and usually well below this figure. A cumulative effect then develops when multiple RFLPs are studied in a panel of patients so that the system becomes poorly informative overall.

Minisatellites, originally identified by Jeffreys *et al.* (1985), consist of a variable number of tandem repeats (VNTRs) of a short DNA core sequence (figure 1.4) and can serve as highly polymorphic single copy sequences within two restriction enzyme recognition sites with probes consisting of the tandem repeats (Nakamura *et al.*, 1987c, 1988a). Therefore, genetic markers based on VNTRs generally show a high level of heterozygosity within the population and are more informative than the biallelic RFLP systems, making them powerful tools for revealing reduction to homozygosity at loci that may be involved in carcinogenesis.

Both RFLPs and VNTRs have been traditionally analyzed by Southern hybridization. However, this method has several disadvantages, chiefly that it requires microgramme quantities of intact, high molecular weight DNA. Such DNA is usually only obtainable from large freshly-frozen tumour specimens taken by surgical resection so that retrospective studies of archival tissues are not possible unless frozen tissue banks are available. The sensitivity of Southern blotting to detect LOH is greatly affected by the level of 'contamination' of normal cells within the specimen, since tumour cells are rarely purified before DNA extraction. As an alternative to Southern analysis, the application of PCR (polymerase chain reaction) to LOH studies has a number of attractions, including the use of mere nanogrammes of DNA which may be of poor quality and the possibility of microdissection of tumour cells from normal cells both from frozen specimens, biopsies, aspirates and, importantly, archival banks of tissue sections (Bianchi *et al.*, 1991; Sundaresan *et al.*, 1993). The study of these banks would permit correlations with survival and disease-free interval not available to studies of recent specimens. Although Southern filters can be stripped and reprobed a number of times, PCR can be multiplexed (Knowles *et al.*, 1993), i.e. several loci can be amplified in the same reaction including control loci used to determine the nature of imbalances. RFLPs would be easily amplified by PCR using appropriate flanking primers and then digested with restriction endonucleases. Minisatellites would be easier to study since the length changes between alleles would be readily visible after amplification using primers

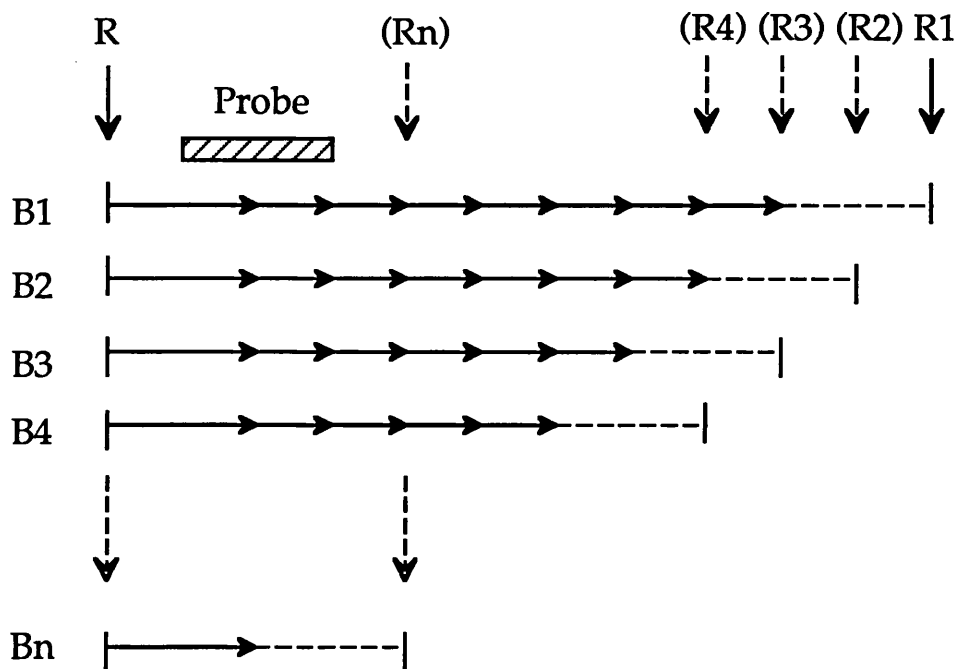
Figure 1.3: Alleles created by an RFLP system. Every individual has a certain number of constant restriction endonuclease sites in their DNA; some individuals have additional sites (the central bracketed restriction endonuclease site), creating sites of polymorphism within the DNA strand. Since the extra site is either present or absent in an individual, by the Hardy-Weinberg equation the frequency of heterozygotes is at most 50%.



R: restriction enzyme recognition site

A1, A2: alleles recognized by the probe

Figure 1.4: Alleles created by a VNTR system. The VNTR sequence is situated between two constant restriction endonuclease recognition sites so that multiple alleles, B1, B2,..., Bn, can be generated through the loss and gain of the tandem repeats. The probe used to detect the locus consists of the tandem repeats of the core sequence creating the polymorphic locus.



R: restriction enzyme recognition site

R1, R2,..., Rn: alternative positions of the right hand site

with respect to the left hand site, due to the changing length
of the allele

B1, B2,..., Bn: alleles detected by the probe

designed from the unique flanking DNA sequences. However, most minisatellite systems generate alleles that are too large for amplification by PCR and in heterozygotes with alleles of vastly different sizes, preferential amplification of the smaller allele occurs (Scharf *et al.*, 1992).

In addition to minisatellites, there is another form of VNTR, microsatellites, which are composed of the simple repeat sequence $(n)_a$, where $n = 1-5$ nucleotides and $a = \sim 10-60$. The human genome contains approximately 50,000 copies of this type of repeat sequence, on average every 30kb (Stallings *et al.*, 1991). Microsatellites show the same degree of variability in the population as minisatellites, although microsatellite alleles differ in length by only a few basepairs. Microsatellite variations are therefore not detectable by conventional Southern blotting but are ideal for PCR amplification followed by detection on sequencing gels (Litt and Luty, 1989; Weber and May, 1989). They are also not so prone to preferential amplification of small alleles in heterozygotes since the size differences between alleles is never vast. However, the use of PCR to amplify any locus is fraught with difficulties, principally the amplification must be quantitative and remain in the exponential phase of the reaction, not being allowed to plateau. Although preferential amplification of different-sized alleles can be avoided in both RFLP and microsatellite systems, the appearance of shadow bands as a result of stuttering in the PCR reaction is common with microsatellites. In particular, amplification of dinucleotide repeats generates additional bands that vary from the actual allele itself by two nucleotides and if conditions and primers are not optimized, the intensity of these shadow bands may actually obscure the real allele (Futreal *et al.*, 1992; Knowles *et al.*, 1993). They are thought to occur by slippage of the *Taq* polymerase enzyme although they are reduced in trinucleotide and tetranucleotide systems.

Loss of heterozygosity (LOH) has been demonstrated for a wide range of both rare and common tumours on many chromosomes, including 1, 3, 5, 6, 11, 13, 16, 17, 18, 21 and 22 (see Green, 1988; Ponder, 1988; Sager, 1989; Stanbridge, 1990; Lasko *et al.*, 1991; Levine, 1993 for review). LOH in the regions 13q14 and 17p13 are probably the most well-defined and are the sites of two tumour-suppressor genes, RB1 and TP53 (also known as p53) respectively.

The RB1 gene, mapping to chromosome 13q14, was the first tumour-suppressor gene to be identified. It undergoes mutation and LOH in second malignancies in familial retinoblastoma patients (Hansen *et al.*, 1985), as well as in breast tumours (T'Ang *et al.*, 1988; Devilee *et al.*, 1989; Varley *et al.*, 1989), and small cell lung carcinomas (SCLC) (Harbour *et al.*, 1988) of non-retinoblastoma patients. In breast

tumours, LOH of the RB1 gene occurs in 25% to 37% of cases (Devilee *et al.*, 1989; Varley *et al.*, 1989).

Chromosome 17p undergoes LOH in tumours of the breast (Mackay *et al.*, 1988b; Varley *et al.*, 1991), colon (Vogelstein *et al.*, 1988; Baker *et al.*, 1989, 1990), bladder (Tsai *et al.*, 1990; Oka *et al.*, 1991) and lung (Yokota *et al.*, 1987, 1990). This allelic loss on the short arm of chromosome 17 has been associated with perturbations in the TP53 gene although this is not yet conclusive and there is evidence for at least two loci on chromosome 17p involved in breast carcinogenesis (Coles *et al.*, 1990; Chen *et al.*, 1991; Andersen *et al.*, 1992; Lindblom *et al.*, 1993). In breast tumours, LOH ranges between 25% and 67% for chromosome 17p (Mackay *et al.*, 1988b; Cropp *et al.*, 1990).

Recently, interest has flared in chromosome 17q with the mapping by genetic linkage of the BRCA1 gene to 17q21 (Hall *et al.*, 1990, 1992; Narod *et al.*, 1991; Easton *et al.*, 1993) thought to be responsible for early-onset familial breast cancer and familial breast-ovarian cancer in some women. LOH studies of 17q actually indicate two regions of loss, one proximal 17q, encompassing the location of BRCA1 (Futreal *et al.*, 1992; Smith *et al.*, 1992) and the other distal 17q (Cropp *et al.*, 1990). Three further studies all show these two regions of deletion to occur independently of one another (Andersen *et al.*, 1992; Cornelis *et al.*, 1993; Lindblom *et al.*, 1993). Importantly, Smith *et al.* (1992) showed that in several families with familial breast-ovarian cancer, allele loss involved the wild-type chromosome as expected for the inactivation of a tumour-suppressor gene where there is an inherited mutation as hypothesized. Chromosome 17q is also the site for a high frequency of LOH in ovarian tumours in both familial and sporadic cases (Foulkes *et al.*, 1993; Jacobs *et al.*, 1993; Phillips *et al.*, 1993).

LOH has been determined in a number of other chromosomal regions in human breast cancer. These include chromosomes 1p, 1q, 3p, 11p, 16 and 18q. Devilee *et al.* (1989) and Ali *et al.* (1989) have reported the loss of alleles on chromosome 3p14-3p21 and 3p21-3p25 respectively. Specifically, THRB (formerly ERBA2), a member of the ERBA steroid/ thyroid hormone receptor family, undergoes LOH (Ali *et al.*, 1989). The tumour-suppressor gene responsible for von Hippel-Lindau disease has recently been cloned (Latif *et al.*, 1993) and mapped to 3p25-p26 (Richards *et al.*, 1993), though whether it is a candidate for the target of deletion on 3p in breast tumours is unknown.

Cropp *et al.* (1990) also identified allelic loss on chromosome 18q in breast tumours. This may be due the presence of the DCC gene on that arm although

this gene is specifically excluded from the deletions mapped in some tumours (Devilee *et al.*, 1991c). The DCC gene is a putative tumour-suppressor gene identified from the analysis of colorectal cancer (Fearon *et al.*, 1990). Currently, the nature of the locus on 11p is unknown. Although the Wilms' tumour-suppressor gene, WT1, mapping to 11p13 has now been identified (Call *et al.*, 1990; Rose *et al.*, 1990), it is not regarded as a candidate in breast tumours and there are several regions on this chromosome involved in breast tumours (Theillet *et al.*, 1986; Ali *et al.*, 1987, 1989; Mackay *et al.*, 1988a; Devilee *et al.*, 1989).

1.1.3.1 Loss of Heterozygosity on Chromosome 1 in Breast Tumours

Before the beginning of the project, there were some preliminary reports of LOH on chromosome 1 in breast tumours (Chen *et al.*, 1989; Genuardi *et al.*, 1989a, 1989b; Merlo *et al.*, 1989; Bièche *et al.*, 1990; Gendler *et al.*, 1990) that were later followed up by more extensive studies. There were thought to be more than one target of LOH on this chromosome in breast tumours: Chen *et al.* (1989) had proposed the existence of a 26cM smallest common region of overlapping deletion in the mid-region of 1q extending between AT3 and D1S53, while both Merlo *et al.* (1989) and Gendler *et al.* (1990) had found significant levels of LOH at MUC1 (DF3) located in band 1q21. Genuardi *et al.* (1989a, 1989b) established a region of high LOH at the tip of the short arm with D1Z2 and D1S57 while Bièche *et al.* (1990) reported LOH further proximal at the MYCL1 locus.

LOH on chromosome 1 in breast tumours was first reported by Chen *et al.* (1989) in which eight loci were assessed, six on the long arm and two on the short arm. Although 48 tumours were studied, LOH on 1q occurred in only nine tumours and formed an overlapping series of deletions. A common region of deletion was identified as the 26-centiMorgan interval between AT3 and D1S53 at 1q23-q32 (figure 1.5), which also formed a peak of LOH, 23-26%, suggestive of a gene targeted for inactivation in this location. There also appeared to be a trend toward less aggressive breast cancers among the cases with allelic loss. Therefore LOH in this region could be an early event in breast tumorigenesis or be associated with a particular breast cancer subtype (Borg *et al.*, 1992b). In a follow-up study, Chen, L.-C. *et al.* (1992) found evidence to suggest that LOH at 1q21-q23 occurred prior to metastatic development in tumours.

Other studies of chromosome 1 followed and implicated additional regions on chromosome 1 as sites for tumour-suppressor genes (figure 1.5). Distal deletions of 1p were identified in 15 of 37 patients (41%) from analysis of D1Z2, a

Legend to figure 1.5: Summary of allelic imbalance (AI) for polymorphic markers mapping to chromosome 1 from the reports discussed in the text (Chen *et al.*, 1989; Genuardi *et al.*, 1989a, 1989b; Merlo *et al.*, 1989; Bièche *et al.*, 1990; Gendler *et al.*, 1990; Larsson *et al.*, 1990; Sato *et al.*, 1990; Devilee *et al.*, 1991b, 1991d; Mars *et al.*, 1991; Borg *et al.*, 1992b). The incidence of LOH at loci studied was not presented by Bièche *et al.* (1993), so that their data can only be used to provide smallest regions of common overlap.

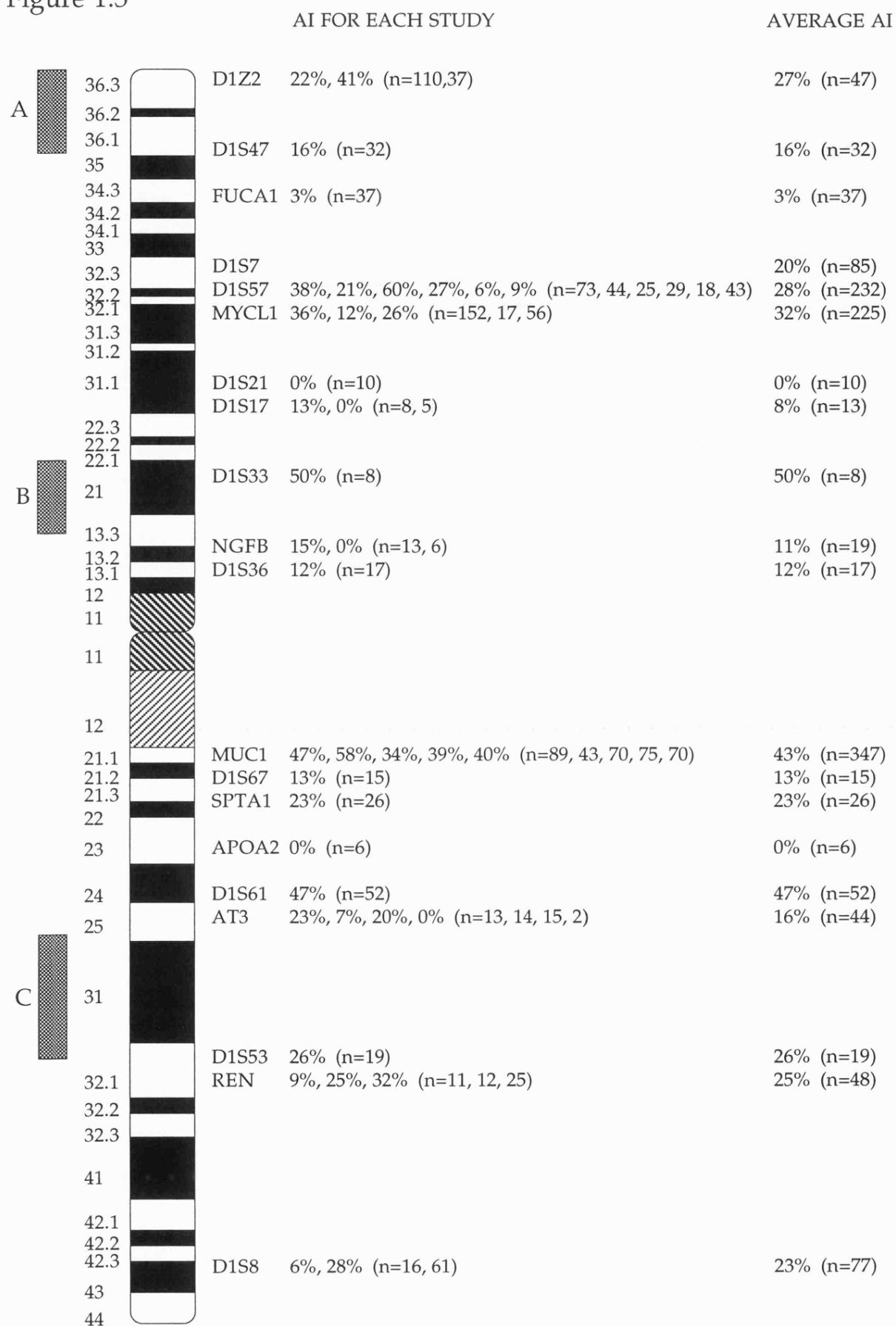
Two studies have identified smallest common regions of deletion: region A limited by the loci D1S80 and D1S95/96 (Bièche *et al.*, 1993), region B bounded by D1S9 and D1S73 (Bièche *et al.*, 1993), and region C with flanking markers AT3 and D1S53 (Chen *et al.*, 1989).

The order of the markers from Dracopoli *et al.* (1991) and Collins *et al.* (1992) is:

pter - D1Z2 - D1S80 - (D1S95, D1S96) - D1S47 - FUCA1 - D1S7 - D1S57 - MYCL1 - D1S21 - D1S17 - D1S9 - D1S73 - NGFB - D1S36 - cen - MUC1 - D1S67 - SPTA1 - APOA2 - D1S61 - AT3 - D1S53 - REN - D1S8 - qter.

The loci D1S95 and D1S96 have not been ordered with respect to others on the chromosome, but map very closely to one another and are thus presented together as the boundary for region A by Bièche *et al.* (1993). While the precise location of D1S33 (1p) is unknown, it does map between D1S17 and NGFB, so that although it appears to correspond to region B, this is not certain.

Figure 1.5



midisatellite situated at the tip of 1p (Genuardi *et al.*, 1989a, 1989b). Analysis of this locus is open to question since the probe detecting D1Z2 creates a complex pattern of bands on Southern filters and haplotypes inherited on each homologue cannot be determined without family linkage studies. Decreases in signals in patients were confirmed when hybridization was repeated in each case after digestion with a different restriction endonuclease. An additional locus was also later scored by the same group who included some of the original panel in this study (Mars *et al.*, 1991). The imbalance at D1S57 increased the overall involvement of distal 1p in breast tumours to 73%. Genuardi *et al.* (1989b) could establish no associations between prognostic factors but LOH at D1Z2 was more frequent in patients with characteristics of hereditary tumours than in patients with sporadic tumours. Mars *et al.* (1991) also identified a possible association between high risk patients and an alteration of the distal part of 1p.

The MUC1 gene encodes a protein which is an epithelial mucin that is expressed in 92% of breast carcinomas as well as in normal lactating breast tissue. LOH at MUC1 was determined as 29% (n=70) and 34% (n=70) by Merlo *et al.* (1989) and Gendler *et al.* (1990) respectively. In both studies examination of loss did not extend to either 1p or distal 1q in sufficient patients to indicate that the region around MUC1 was specifically targeted for deletion in breast tumours.

In a limited study of the three loci D1Z2, D1S57 and MUC1 on a panel of 124 patients (Borg *et al.*, 1992b), LOH was found to occur on distal 1p (D1Z2 and D1S57) independently of LOH on 1q (MUC1). LOH at the MUC1 locus has been correlated with early disease recurrence (i.e. poor prognosis) while independently, LOH on 1p correlated with the presence of lymph node metastasis, large tumour size and DNA aneuploidy (Borg *et al.*, 1992b).

Devilee *et al.* (1991b, 1991d) established high rates of allelic imbalance on chromosome 1 in a panel of 84 breast tumours, including two independent regions of allelic loss, one at D1S33, 1p21-p31 and the other on the long arm, q24-q32, between D1S61 and REN. Notably in about half the cases, allelic imbalance was consistent with a gain in copy number (GCN) of one allele, confirmed by fluorescence *in situ* hybridization to interphase nuclei from some of the tumours. Allelic imbalance (AI) is now used as a blanket term for LOH, GCN and all other changes in intensity of one or both alleles in the tumour DNA track with respect to those in the constitutional DNA track of an autoradiograph. Cornelisse *et al.* (1992) investigated the relationship between allelic imbalance, DNA aneuploidy and several clinical parameters after obtaining extensive data on each tumour for all chromosomes to produce an allelotype (Devilee *et al.*,

1991d). Although AI on 13 different chromosome arms, including 3p, 11p and 17p, correlated significantly with multiple AI events, the AI at 1q and 16q did not show any such correlation. This suggests that AI on 3p, 11p and 17p occur late and are progression-related events, while AI on 1q and 16q would represent earlier events.

A preliminary study of LOH at MYCL1 (1p32) showed 36% loss (n=152) which was associated with more aggressive tumours (Bièche *et al.*, 1990). Subsequently, a more detailed analysis of 22 loci on 1p, with six loci on 1q, was made in 74 patients (Bièche *et al.*, 1993). This second study identified two independent regions of LOH on the short arm, one at 1p32-pter, where the smallest common region of deletion included the loci D1S95/96 and D1S80, and the second at p13-p21, with this smallest commonly deleted region including the loci D1S9 and D1S73. No further attempt at correlation with clinical parameters was reported.

Three allelotype studies of breast tumours have now been published (Larsson *et al.*, 1990; Sato *et al.*, 1990; Devilee *et al.*, 1991d). The frequencies of LOH on 13q, 16q, 17p and 22q were found to be similar between the studies. However, neither of the studies by Larsson *et al.* (1990) nor Sato *et al.* (1990) supported the frequencies of AI found by Devilee *et al.* (1991d) at 1q, 3p and 18q. The use of widely spaced polymorphic markers by the different groups may explain the observed differences, while heterogeneity with respect to chromosome involvement may also exist in the relatively small panels of breast tumours each group examined (Devilee *et al.*, 1991d).

1.1.3.2 Loss of Heterozygosity on Chromosome 1 in Other Tumours

LOH has also been detected on chromosome 1 in other tumours. The first description of allele loss on chromosome 1 in any tumour was reported in malignant melanoma (Dracopoli *et al.*, 1985) at the distal end of the short arm, later found to occur late in melanoma tumour progression (Dracopoli *et al.*, 1989). Other studies have highlighted the importance of 1p allele loss in tumours.

The deletion of chromosome 1 sequences from neuroblastoma cells is well documented (Fong *et al.*, 1989, 1992; Weith *et al.*, 1989; Takayama *et al.*, 1992; White *et al.*, 1993). These studies have defined a consensus deletion in neuroblastomas mapping to 1p36.1-p36.2 (Weith *et al.*, 1989). This is believed to be the site of a tumour-suppressor gene involved in neuroblastomas but is also consistent with the distal 1p site in breast tumours. The loss of these distal 1p

sequences has been correlated with MYCN amplification and although the loss can occur in early stages of the disease, it usually is found in tumours of advanced clinical stages, suggesting an association with particularly aggressive neuroblastoma (Fong *et al.*, 1989, 1992). Two more recent studies have provided conflicting evidence for the influence of genomic imprinting on the parental origin of genes involved in LOH on 1p. Caron *et al.* (1993) found that the maternal allele of polymorphic markers on distal 1p was preferentially lost, while Cheng *et al.* (1993) found no significant preference for loss of either allele despite a preference for amplification of the paternal MYCN allele on chromosome 2.

The loss of 1p loci has also been established in medullary thyroid tumours and pheochromocytomas which arise sporadically or as part of the dominantly-inherited cancer syndrome multiple endocrine neoplasia type 2 (MEN2). Study of both sporadic and syndrome cases by a number of groups (Mathew *et al.*, 1987; Tsutsumi *et al.*, 1989; Khosla *et al.*, 1991; Moley *et al.*, 1992; Mulligan *et al.*, 1993a) implicates the importance of deletion of sequences on 1p, although no smallest common region of deletion has been satisfactorily established. The gene responsible for the MEN2 syndrome has recently been identified (Mulligan *et al.*, 1993b), as the proto-oncogene RET which maps to chromosome 10q11.2.

Comparisons of melanoma, neuroblastoma and MEN2 tumours suggest that the frequent loss of 1p in these malignancies is a common late event of neuroectodermal tumour progression (Dracopoli *et al.*, 1989; Kubo *et al.*, 1991). Distal 1p loss may be a marker of tumours arising from embryonic neural crest tissue (Harnett *et al.*, 1991), the allelic loss in these tumours targeting the same sequences. Conceivably, this locus is also the target of distal 1p deletion in breast tumours.

LOH of MUC1 has been found not to be restricted to breast tumours but has also been identified in gastrointestinal tumours (Fey *et al.*, 1989; Sano *et al.*, 1991). In the latter study, LOH at MUC1 was detected only in advanced well-differentiated adenocarcinomas, and not detected in cases of early well-differentiated adenocarcinoma or in poorly-differentiated adenocarcinomas of any stage. LOH was also detected at D1S7 on the short arm of chromosome 1, regardless of stage and histological type (Sano *et al.*, 1991).

1.1.4 Cytogenetic Studies of Tumours

Most, if not all, tumours have structural and/or numerical chromosomal aberrations, some of which are consistently associated with particular tumours. A number of specific chromosomal abnormalities have been well characterized, notably the Philadelphia (Ph') chromosome, an unusually small chromosome observed in the leukaemic cells of chronic myeloid leukaemia (CML) patients (for review see Rowley, 1984, 1990). The two genes that are invariably involved in the reciprocal translocation $t(9;22)(q34;q11)$ have been identified as the proto-oncogene ABL1, mapping to 9q34, and the BCR gene (breakpoint cluster region), mapping to 22q11. The translocation results in a fusion protein responsible for transformation of the cells. Such an acquired specific chromosomal abnormality and other less well understood alterations can be placed into three categories (Mitelman and Heim, 1988):

- i) Primary abnormalities, which may be found as the sole abnormality within a tumour cell, and will be present in the earliest phases of the disease.
- ii) Secondary abnormalities, which may accrue in later phases, often as the result of reduced genomic stability in the neoplastic cells. These changes are important in creating the genetic variability from which clonal evolution occurs via selective competition. Although often non-random, they are less specific than primary changes and, by definition, do not occur alone.
- iii) Cytogenetic noise, which includes those changes without a long-term selective value, many of which are therefore nonclonal and generally have no significant correlation with the disease. The more genetically unstable a tumour cell population is, the greater the likelihood that many of the ensuing rearrangements will fail to be of consequence in tumour progression.

Although leukaemias constitute approximately 10% of all human neoplasia, they provide the information for more than 80% of available karyotype data. It is more difficult to study solid tumours by traditional karyotyping methods than haematopoietic tumours for a variety of reasons. Most leukaemias and lymphomas have relatively simple karyotypes (usually near-diploid) in which a characteristic change is fairly easy to detect. Those of solid tumours are often far more complex, undergoing extreme numerical and structural changes that tend to mask the primary karyotypic event and it is almost impossible to attach pathogenetic significance to the various changes seen. The study of solid tumours is also impeded because it can be difficult to obtain sufficient metaphases

for analysis, the mitotic index (MI) of solid tumours often being very low (Teyssier, 1989); the quality of the chromosomes eventually obtained may be poor with individual chromosomes appearing 'fuzzy'. The presence of cytogenetically normal cells in solid tumours is common, since stromal and supporting tissue elements in carcinomas and sarcomas are of normal tissue origin, an important factor in many solid tumours including those of the breast, (Sandberg *et al.*, 1988). Unlike haematopoietic tumours, solid tumours must first be disaggregated before karyotyping can be carried out (Tanaka and Testa, 1987). In order to overcome the difficulties experienced in direct karyotyping of solid tumours which often lead to only partial karyotypes, cell lines can be established in tissue culture before karyotyping by a number of different protocols. This will increase the number of metaphases available for analysis, but it can inadvertently lead to selection of particular sub-populations of tumour cells as one or several become the dominant cell type in culture (Mars and Saunders, 1990). Also, further chromosomal rearrangements may occur during culture which were not originally present in the tumour cell. Therefore, the results obtained from cultured tumour cells may not be truly representative of the original tumour. Solid tumours are therefore processed by a variety of methods, some by direct karyotyping, others by cell culturing protocols, either short or long term. Within these three broad divisions, different research groups have established their own particular protocols to obtain results so that these studies are largely not comparable.

Consequently, consistent chromosomal changes have been observed in relatively few solid tumours; those most studied are the childhood tumours, in which there is known to be a strong genetic element. Retinoblastoma, a rare ocular tumour, is one of a very few tumours for which there is a primary genetic lesion identified. Approximately 40% of patients with retinoblastoma carry a germ-line mutation at the retinoblastoma (RB1) locus on chromosome 13q14 and most of these patients develop bilateral tumours. However, non-hereditary retinoblastoma, occurring in the remaining 60% of patients, is always unilateral (Knudson, 1971; Cowell, 1991). In addition to deletions encompassing chromosome 13q14 which may extend along the entire length of the chromosome, three other chromosomal changes have been identified in the karyotypes of retinoblastomas, namely, isochromosome 6p, trisomy 1q and trisomy 17q (Gardner *et al.*, 1982; Benedict *et al.*, 1983). From the consensus region of deletion at chromosome 13q14, a tumour-suppressor gene, RB1, has been cloned and extensively characterized, but no further genes that may be associated with the genesis or progression of retinoblastoma mapping to the other consensus regions have been reported. The RB1 locus itself undergoes

submicroscopic and even intragenic loss of material in the majority of retinoblastoma tumours (Cavenee *et al.*, 1983; Lee *et al.*, 1987). It has since been found that the RB1 gene undergoes similar loss in a variety of other tumours, including those of the breast, bladder, lung and pancreas in non-retinoblastoma patients (T'Ang *et al.*, 1988; Yokota *et al.*, 1988; Varley *et al.*, 1989; Bookstein *et al.*, 1990), but not in colorectal carcinomas (Vogelstein *et al.*, 1988).

Neuroblastoma is the most common solid tumour in children, with a subset of patients exhibiting a predisposition to develop the disease which follows an autosomal dominant pattern of inheritance. The most characteristic cytogenetic abnormality is deletion of chromosome 1p (Brodeur *et al.*, 1977; Gilbert *et al.*, 1984) which is associated with advanced stages of disease (Christiansen *et al.*, 1992) and the region most commonly deleted is between 1p32 and 1pter (Brodeur, 1990). Weith *et al.* (1989) have defined a smaller consensus deletion mapping to 1p36.1-p36.2 using 13 cases undergoing allelic loss on chromosome 1p. Alterations to chromosome 17 have also been observed at a lower frequency in neuroblastomas. Constitutional chromosome alterations have been identified in only two neuroblastoma patients: a translocation t(1;17)(p36;q12-21) identified by Laureys *et al.* (1990) and an interstitial deletion involving 1p36.1-p36.2 (Biegel *et al.*, 1993) which support the localization of a neuroblastoma tumour-suppressor gene within the sub-bands 1p36.1-p36.2 determined by the allelic loss consensus (Weith *et al.*, 1989).

1.1.4.1 Breast Tumours

Cytogenetically, breast tumours are probably the most well studied of all the solid tumours. Although the information is usually of limited value as discussed above, what is available does show that breast tumour cells are often highly aneuploid and can contain a large number of marker chromosomes, whose identification and derivation are frequently indeterminate, despite their consistent appearance and presence within the cells of any one tumour sample. Specific subsets of these marker chromosomes occur in tumour cell sub-populations, with a number of subsets occurring throughout the whole of the tumour (Jones Cruciger *et al.*, 1976; Pathak *et al.*, 1979). Homogeneously staining regions (HSRs) and double minute chromosomes (DMs) are also characteristic of breast tumours (Barker and Hsu, 1979; Kovacs, 1979; Barker *et al.*, 1980) and are thought to be associated with the amplification of oncogenes or drug resistance genes (Kinzler *et al.*, 1986; Fukumoto *et al.*, 1993).

A significant proportion of primary breast cancers do have near-diploid karyotypes, in some cases these cells comprising the majority of the tumour mass (Rodgers *et al.*, 1984; Gerbault-Seureau *et al.*, 1987; Zhang *et al.*, 1989). A large proportion of the human chromosome complement is frequently altered, notably chromosomes 1, 3, 5, 6, 8, 11, 12, 13, 14, 16 and 17. Chromosome 1 structural changes are generally considered to be significant in breast tumours since chromosome 1 is the most frequently rearranged of all the human chromosomes (Mitchell and Santibanez-Koref, 1990). Indeed, chromosome 1 changes are common in most, and probably all, forms of cancer (Atkin, 1986). Chromosome 1 is one of the few chromosomes consistently identifiable as a component of marker chromosomes in breast tumours, particularly 1p13 to 1q12 (Gebhart *et al.*, 1986). Critical assessment of both new data and that recorded in the Cancer Chromosome Registry at the University of Lund was carried out by Mitchell and Santibanez-Koref (1990). After statistical analysis, in which the size of each cytogenetic band was taken into account, it was concluded that changes to 1p13 are the single most common lesion in breast cancer, followed by changes to 1q21, 1q23, 1p11 and 1p22 in descending order. The significance of these observations has been confirmed by a larger study of the literature (Mitelman and Heim, 1988) in which 71 of the 329 cytogenetic bands of the human genome were found to be consistently involved in primary neoplasia-related rearrangements. Of these 71 bands, six are on chromosome 1: 1p36, 1p32, 1p11, 1q21, 1q23 and 1q32.

The karyotypes of over 300 primary breast tumours have been reported in the literature. These have been analyzed both by direct karyotype analysis of the tumour and after short- and long-term culture. A large proportion of the tumours were found to have undergone changes in ploidy becoming triploid and even tetraploid. In addition the tumours display structural rearrangements to specific chromosomes including gain of 1q often within the marker chromosome population or as isochromosome 1q (Kovacs, 1981; Hill *et al.*, 1987; Dutrillaux *et al.*, 1991). In one directly-karyotyped hypertriploid tumour, nine copies of 1q were counted in each cell: in addition to trisomy of chromosome 1, five marker chromosomes of three different origins involving 1q were identified (Kovacs, 1981). Those tumours that were either diploid or pseudo-diploid in nature showed a variation in the number of structural rearrangements although apparently normal karyotypes have also been observed (Zhang *et al.*, 1989). Single clonal changes involving chromosome 1 in unbalanced translocations have been identified (Zhang *et al.*, 1989) as well as other changes to chromosome 1 in more complex cases. As in polyploid tumours, the net gain of 1q is a common event in diploid and pseudo-diploid cases, often associated with the formation of marker chromosomes and isochromosomes (Jones Cruciger *et al.*,

1976; Kovacs, 1978; Rodgers *et al.*, 1984; Gebhart *et al.*, 1986; Ferti-Passantonopoulou and Panani, 1987; Gerbault-Seureau *et al.*, 1987; Hainsworth *et al.*, 1991; Pandis *et al.*, 1992, 1993). Other common events involving chromosome 1 include partial or complete deletions of 1q (Gebhart *et al.*, 1986; Hainsworth *et al.*, 1991) or 1p (Gebhart *et al.*, 1986; Ferti-Passantonopoulou and Panani, 1987; Gerbault-Seureau *et al.*, 1987) and unbalanced translocations of 1p with clusters of breakpoints at bands p36, p35, p32 and p22 (Gerbault-Seureau *et al.*, 1987; Hainsworth *et al.*, 1991, 1992). These 1p translocations were found to correlate with poor prognosis in one particular study (Hainsworth *et al.*, 1992); correlations to clinical parameters were not looked for by Gerbault-Seureau *et al.* (1987) or Hainsworth *et al.* (1991).

Metastases and pleural effusions derived from breast cancer patients have also been studied by direct karyotyping or after culturing, and stable cell lines have also been established from these samples. As with primary tumours, these cells can also show structural as well as numerical changes to chromosomes, with chromosome 1 principally involved in the genesis of multiple marker chromosomes (Bertrand *et al.*, 1979; Pathak, 1980; Satya-Prakash *et al.*, 1981; Gebhart *et al.*, 1986; Bello and Rey, 1989; Sasi *et al.*, 1991). Breakpoints either side of the centromere on chromosome 1 give rise to partial or complete deletions of 1p and excess 1q (Bello and Rey, 1989; Sasi *et al.*, 1991).

Two studies were made of fibroadenomas using short-term culture techniques (Zhang *et al.*, 1989; Calabrese *et al.*, 1991). Most of these showed a normal karyotype and those with structural alterations were all diploid in nature. In a single patient a clonal event was found to involve chromosome 1 in a complex reciprocal translocation and inversion rearrangement with chromosome 20 (Calabrese *et al.*, 1991).

Therefore from the studies of fibroadenomas, primary carcinomas, metastases and pleural effusions, there is an overwhelming body of evidence that implicates changes to chromosome 1, principally as net loss of the short arm and gain of the long arm, in breast disease. The high frequency of 1q alteration in carcinomas with few anomalies suggests trisomy 1q is an early chromosomal change (Dutrillaux *et al.*, 1990; Pandis *et al.*, 1993). This appears to be borne out by the single clonal event involving chromosome 1 in a fibroadenoma (Calabrese *et al.*, 1991) and an isochromosome of 1q present in direct preparations of a hypodiploid primary breast carcinoma which remained stable in cells cultured from that tumour over a period of five years (Gebhart *et al.*, 1986). Based on the results with pleural effusions, suggestions have been made that alterations to 1q provide

some proliferative advantages for different human tumours (Pathak, 1980; Satya-Prakash *et al.*, 1981). It may be that while not a primary event or involved in malignant transformation, gain of 1q aids tumour progression early on so that it can occur in benign as well as malignant breast disease by affecting growth control. This appears to be in agreement with the observations of somatic cell hybrids in which chromosome 1 correlated with negative growth control and/or cellular senescence (Benedict *et al.*, 1984; Stoler and Bouck, 1985; Bouck *et al.*, 1986; Sugawara *et al.*, 1990). This same argument has been recently made for rearrangements of 1p (Sreekantaiah and Sandberg, 1991) which have been observed in a variety of benign neoplasms, notably a deletion involving the distal chromosome 1p was observed in a non-malignant human mammary epithelial cell line (Nielsen and Briand, 1989).

All the aforementioned tumours were from previously untreated patients, but Pathak (1980) reported the karyotype of one pleural effusion from a patient previously treated with chemo- and radiation-therapy. The stemline chromosome number was 35, one of the lowest in human neoplasms, and contained a marker chromosome derived from a 1q translocation product. Zhang *et al.* (1989) also analyzed a breast tumour heavily-treated with radiation and chemotherapy to confirm that their culturing protocol did not eliminate karyotypically abnormal cells. The many gross chromosomal changes within this tumour were maintained.

Analysis of chromosome instability in the lymphocytes of breast cancer patients has suggested the specific involvement of chromosome 1 in constitutional abnormalities. Lymphocyte cultures of ten breast cancer patients and ten normal individuals as controls showed aberrant metaphases in both groups (7.36% and 3.76%, respectively) with a clustering of breakpoints on a small number of chromosomes including chromosome 1 at p22 and q32 (Barrios *et al.*, 1991). In a much larger study, lymphocyte cultures were prepared from 76 breast cancer patients, 68 predisposed family members, with 40 normal individuals and an additional 30 lung cancer patients as controls (Pathak *et al.*, 1991). There was a marked clustering of alterations on 1q (q11-22) in breast cancer patients and some family members. Alterations in 1q were observed in 1-3% of metaphases in each of ~20% of cases, and included translocations to other chromosomes; 1q deletions and pericentric inversions were also observed. Although 2/40 (5%) of normal controls also had 1q alterations in 1-3% of metaphases, none of the lung cancer controls showed any such changes. Pathak *et al.* (1991) concluded 1q rearrangements may be one of the primary lesions associated with the development of breast cancer.

A major difficulty in the study of many solid tumours, and notably breast tumours, is the determination of a progression of the specific changes required for tumourigenesis. Although certain genetic events are recognizable in many tumours, it is difficult to determine the order, if there is one, in which they occur. In colorectal carcinomas, however, it has been possible to establish a model for the progression of the disease from adenoma to carcinoma (Fearon and Vogelstein, 1990). The model suggests that mutations in at least four or five genes are required for the formation of a malignant tumour, while fewer changes suffice for benign tumourigenesis, these genetic alterations often occurring according to a preferred sequence but not in a strict order. However, it is the total accumulation of changes, rather than their strict order of occurrence with respect to each other, that is responsible for determining the tumour's biologic properties. Unlike colorectal cancer, a clear adenoma-to-carcinoma sequence has not been recognized for breast cancer, although the association of atypical hyperplastic lesions with an increased risk for breast cancer is suggestive in this respect (Dupont and Page, 1985).

1.1.4.2 Fluorescence *In Situ* Hybridization

The techniques of karyotype analysis and molecular genetics have yielded a vast amount of information about solid tumours, but there are limitations to both these techniques. Karyotype analysis of solid tumours can only reliably detect those changes that affect regions greater than 30Mb of DNA (Sandberg *et al.*, 1988), while the average size of a chromosomal band is 10Mb. Therefore, breakpoints mapped to the same region of the chromosome in several tumours may or may not be targeting the same sequences. The technique does not preserve tissue architecture since the cells are first disaggregated in order to produce metaphases of individual cells. As many as 100 metaphases are analyzed to obtain an accurate picture of chromosome rearrangements that have taken place and the clonal nature of each tumour, even though the original distribution of these cell types within the tumour has been lost. While the high sensitivity of molecular techniques allows the study of gene sequences, no information is obtained at the single-cell level, and heterogeneity within a population of cells is often difficult to detect. *In situ* hybridization can to some extent bridge the gap between the two techniques, and is widely used to visualize specific nucleic acid sequences in preparations of chromosomes, single cells or tissue sections. Initially, probes were labelled isotopically and detected post-hybridization by autoradiography. However, the advent of non-isotopic protocols (primarily in the form of

fluorescence *in situ* hybridization, FISH) has permitted application to a broad spectrum of research-based as well as clinical problems (Lichter and Ward, 1990).

In interphase nuclei, probes hybridizing to the blocks of highly-repetitive satellite sequences situated at the centromeres of chromosomes are detected as discrete spots (Pinkel *et al.*, 1986; Moyzis *et al.*, 1987; Devilee *et al.*, 1988; Greig *et al.*, 1989; van Dekken *et al.*, 1989). Therefore, these probes can be applied to the analysis of tumour nuclei without the need for high-quality metaphase spreads. The number of signals per nucleus can be determined and averaged in a tumour specimen by the study of a large number of nuclei, according to set criteria (Cremer *et al.*, 1988; Hopman *et al.*, 1988; Nederlof *et al.*, 1989; Dhingra *et al.*, 1992; Kim *et al.*, 1993). Polysomy of chromosome 1 (especially 1q) has been determined in interphase nuclei of breast tumours (Devilee *et al.*, 1988; Viegas-Péquignot *et al.*, 1989; Balazs *et al.*, 1991). These three studies used either cell line material or scrapings of cells directly from the fresh tumour that were fixed and dropped onto microscope slides. In a more recent study to characterize some of the marker chromosomes identified by traditional karyotyping from metaphase spreads (Dutrillaux *et al.*, 1990, 1991), the original slide specimens were re-used for FISH with satellite sequences as probes (Kokalj-Vokac *et al.*, 1993). Complex rearrangements detected karyotypically could be characterized on both metaphase spreads and interphase nuclei and their origins established. Other studies utilizing FISH to single-cell preparations of breast tumours have detected alterations at the sites of specific genes. Deletion of the D17S5 locus mapping to chromosome 17p13 was detected by FISH and confirmed the LOH events identified in these tumours by Southern analysis at this locus (Matsumura *et al.*, 1992). The data suggest that the dominant mechanism of allele loss at this site in breast cancer is a physical deletion from the chromosome (figure 1.1.b.i, iv, and v) rather than by alternative mechanisms for uncovering inactivated tumour-suppressor alleles, such as a second point mutation (1.1.b.vi), mitotic recombination (figure 1.1.b.iii) or non-disjunction, loss of the wild-type homologue and reduplication of the remaining homologue (figure 1.1.b.ii). Similar analysis of the RB1 gene in breast tumours by FISH has not yielded the same result; analysis of clinical breast cancer samples showed that most cells contained two copies of the RB1 gene, despite LOH indicated by Southern analysis (Kallioniemi, A. *et al.*, 1992). Although it was suggested by the authors that LOH must occur by a mechanism other than physical deletion, it must be noted that no mention was made of a control locus to determine DNA loading between the normal and tumour lanes, although laser scanning densitometry was carried out. Therefore, the allelic imbalances observed by Southern analysis may not have been strictly LOH. Amplification of proto-oncogenes has also been determined by

FISH. The ERBB2 gene sequences were shown to be amplified in touch preparations of uncultured breast tumours (Kallioniemi, O.P. *et al.*, 1992). The distribution of these amplified sequences was found to be clustered in the interphase nuclei studied suggesting that an intrachromosomal amplification had taken place. Amplification of chromosome 11q13 sequences has been studied in breast tumour cell lines (Roelofs *et al.*, 1993). The amplicons were all found to have occurred *in situ*, i.e. all were located on chromosome 11 or derivatives of this chromosome. Therefore a translocation event onto another chromosome or onto an episome (an extrachromosomal structure) did not initiate the progression of events required to produce the amplicon.

The success of interphase cytogenetics on single-cell preparations from a variety of tumours has also been repeated with routinely-processed paraffin sections of tissues. The reliability of interphase cytogenetics has been demonstrated with tissue sections from a variety of sources, including normal tissues (Burns *et al.*, 1986; Pringle *et al.*, 1987), but also tumours of the breast (Dhingra *et al.*, 1992), colon (Steiner *et al.*, 1993), lung (Kim *et al.*, 1993), bladder (Hopman *et al.*, 1991), as well as other urological tumours (van Dekken *et al.*, 1992), germ cell tumours (Emmerich *et al.*, 1989; Looijenga *et al.*, 1993) and melanomas (de Wit *et al.*, 1992).

Another technique applied to the study of tumours is flow cytometry (FCM), which measures two important parameters. First, FCM can determine the percentage of cells in the S-phase of the cell cycle to indicate the rate of tumour growth. Secondly, it can assess the degree of aneuploidy of the cells, which appears to correlate with malignant aggressive tumour behaviour (McGuire and Dressler, 1985; Owainati *et al.*, 1987). Therefore, in concert with FCM, FISH can provide a picture of the nature of specific chromosome rearrangements and the mechanisms by which these rearrangements have arisen. It is also possible to determine in each cell whether changes in copy number of a particular chromosome are specific to that chromosome or occur as a result of ploidy changes involving the entire genome. The overall effect of these alterations on tumour heterogeneity can also be investigated, to determine how adjacent sub-clones of tumour cells are related as well as how new sub-clones arise in the tumour. The arrangement of normal cells engulfed by the tumour spread can also be established. Investigation of heterogeneity is particularly applicable to tissue sections since histological preservation of tissue architecture is maintained.

1.2 The X Chromosome and Cancer

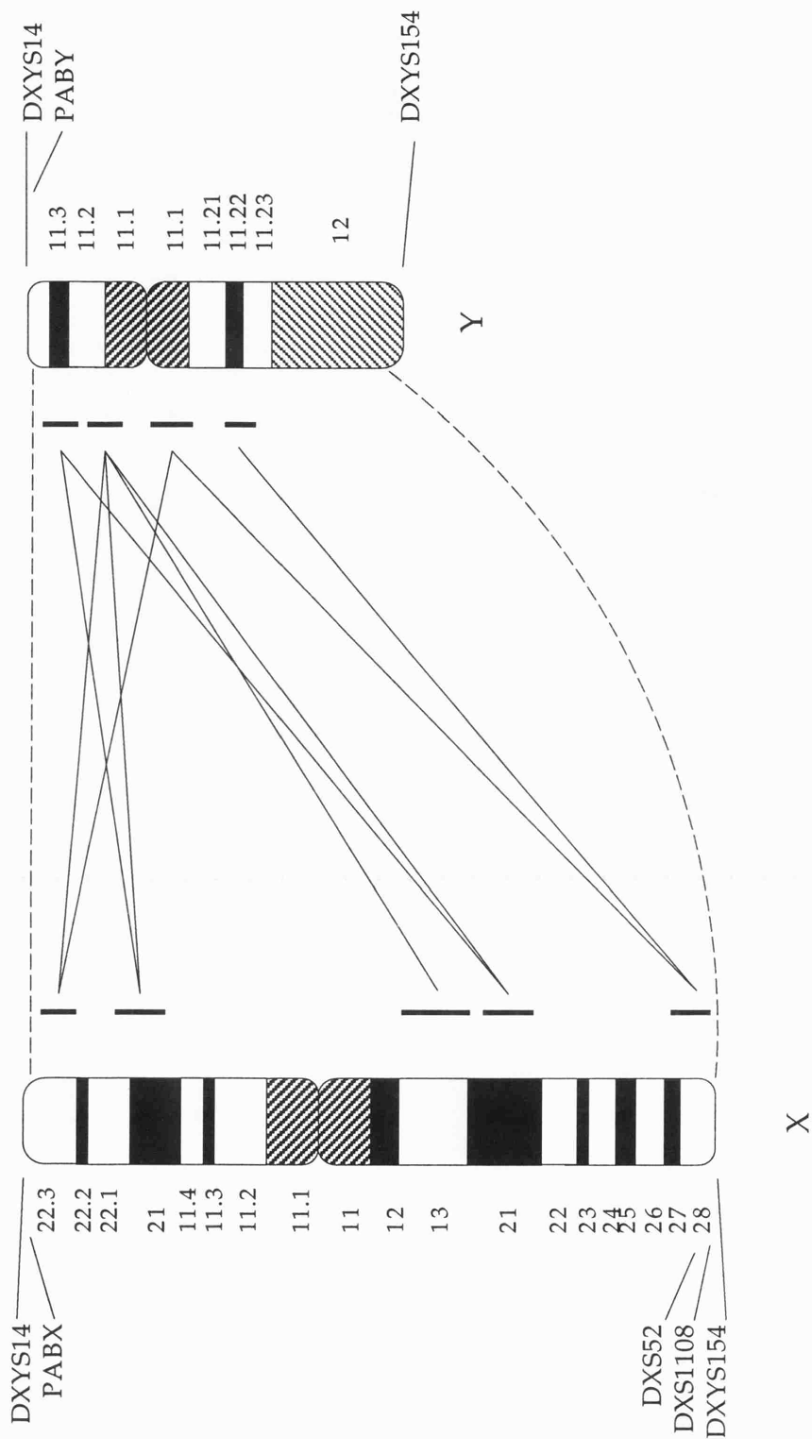
Tumour-suppressor genes encoded on the autosomes (chromosomes 1 to 22) are consistent with Knudson's two-hit hypothesis of inactivation in tumours (Knudson, 1971). However, the presence of a tumour-suppressor locus on either of the sex chromosomes, X and Y, raises difficulties with respect to gene dosage. In the female, one X chromosome is inactivated in all somatic cells to form a highly-condensed structure known as the Barr body or sex chromatin (Barr and Bertram, 1949; Ohno and Makino, 1961) by random selection of either homologue (Lyon, 1961) at an early stage in the developing embryo. In normal male cells, no such feature exists as both the X and Y chromosomes remain active. The mechanism of X-inactivation which renders genes transcriptionally silent is still unknown, but there is considerable data for the presence of an inactivating centre, XIC, situated on Xq13 from which the inactivation spreads out across the chromosome and is maintained by it (Lyon, 1993). There is also conflicting evidence for the involvement of DNA methylation at cytosine residues in the maintenance of X-inactivation (Mohandas *et al.*, 1981; Miller *et al.*, 1982). Reactivation of inactive X chromosomes has been reported following 5-azacytidine treatment to hypomethylate DNA in a mouse-human somatic cell hybrid clone (Mohandas *et al.*, 1981). However by using antibodies specific for 5-methylcytosine, Miller *et al.* (1982) found no detectable difference in the extent of methylation between the active and inactive X chromosomes in simian and human females. A strong candidate gene, XIST, has been cloned and found to be transcribed from the inactive X but not from the active copy (Brown *et al.*, 1991). The RNA transcript appears not to be translated but co-localizes with the Barr body within the nucleus suggesting a role as a structural RNA involved in rendering the second X chromosome inactive (Brown *et al.*, 1992).

Although non-homologous pairing of the autosomes may occur during pachytene of male meiosis, their pairing during zygotene is confined to homologous associations. Similarly, although the sex chromosomes extensively associate during pachytene along almost the entire length of the Y chromosome (Darlington *et al.*, 1934; Chandley *et al.*, 1984), later in zygotene pairing is restricted to a small region situated at the tips of the short arms of X and Y, in bands Xp22.3 and Yp11.3 respectively (Chandley *et al.*, 1984). Burgoyne (1982) proposed the 'X-Y crossover model' in which the specific pairing in zygotene is a result of genetic homology between the sex chromosomes where a single obligatory crossover event takes place. The segment of X which pairs with Y is protected from subsequent X-inactivation and genes distal to the crossover appear to be pseudoautosomally inherited, transmitted to both male and female offspring and

so termed pseudoautosomal genes. This model has now been confirmed by genetic analysis and the pseudoautosomal region (PAR) has been shown to be composed of strictly homologous sequences extending from the telomeres of Xp and Yp for ~2.6Mb (Brown, 1988; Rappold and Lehrach, 1988; Petit *et al.*, 1989) until the pseudoautosomal boundary, termed PABX and PABY for the X and Y chromosomes respectively. On the Y chromosome, PABY is characterized by an *Alu* repeat sequence that is not present on the X chromosome (Ellis *et al.*, 1989). The actual boundary is formed by abrupt sequence non-identity between the sex chromosomes and is not a specific genetic structure (Ellis and Goodfellow, 1989). A single obligatory X-Y crossover event within the PAR occurs during male meiosis and results in a gradient of sex linkage (Rouyer *et al.*, 1986), such that the MIC2 gene sequences situated close to the pseudoautosomal boundary segregate with sex in >90% of male meioses (Goodfellow *et al.*, 1986) while the most telomeric locus, DXYS14, shows no sex linkage with a frequency of recombination close to 50% (Cooke *et al.*, 1985; Simmler *et al.*, 1985; Rouyer *et al.*, 1986). A second pseudoautosomal region has been proposed, situated at the tips of the long arms of the sex chromosomes, in bands Xq28 and Yq12 respectively, closely linked to DXS52 on the X chromosome (Freije *et al.*, 1992). The sequence homology extends over 400kb with genetic exchange between the X and Y chromosomes demonstrated for the microsatellite locus DXYS154 within pedigrees from the Centre d'Etude du Polymorphisme Humain (CEPH) with recombination events between DXS1108 and DXYS154 identified (figure 1.6). This second PAR is consistent with the previous observation of pairing between the tips of Xq and Yq in ~50% of spermatocytes (Chandley *et al.*, 1984).

Several genes have been mapped to the primary PAR and there is now growing evidence to support part of the 'X-Y crossover' model (Burgoyne, 1982) as three of these have been shown to escape X-inactivation in the female (Goodfellow *et al.*, 1984; Ellison *et al.*, 1992a, 1992b; Schiebel *et al.*, 1993; Slim *et al.*, 1993; Smith *et al.*, 1993). The second PAR is not characterized by any expressed sequences as yet (Freije *et al.*, 1992). A number of genes within the sex-specific sequences of the X chromosome have also been found to escape X-inactivation and are interspersed among genes known to be X-inactivated at variable locations along the sex chromosomes (Ellis and Goodfellow, 1989; Brown and Willard, 1990; Wang *et al.*, 1992). Anonymous DNA sequences have also been reported to be shared by the sex chromosomes, leading to the identification of large regions of cross-homology between X and Y which are broken up by inversions and translocations (figure 1.6).

Figure 1.6: Summary of the pattern of homologies between the human X and Y chromosomes, after Lambson *et al.* (1992). The pseudoautosomal region and the secondary pseudoautosomal regions are indicated by the broken lines between the tips of the p and q arms of the two chromosomes. Additional regions of homology were identified during the study of polymorphisms ranging along the chromosomes. Loci involved in defining the two pseudoautosomal regions are listed in order alongside each chromosome.



Thus, it is conceivable that tumour-suppressor loci conforming to Knudson's model (Knudson, 1971) could exist on the sex chromosomes, most likely as pseudoautosomal loci but also possibly as X-linked loci that escape X-inactivation with active Y-encoded homologues.

The X chromosome has been implicated in many genetic diseases and disorders, for which an increasing number of the genes responsible are being isolated, but cancer studies have not focused much attention on this chromosome. There is no evidence that the few X-located genes identified with homology to proto-oncogene families have oncogenic potential (Miyoshi *et al.*, 1984; Huebner *et al.*, 1986; Rao *et al.*, 1989; Vetrie *et al.*, 1993). However, the human oncogene, DBL, isolated from a diffuse B-cell lymphoma (Eva and Aaronson, 1985), is an exception. The DBL proto-oncogene, situated at Xq26-q27.2, is activated by undergoing recombination at its 5' end with sequences mapped to chromosome 3p13-qter and at its 3' end with sequences at chromosome 16p13-q22. Another oncogene, MCF2, isolated from the human mammary carcinoma cell line MCF-7, also contains sequences derived from Xq27 (Fasano *et al.*, 1984). MCF2 is generated by translocation of chromosome 3 sequences onto the 5' end of the X27 gene. Comparison of restriction maps (Noguchi *et al.*, 1987; Eva *et al.*, 1988) and the sequences of DBL and MCF2 (Noguchi *et al.*, 1988; Ron *et al.*, 1988) shows that they are derived from the same gene on the X chromosome (Tronick *et al.*, 1989). The sequences diverge 5' of the point of recombination with chromosome 3 sequences, suggesting that different sequences are targeted for recombination on chromosome 3 to produce the two activated oncogenes.

The X chromosome is rarely mentioned in cytogenetic studies of tumours compared to other chromosomes, although losses and gains of sex chromosomes are characteristic of many tumours (Sandberg, 1983) and sex chromosome loss has also been demonstrated in the leukocytes of normal individuals, increasing with age (Jacobs *et al.*, 1963, 1964; Sandberg *et al.*, 1967). The X chromosome is involved in a consistent translocation in synovial sarcomas, t(X;18)(p11.2;q11.2). This is regarded as a primary alteration (Turc-Carel *et al.*, 1987) and the breakpoint maps within the OATL1 gene cluster on the X chromosome (de Leeuw *et al.*, 1993). Although the gene(s) involved have not yet been identified, a yeast artificial chromosome (YAC) spanning the breakpoint has been isolated (de Leeuw *et al.*, 1993). There is evidence that Xp11.2 is subject to a recurring breakpoint (Tomlinson *et al.*, 1991), since translocations involving this band and a number of other chromosomes including chromosome 1, have been reported (Kajii *et al.*, 1985; de Jong *et al.*, 1986; Jenkyn and McCartney, 1986; Castedo *et al.*, 1989; de Bustamante *et al.*, 1990; Chen, Z. *et al.*, 1992; Meloni *et al.*, 1992, 1993). Whether

the translocation involves the active or inactive X chromosome in these cases is unknown.

Reactivation of inactive X chromosomes has been reported in a mouse-human somatic cell hybrid clone through selection for the HPRT gene following 5-azacytidine treatment to hypomethylate DNA (Mohandas *et al.*, 1981). Whilst no observation of such a reactivation event has been made in human tumour cells, reports have suggested that reactivation of the inactive X may occur in some tumours to account for the presence of only active X chromosomes (Straub *et al.*, 1969; Ghosh *et al.*, 1979; Camargo and Wang, 1980). However, additional studies indicate that reactivation of the inactive X may not be the cause of the presence of only active X chromosomes in tumour cells, but rather that loss of the inactive X and duplication of the active X take place (Wang *et al.*, 1990).

Prior to the implementation of reliable karyotyping, the nature of sex chromatin of tumour cells was extensively studied. A number of neoplasms originating in human females have significantly lower incidence of SCB (sex-chromatin body) positive cells than in their respective normal female tissues (Sandberg, 1983). In breast cancer, an increased frequency of polyploidy was found to be associated with a lower SCB count and cancers that were more undifferentiated were aneuploid and had a lower SCB frequency (Kallenberger *et al.*, 1968). Further studies have shown that although tumour grade appears not to be related to SCB frequency, there is a positive correlation between an SCB frequency close to that of normal female tissue and disease-free survival (Savino and Koss, 1971; Ghosh and Shah, 1975; Kirucuta and Olinici, 1975). However, the SCB frequency in normal female tissues is controlled by many factors, one of which is variation in the oestrogen levels during the menstrual cycle (Schmidt *et al.*, 1966). Significant loss of SCB in oestrogen-treated HeLa cells compared to controls suggests a possible reactivation of the inactive X by the steroid (Ghosh *et al.*, 1979). Therefore SCB incidence may have some still-undefined association with oestrogen receptor status in tumours (Ghosh and Shah, 1981).

Congenital conditions accompanied by loss or gain of one or more X chromosome(s), such as Turner syndrome (XO females), Klinefelter syndrome (XXY males) and triple X syndrome (XXX females), true hermaphrodites (XX males), and their accompanying mosaic variations, may be associated with a unique or increased incidence of neoplasia. Most of these syndromes are characterized by abnormal gonadal anatomy and function, though they may be accompanied by other phenotypic abnormalities unrelated to the sex organs. Neoplasia in these subjects tends to involve the gonads and related organs.

However, it is not certain whether the development of such neoplasia is the result of genetic factors primarily or of a radically modified hormonal environment resulting from the abnormal gonadal development and physiology (Sandberg, 1983). There are no data to indicate any apparent increased frequency over the normal population of tumours in individuals with Turner syndrome, triple X syndrome or true hermaphrodites. This is also true for the incidence of leukemia, lymphoma and extragonadal (mediastinal) germ cell tumours in Klinefelter males. It has been suggested that Klinefelter patients have an increased incidence of breast cancer over the male population (Bauer and Erickson, 1955) with a rate approaching that in normal females (Jackson *et al.*, 1965); the normal ratio of male to female breast cancer being 1:100. Other reports of breast cancer in Klinefelter syndrome and its variants have also been made, totalling 27 individuals (summarized by Evans and Crichlow, 1987), including a report of a family study of one Klinefelter patient with breast cancer which showed a high incidence of cancers among his first degree relatives, notably a sister who developed unilateral breast cancer at 41 years of age (Lynch *et al.*, 1974). Despite the claim of Jackson *et al.* (1965), the data are not substantial enough to draw firm conclusions on the incidence of breast cancer in Klinefelter males compared to normal females. However, that said, statistical analysis of reported cases does suggest Klinefelter males may have an increased risk, but only of ~3% (Evans and Crichlow, 1987), compared to ~9% for normal females and ~0.1% in normal males.

Cultured cells of patients with various forms of gonadal dysgenesis, including X chromosome abnormalities, have an increased susceptibility to transformation by SV40 virus (Mukerjee *et al.*, 1970; Lynch *et al.*, 1974) and to the effects of X-irradiation (Sandberg, 1983). Whether this is a reflection of an actually increased tendency toward malignant transformation *in vivo* has not been established.

The X chromosome is not implicated as playing a role in malignant transformation or progression from experiments with a human renal cell carcinoma cell line, YCR. Transfer of the X chromosome by microcell into YCR cells had no effect on their tumourigenicity or tumour-growth rate in nude mice (Shimizu *et al.*, 1990). The long arm of the X chromosome has been used as a carrier since it encodes the HPRT gene for use as a selectable marker to introduce portions of chromosomes into cell lines as translocation products, e.g. chromosome 1q to regionalize a locus involved in cellular senescence (Sugawara *et al.*, 1990; see above). There is no description of introducing intact copies of the X chromosome into other cell lines, so that the X chromosome has not been

studied in detail in a variety of cell lines and the lack of functional suppression in YCR cells may be due to tissue specificity.

The X chromosome has not been associated with tumourigenesis through the study of loss of heterozygosity in human tumours (Seizinger *et al.*, 1991). Allelotype studies of tumours of the colon (Sasaki *et al.*, 1989; Vogelstein *et al.*, 1989), lung (Tsuchiya *et al.*, 1992), breast (Sato *et al.*, 1990) and ovary (Sato *et al.*, 1991b) do not include polymorphic markers mapping to the sex chromosomes. However, two allelotype studies of breast cancer have used probes mapping to the X chromosome. In the first, 52 breast carcinomas were assessed for LOH on all chromosomes with LOH on chromosome Xq found to be 25% (n=20) (Larsson *et al.*, 1990). In the second study, 60 breast tumours were screened with polymorphic loci from all chromosomes and LOH ranged from 16% on Xp to 9% on Xq (n=44) (Devilee *et al.*, 1991d). A recent allelotype of 37 ovarian tumours used a single locus from the X chromosome mapping to the pseudoautosomal region and determined LOH as 28% (n=29) (Cliby *et al.*, 1993).

Allele loss at various polymorphic markers on the X chromosome has been described in a number of other reports for melanoma, germ cell tumours and a common region of deletion identified in a study of ovarian tumours. Of the six cell lines derived from six independent metastases from a melanoma patient, all showed the same loss of alleles of chromosome X markers (Dracopoli *et al.*, 1987). Southern analysis of polymorphic markers on the sex chromosomes in 31 testicular germ cell tumours showed that changes in the sex chromosomes occurred in a substantial proportion of the tumours (Peltomäki *et al.*, 1991). Specifically, loss of Y chromosome loci occurred in 39% of the patients, usually involving the entire chromosome, with a concomitant gain of X chromosomal dosage in two of these patients; LOH in the pseudoautosomal region was found in 26% of tumours, two of these showing no changes outside the region. After extensive analysis of the X chromosome had commenced in this project, LOH on X was reported in a panel of ovarian cancer patients at a rate of 60% (n=15) with a common region of deletion established using a number of sex-specific probes on Xp between DXS7 and DXS84 in the region Xp21.1-p11.4 (Yang-Feng *et al.*, 1992). Finally, during routine analysis of CEPH pedigree DNAs with a number of minisatellites, two pedigrees showed abnormal transmission of alleles from grandparents to grandchildren, with the apparent absence of the alleles in the mother (Royle *et al.*, 1993). The reduction to hemi- or homozygosity (i.e. LOH) of the distal chromosome 19q region occurred in one, while one entire copy of the X chromosome was thought to have been lost in the second case. These losses of chromosome material probably occurred when the lymphoblastoid cell line from

the mothers were established in culture and suggest that loss of these regions of the genome may have conferred a growth advantage on the cells.

Thus, there is little indication in the literature that the X chromosome is likely to harbour tumour-suppressor genes. However, this does not appear to be due to studies failing to find evidence of tumour-suppressor function, but instead due to studies in general failing to assess sequences on the X chromosome for such a function; the suppression studies involving X chromosome sequences are by no means definitive.

1.3 Aims of the Project

Before the beginning of the project, there had been preliminary reports of LOH on chromosome 1 in breast tumours (Chen *et al.*, 1989; Genuardi *et al.*, 1989a, 1989b; Merlo *et al.*, 1989; Bièche *et al.*, 1990; Gendler *et al.*, 1990). These suggested there were at least two targets of LOH on this chromosome in breast tumours: a defined 26cM smallest region of overlap (SRO) in the mid-region of 1q (Chen *et al.*, 1989) and three other regions of high LOH, namely proximal 1q (Merlo *et al.*, 1989; Gendler *et al.*, 1990), the tip of the short arm (Genuardi *et al.*, 1989a, 1989b) and mid-region of 1p (Bièche *et al.*, 1990). Significantly, all these reports described only LOH i.e. deletions, while the extensive cytogenetic data available at that time had already established that chromosome 1 was often subject to increases in copy number, notably involving the long arm (Jones Cruciger *et al.*, 1976; Kovacs, 1978; Rodgers *et al.*, 1984; Gebhart *et al.*, 1986; Gerbault-Seureau *et al.*, 1987; Hill *et al.*, 1987). The tumour-suppressing capabilities of chromosome 1 in a number of different cell types suggested that there were one or more tumour-suppressor loci present on the chromosome (Benedict *et al.*, 1984; Stoler and Bouck, 1985; Bouck *et al.*, 1986; Kugoh *et al.*, 1990; Oshimura *et al.*, 1990; Sugawara *et al.*, 1990; Yamada *et al.*, 1990a, 1990b), and these loci could be the target(s) for LOH.

Therefore the initial and main aim of the project was to confirm the number of regions on chromosome 1 targeted for deletion in breast tumours and to also attempt to map the limits of each independent region of deletion. Since Chen *et al.* (1989) had already identified an SRO by deletion mapping in a small number of breast tumours, my aim was to try to reduce the size of this region in particular. By employing controls, it was hoped to distinguish between the loss (deletion) and gain (increased copy number) of sequences on chromosome 1 so that the 'loss of heterozygosity' studies, or more accurately, the allelic imbalance

studies, would more closely reflect the karyotype data available for breast tumours in the literature.

During the project, the focus was altered in two new ways. After LOH was found on the X chromosome, it was decided that the project should also try to characterize the alterations taking place on this chromosome, in particular mapping the deletions extending into the sex-specific sequences of Xp. Since there was no previously published LOH data on the X chromosome, the frequency of loss in the pseudoautosomal region was particularly important to determine if this was non-random and therefore of possible importance in breast tumourigenesis. Thus, data would be generated for experiments to isolate candidate sequences for functional studies. Once it became clear that chromosome 1 was not undergoing simple deletions, unlike the X chromosome, and therefore unlikely to yield strong candidate regions for harbouring tumour-suppressor loci, an attempt was made to confirm the copy number changes found for chromosome 1 in some patients. Fluorescence *in situ* hybridization of satellite DNA to sections of formalin-fixed paraffin-embedded tumour and normal breast tissue was developed to count the number of signals representing chromosome 1q12 in the nuclei for comparison with the Southern hybridization data.

1.4 Strategy

The initial step was to establish a panel of female breast cancer patients with a breast tumour specimen and blood sample held in the laboratory. For each of these patients, DNA could then be extracted from the tumour and lymphocytes so that the genotype of the tumour could be compared to that of the normal constitutional DNA of the patient, of which lymphocyte DNA was the easiest to obtain. The size of the panel was important in order to generate results that would stand up to statistical analysis and could be representative of breast cancer patients in general. It was also important to have a representative panel in that patients were not pre-selected with regard to any clinical parameter and three largely consecutive groups of patients were obtained for study from two different clinicians, Dr. Rosemary Walker, Department of Pathology, University of Leicester and Dr. Anthony Howell, Department of Medical Oncology, Christie Hospital, Manchester.

In addition to the panel, a bank of probes was established recognizing polymorphic loci on chromosome 1, primarily to the long arm. Of the loci presently mapped to chromosome 1, only a subfraction have been assessed for LOH, some of which have been repeatedly assessed (figure 1.5). Loci studied include the classic RFLPs as well as VNTRs. Since VNTRs generate more data due to their high levels of polymorphism, it was decided to concentrate on the analysis of VNTRs where possible and for comparison some loci used by other groups were included. One of the minisatellites used was subsequently re-assigned to the pseudoautosomal region of the sex chromosomes. This is a region of almost perfect sequence homology situated at the tips of the short arms of both the X and Y chromosomes. The level of LOH at this locus prompted a larger study of the X chromosome in the panel; polymorphic loci mapping to the sex-specific region of the Y chromosome were not subjected to analysis since the panel consisted entirely of female patients.

In addition to polymorphic markers mapping to chromosomes 1 and X, five other polymorphic loci were studied; the minisatellite situated in the sixteenth intron of the RB1 gene on chromosome 13q14; the minisatellite at D17S5 localized to chromosome 17p13; the minisatellite at D2S44 mapping to chromosome 2p; the minisatellite at D7S21 on chromosome 7p; and the minisatellite at the locus D20S26 on chromosome 20q. The use of RB1 VNTR and D17S5 would also help determine if there was any unforeseen bias in the patients picked for study; if the level of LOH at these loci was consistent with the levels described in the literature, then bias was unlikely. Also, it would be possible to identify any correlations between the incidence of LOH at 1p, 1q, Xp, Xq, RB1 and D17S5. D2S44, D7S21 and D20S26 were used as control loci to establish the nature of imbalance observed in the patients. Chromosome 2 and also chromosome 20 rarely undergo structural or numerical rearrangements in breast tumours, except as background events when the tumour cells undergo ploidy changes and, furthermore, alteration to neither chromosome is associated with a common solid tumour (Mitelman *et al.*, 1991). LOH has not been reported at significant levels for loci on either chromosome in allelotypes and are not regarded as sites of LOH (Seizinger *et al.*, 1991). While there has been some interest in LOH on the long arm of chromosome 7 in breast tumours (Bièche *et al.*, 1992), this has been largely refuted by others (Ali *et al.*, 1987; Lundberg *et al.*, 1987; Larsson *et al.*, 1990; Sato *et al.*, 1990; Devilee *et al.*, 1991d; Cooke *et al.*, 1993) and the chromosome is not regarded as a site of significant LOH (Seizinger *et al.*, 1991). Low levels of imbalance were detected in the panel with D2S44, D7S21 and D20S26, so they were used to complement each other in the determination of the

nature of AI on the chromosomes of interest, although D2S44 proved the most useful.

The results of the Southern analysis of the panel showed that chromosome 1 was subject to an array of complex rearrangements, consistent with cytogenetic data published for other breast tumours. In contrast, simple deletions predominated on the X chromosome, often too small to be detectable by the karyotype analysis carried out on other breast tumours. To try to obtain a clearer picture of the events involving chromosome 1 that occur in tumours from the panel, some form of cytogenetic data was required. Since karyotyping of the tumour specimens was not possible, it was decided to use fluorescence *in situ* hybridization. Formalin-fixed paraffin-embedded sections of tumours from nine members of the panel were obtained from Dr. Walker for FISH using a satellite III probe mapping to 1q12, D1Z1, and an α -satellite probe mapping to the centromere of chromosome 2, D2Z1. A dual hybridization and detection protocol was developed on lymphocyte splash preparations from normal individuals and subsequently adapted for tissue sections. The technique employed biotinylated probes, detected with the fluorochrome fluorescein isothiocyanate (FITC) and digoxigenylated probes, detected by the fluorochrome tetramethyl rhodamine isothiocyanate (TRITC). Therefore, the copy number (somy) of chromosome 1 could be directly compared with that of a (control) chromosome rarely involved in rearrangements in the tumour cells. Unfortunately, although the dual hybridization technique was established, the probe for D2Z1 could not be reliably applied to breast tissue sections in conjunction with the probe for D1Z1. Thus single probe hybridizations of D1Z1 were carried out on sections of the nine tumours as well as on sections of normal breast tissue (serving as a positive control). The results closely mirrored expectations from the Southern hybridization data available for each of the tumours analyzed when those of the normal tissue sections were incorporated.

Once all the data had been gathered for AI on chromosomes 1 and X, statistical tests were employed to test for associations between the findings and the clinico-pathological data available for the patients. All possible combinations of the datasets were analyzed by the chi-square test and its mathematical corrections were applied as necessary. Exact probability (Fisher-Urwin test) was subsequently calculated to confirm the findings for those few cases showing association as well as the more frequent borderline cases. However, in the majority of cases, the two datasets under test of association were found to be independent of one another.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

General laboratory chemicals were supplied by Fisons (Loughborough), BDH (Poole), Serva (*via* Universal Biologicals, London) or Sigma (Poole) unless otherwise stated and were of analytical grade or equivalent. Bacterial culture media were obtained from Difco (East Molesley) and animal cell culture media from Gibco BRL (Paisley). Radiolabelled nucleotides were supplied by Amersham International (Amersham). Millipore Super-Q system (Millipore Corporation, Bedford) treated water was used for all solutions.

Calcium chloride (1M): 21.9g of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (BDH, for transformation of competent cells only) were dissolved in 100ml of Q water, sterilized by autoclaving and frozen in 10ml aliquots at -20°C .

DAPI (10mg/ml): 10mg of 4',6-diamino-2-phenylindole (Gibco BRL) were dissolved in 1ml PBS and stored in an opaque container at 4°C .

Denhardt's solution (100x): 2% (w/v) each of Ficoll 400 (Pharmacia), polyvinylpyrrolidone (Sigma) and BSA (fraction V, Sigma) were dissolved in Q water. The solution was divided into 20 ml aliquots and stored at -20°C .

Dextran sulphate (40%): dextran sulphate (Pharmacia) was dissolved at 40% in 100ml Q water; autoclaved for five minutes only and stored at 4°C .

dNTP solutions (0.1M): powdered dNTPs (Pharmacia) were dissolved in the specified volume of TE and stored at -20°C : 10 μg of dATP, 186.9 μl ; 5 μg of dTTP, 103.7 μl ; 5 μg of dGTP, 98.6 μl . The TE used was specifically for random-primed oligonucleotide synthesis of DNA probes: 3mM Tris-HCl (pH 7.0), 0.2mM EDTA (pH 8.0)

EDTA (0.5M, pH 8.0): 186.1g of EDTA (Fisons) were dissolved in 1l of Q water adjusted to pH to 8.0 with NaOH pellets and 10M NaOH. Following sterilization by autoclaving, the solution was stored at room temperature.

Ethidium bromide (10mg and 5mg/ml): ethidium bromide (Serva) was dissolved at the specified concentration in Q water and stored in opaque plastic bottles at room temperature.

Ethanol (100%, 80%, 70%): ethanol (Fisons) was diluted as required with Q water and stored at room temperature.

Fixative (3:1): three volumes of methanol (Fisons) were mixed with one volume of glacial acetic acid (Fisons) and kept on ice. The solution was freshly-prepared and not kept.

Glycerol (45%): glycerol (Fisons) was dissolved at a concentration of 45% (w/v) in Q water and filter sterilized. The solution was stored at room temperature.

HEPES (2M, pH 6.6): 9.53g of HEPES (Sigma) were dissolved in a final volume of 20ml Q water after adjusting the pH to 6.6 with 5M NaOH. After filter sterilization, the solution was stored at 4°C.

Hydrochloric acid (0.25M): 21.55ml of concentrated HCl (Fisons) were added to 978.45ml of Q water. The solution was stored at room temperature.

Magnesium chloride (1M): 20.3g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Fisons) were dissolved in 100ml of Q water, sterilized by autoclaving and stored at room temperature.

P buffer: 50mM Tris-HCl (pH 7.5), 5mM EDTA (pH 8.0); sterilized by autoclaving and stored at room temperature.

Paraformaldehyde fixative (4%): 100ml PBS were heated to boiling point and 4g paraformaldehyde (Sigma) added. The solution was freshly made just before use, allowed to cool to room temperature and not kept.

PBS: Dulbecco's modified PBS (without Mg^{2+} or Ca^{2+}) was prepared by dissolving one tablet (ICN Flow) in 100ml of Q water. Following sterilization by autoclaving, the solution was stored at room temperature.

PBS-glycine: 0.2g glycine (Sigma) were added to 100ml PBS. The solution was autoclaved and stored at room temperature.

PCI: 50% (v/v) phenol (liquified, containing 0.1% (w/v) 8-hydroxy-quinoline and equilibrated against 100mM Tris-HCl, pH 7.6, Fisons), 48% (v/v) chloroform (Fisons), 2% (v/v) isoamyl alcohol (Fisons); equilibrated against 10mM Tris-HCl (pH 8.0) and kept in the dark at 4°C.

Potassium chloride (1M): 7.46g of KCl (Fisons) were dissolved in 100ml of Q water, sterilized by autoclaving and stored at room temperature.

Preincubation buffer: 4x SSC (pH 7.0), 0.05% (w/v) Tween-20, 5% (w/v) Marvel nonfat dried skimmed milk (Sainsbury's); freshly made just before use and not kept.

Propidium iodide (2mg/ml): propidium iodide (Calbiochem) was dissolved at the specified concentration in PBS and stored in opaque plastic bottles at 4°C.

SE buffer: 150mM NaCl, 100mM EDTA (pH 8.0). The solution was autoclaved and stored at room temperature.

SDS (10%): 50g of SDS (Calbiochem) were dissolved in 500ml of Q water and stored at room temperature.

Sodium acetate, (3M, pH 5.6): 40.8g of $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ (Fisons) were dissolved in 100ml of Q water, after adjusting the pH to 5.6 with glacial acetic acid. The solution was sterilized by autoclaving and stored at room temperature.

Sodium acetate, (0.2M, pH 7.0): 2.72g of $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ were dissolved in a final volume of 100ml of Q water after the pH had been adjusted to 7.0. Following sterilization by autoclaving, the solution was stored at room temperature.

(tri)-Sodium citrate (1M): 29.41g of $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ (Fisons) were dissolved in 100ml of Q water, sterilized by autoclaving and stored at room temperature.

Sodium chloride (4M): 23.4g of NaCl (Fisons) were dissolved in 100ml of Q water and sterilized by autoclaving. The solution was then stored at room temperature.

Sodium hydrogen phosphate (0.5M): 89g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (Fisons) were dissolved in 1l of Q water. After sterilizing by autoclaving, the solution was stored at room temperature.

Sodium hydroxide (10M, 5M): 200g or 100g of NaOH (Fisons) were added slowly to 400ml of Q water. After adjusting the volume to 500ml, the solutions were autoclaved and stored at room temperature.

Southern denaturation solution: 0.5M NaOH, 1.5M NaCl; stored at room temperature.

Southern neutralization solution: 0.5M Tris-HCl (pH 7.4), 3M NaCl; stored at room temperature.

SSC (20x): 3M NaCl, 0.3M tri-sodium citrate; pH was adjusted to 7.0 with NaOH, autoclaved and stored at room temperature.

TAE (10x): 48.4g of Trizma base (Sigma) and 20ml of 0.5M EDTA (pH 8.0) were dissolved in 1l of Q water after the pH had been adjusted to 7.5 with glacial acetic acid.

TE (1x): 10mM Tris-HCl (pH 7.2), 1mM EDTA (pH 8); autoclaved and stored at room temperature.

TNB: 0.1M tris-HCl pH 7.5, 0.15M NaCl, 0.5% Blocking Reagent (Boehringer); freshly made, pre-warmed to 37°C and not kept.

TNE (10x): 500mM Tris-HCl (pH 7.5), 1M NaCl, 50mM EDTA (pH 8.0); autoclaved and stored at room temperature.

TNT: 0.1M tris-HCl pH 7.5, 0.15M NaCl, 0.05% Tween-20; freshly made and not kept.

Tris-HCl (1M): 121.1g of Trizma base (Sigma) were dissolved in 1l of Q water after adjusting the pH as required with concentrated HCl. Following sterilization by autoclaving, the solution was stored at room temperature.

Tween-20 (10%): 10g (w/v) Tween-20 (BDH) were dissolved in 100ml Q water, filter sterilized and stored at room temperature.

2.1.2 Enzymes, Proteins and Antibodies

Restriction endonucleases were purchased from Gibco BRL, New England Biolabs (*via* CP Laboratories, Bishop's Stortford) or Pharmacia P-L Biochemicals (Milwaukee). DNA polymerase I (Klenow fragment) was supplied by Amersham. *Taq* DNA polymerase was obtained from Amersham or Northumbria Biologicals Limited (Northumbria). Proteinase K was purchased from Boehringer Mannheim (Lewes). Spermidine, pronase E and RNase A (pancreatic RNase) were obtained from Sigma. DNaseI was supplied by Pharmacia. BSA (enzyme grade) was obtained from Gibco BRL. DNase-free RNase A was prepared by the methods described in Sambrook *et al.* (1989). FITC-avidin DN and biotinylated-anti-avidin D were obtained from Vector Laboratories (Peterborough), mouse anti-digoxigenin antibody from Boehringer and the rabbit anti-mouse antibody-TRITC conjugate and goat anti-rabbit antibody-TRITC conjugate came from Sigma.

2.1.3 Bacterial Strains and Culture Conditions

Two strains of *Escherichia coli* K12 were used:

- a) JM83 *recA*⁻: F⁻, *ara*, Δ (*lac-proAB*), *rpsL*, ϕ 80*dlacZ* Δ M15, *recA1*.
- b) DH5 α : F⁻, ϕ 80*dlacZ* Δ M15, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (*rK*⁻, *mK*⁺), *supE44*, *relA1*, *deoR*, Δ (*lacZYA-argF*)U169.

Bacteria were grown using the following media:

- a) Luria agar (solid media): 1% (w/v) tryptone; 0.5% (w/v) yeast extract; 0.5% (w/v) NaCl; 1.5% (w/v) agar.
- b) Luria broth (liquid media): 1% (w/v) tryptone (w/v); 0.5% (w/v) yeast extract (w/v); 0.5% (w/v) NaCl (w/v).
- c) 2x YT broth (liquid media): 1.6% (w/v) tryptone; 1% (w/v) yeast extract; 0.5% (w/v) NaCl.

2.1.4 Tissue Samples

Samples of normal human placentae were obtained from the maternity unit at the Leicester Royal Infirmary. Small pieces (1cm³) were cut from a freshly-delivered placenta (less than 30 minutes post-partum) and placed in liquid nitrogen before long-term storage at -70°C.

Primary human breast carcinoma samples from female patients were frozen in liquid nitrogen immediately after surgical resection, and subsequently stored at -70°C. A sample of 16-20ml of whole blood in EDTA was obtained from each of the breast cancer patients and stored at -20°C before transfer to -70°C. 5µm tissue sections were cut from formalin-fixed paraffin-embedded breast tumours and adhered to saline-treated microscope slides. These specimens were supplied by Dr. Rosemary Walker, Department of Pathology, University of Leicester. Extensive clinical data on each of the patients was also provided Rosemary Walker.

2.1.5 Deoxyribonucleic Acids

Aliquots of tumour and lymphocyte DNA from 15 female breast cancer patients were supplied by Dr. John Armour, Department of Genetics, University of Leicester. Of these, 11 originally came from Rosemary Walker and the remaining four from the Royal Marsden Hospital, London. An additional 20 paired DNA samples from female breast cancer patients were provided by Dr. Anthony Howell, Department of Medical Oncology, Christie Hospital, Manchester. Clinical data on these patients was also available.

DNA probes were obtained from a variety of sources for Southern hybridization (see table 2.1) and fluorescence *in situ* hybridization (see table 2.2).

2.2 Methods of Sterilization

Autoclaving: Autoclaves used to sterilize media and disposable plastic-ware were as follows: a Cabburn 8cu.ft. capacity autoclave (Cabburn Sterilisers, Shoeburyness) set to attain a temperature of 121°C for 30 minutes; a model ST19 portable electric autoclave (Dixon's surgical instruments, Wickford) set to attain a temperature of 121°C for 20 minutes.

Dry sterilization: A B&T "Unitemp" sterilizing cabinet (Laboratory thermal equipment, Oldham) was used to sterilize glass-ware. The cabinet was set to attain a temperature of 160°C for six hours.

Filter sterilization: Small volumes (up to 50ml) were filter sterilized by passing through Acrodiscs (Gelman Sciences, Ann Arbor) with a pore size of 0.2µm.

Larger volumes (50-500ml) were filter sterilized using Nalgene 0.2µm vacuum filter sterilizing units (Nalgene, Rochester, New York).

2.3 Experimental Protocols

2.3.1 Preparation of Genomic DNA

Purification of Genomic DNA from Human Tissues

Two methods were used to extract genomic DNA from human tissues: standard phenol-extraction based protocols and Qiagen-based protocols. Subsequent to purification by both methods, the quantity and quality of the DNA was checked by spectrophotometric analysis and agarose gel electrophoresis respectively.

Standard Procedures

a) From human placentae and breast tumours. Pieces of tissue (<1cm³) were powdered in a Mikro Dismembrator II (FT Scientific Instruments, Tewksbury) at liquid nitrogen temperature in a Class II microbiological safety cabinet. The powder was then added to 5-20ml of SE buffer plus $\frac{1}{10}$ volume of 10% SDS and $\frac{1}{40}$ volume of proteinase K (20mg/ml) in Corex tubes (DuPont Scientific Instruments, Delaware), mixed gently and incubated overnight at 50°C. $\frac{1}{2}$ volume of PCI was added and after mixing gently, the emulsion was spun at 10,000 rpm in an HB-4 rotor (DuPont) within a Sorval RC-5B centrifuge (DuPont) for two minutes at 4°C. The aqueous phase was transferred to a fresh Corex tube and the phenol was re-extracted with $\frac{1}{4}$ volume of SE buffer. Both aqueous phases were pooled and dialyzed overnight at 4°C in 1x TE. The volume of solution was reduced on a bed of PEG 6000 flakes (Serva) to 2-5ml and transferred to a Corex tube. 4M NaCl was added to a final concentration of 10mM NaCl as well as $\frac{1}{100}$ volume 10mg/ml pancreatic RNase A (DNase-free), and the solution incubated at 37°C for 30 minutes. $\frac{1}{10}$ volume 10% SDS, $\frac{1}{10}$ volume 10x TNE, $\frac{1}{200}$ volume 20mg/ml pronase E were added and the tube incubated at 37°C for 30 minutes. Phenol extraction was repeated as before using PCI and the aqueous phase obtained was dialyzed for 24 hours at 4°C in 1x TE and then overnight in Q water only at 4°C. The DNA solution was reduced in volume on a bed of PEG 6000 as before to 1-3ml, transferred to one or more 1.5ml microfuge tubes and stored at -20°C.

b) From whole blood. 8-10ml frozen whole blood were thawed out at room temperature in a Class II microbiological safety cabinet, transferred to a Nunc universal tube (Gibco BRL), topped up to 25ml with 1x SSC and the lymphocytes purified by several rounds of centrifugation using a minifuge RF (Hereaus Sepatech, Brentwood), 4,500 rpm for ten minutes at 4°C and resuspension in 1x SSC. The purified lymphocytes were finally resuspended in 2-3ml 1x SSC, and 5ml 0.2M NaOAc, pH 7.0 were added. The suspension was transferred to a Corex tube and 1ml 10% SDS added and the tube gently mixed. After 5ml PCI were added, the tube was stoppered and the contents allowed to mix gently on a vertical blood tube rotator (Stuart Scientific, re-gearred for 20 rpm) for 2-3 hours. The emulsion was spun at 10,000 rpm in an HB-4 rotor within a Sorval RC-5B centrifuge for two minutes, 4°C. The aqueous phase was transferred to a fresh tube and the phenol was re-extracted with $\frac{1}{4}$ volume of SE. Both aqueous phases were pooled and dialyzed overnight at 4°C in 1x TE. The volume of solution was reduced on a bed of PEG 6000 flakes to 2-5ml. If the DNA solution was pure, it was transferred to one or more 1.5ml microfuge tubes and stored at -20°C, otherwise the solution was treated with RNase A and pronase E as for the breast tumour DNA extraction protocol before final purification and storage at -20°C in 1.5ml microfuge tubes.

Qiagen Protocols

Latterly Qiagen kits (Hybaid, Teddington) utilizing Qiagen columns were used to extract genomic DNA from human tissue according to the manufacturer's instructions. The whole blood DNA kit was used on 8-10ml samples of frozen whole blood from patients and the cell culture DNA kit was applied to dismembrated (powdered) breast tumour tissue. The DNA obtained was stored at -20°C in 1.5ml microfuge tubes.

Preparation of Sheared Salmon Testes DNA

1g salmon testes DNA (type III, Sigma) was dissolved in 500ml Q water and 20ml 0.5M EDTA, pH 8.0 in a boiling water bath. 15ml 10M NaOH were added, the solution mixed well and the pH was confirmed to be 14.0, after which it was boiled for 20 minutes and then cooled on ice. 20ml 1M Tris-HCl, pH7.5 were added and the pH adjusted to between 7.0 and 8.0 with concentrated HCl. After 80ml PCI were added, the emulsion was mixed vigorously, divided into several 250ml polyallomer bottles and spun at 6,000 rpm in a GSA rotor within a Sorval

RC-5B centrifuge for five minutes at 4°C. The aqueous phase was removed to fresh bottles and two volumes IMS (industrial methylated spirits) added and the bottles well mixed. The DNA was pelleted at 6,000 rpm in a GSA rotor for ten minutes, 4°C. The supernatant was drained and allowed to dry overnight. The DNA was resuspended in a total of 50ml Q water, its concentration estimated by spectrophotometric analysis and adjusted to 5mg/ml, before storage at -20°C.

2.3.2 Preparation of Plasmid DNA

Transformation of Competent *E. coli* Cells

E. coli cells were made competent for the uptake of DNA and transformed by a variation of the method described by Mandel and Higa (1970). Briefly, an overnight culture of bacteria was diluted $1/100$ into 100ml of fresh medium and grown until mid log phase ($A_{550} = 0.4-0.5$), chilled on ice for ten minutes and centrifuged to pellet the cells. The cells were resuspended in 50ml of ice-cold 0.1M $MgCl_2$ and pelleted, resuspended in 25ml of freshly diluted ice-cold 0.1M $CaCl_2$ and incubated on ice for 20 minutes, and pelleted again. Finally, the cells were resuspended in 5ml of ice-cold 0.1M $CaCl_2$ and kept on ice until required. For freezing competent cells, the final pellet was resuspended in 5ml of ice-cold 0.1M $CaCl_2$ containing 12.5% (v/v) glycerol, snap frozen on dry ice and stored at -70°C. These cells were then thawed on ice when required.

Transformation of competent cells was performed as follows: 1-100ng plasmid (1-10 μ l) and 100 μ l 0.1M $CaCl_2$ were added to 100 μ l of competent cells. After mixing, the cells were incubated on ice for 30 minutes, subjected to heat shock at 42°C for three minutes then incubated on ice for five minutes. 800 μ l of Luria broth was added to the cells which were then incubated at 37°C for 30 minutes prior to plating on Luria-plates containing appropriate antibiotics (usually ampicillin, 50 μ g/ml) to select for cells containing the plasmid. The plates were allowed to dry, then inverted and incubated overnight at 37°C.

Large Scale Preparation of Plasmid DNA

Two methods were used for large scale plasmid DNA purification: a caesium chloride-based protocol and a Qiagen-based protocol. Subsequent to purification by both methods, the quantity and quality of the DNA was checked by spectrophotometric analysis and agarose gel electrophoresis respectively.

a) Detergent lysis followed by CsCl/EtBr equilibrium density centrifugation was essentially as described in Sambrook *et al.* (1989). Briefly, bacterial cultures were lysed with Triton-X 100 and supercoiled plasmid DNA separated from other nucleic acids by centrifugation in a solution of CsCl/EtBr at 39,000 rpm for 48 hours in a T1270 rotor (DuPont) in a Ultracentrifuge (DuPont). After isolation of the supercoiled plasmid DNA, isopropanol extraction followed by dialysis removed EtBr and CsCl from the sample respectively. After ethanol precipitation, the DNA was resuspended in Q water in 1.5ml microfuge tubes.

b) Alkaline lysis and purification of DNA by the use of Qiagen columns (Hybaid, Teddington) was performed according to the manufacturer's instructions. The DNA obtained was resuspended in Q water in 1.5ml microfuge tubes.

Small Scale Purification of Plasmid DNA

Isolation of plasmid DNA from 1.5ml of a 5ml 5-20 hour bacterial culture was performed essentially according to the method of Birnboim and Doly (1979), with the exclusion of lysozyme which was found to be unnecessary.

2.3.3 Polymerase Chain Reaction (PCR) Methods

PCR was used for amplifying the VNTR at the DXYS14 locus from normal human genomic DNA to prepare a probe for Southern hybridization. Synthetic oligonucleotides used: 5'-A G G T C C C G A T A G G A G G A-3' and 5'-T C C T C C T C T T A G G A C C A T-3'.

Amplification was performed using a Perkin Elmer Cetus DNA thermal cycler (model No. N8010177, Perkin Elmer Corporation, Norwalk). The buffer used was supplied with the Taq DNA polymerase and dNTPs (Pharmacia, ultra pure) were used at 0.2mM concentration. Primers were present at 0.4μM each and 200-300ng of template DNA were used. Reaction volumes were 100μl containing 2.5U of *Taq* polymerase and overlaid with 100μl of mineral oil (Fisons). The thermal cycler was programmed for an initial 94°C melt for four minutes (since genomic DNA was the template used) followed by 30 cycles of: 94°C melt for one minute, 54°C anneal for one minute and extension at 72°C for three minutes, with a final ten minutes at 72°C followed by a 4°C soak.

2.3.4 Gel Electrophoresis

Deoxyribonucleic acids were separated by electrophoresis through horizontal agarose slab gels (Seakem HGT or NuSieve GTG; both purchased from Flowgen, Sittingbourne), which varied between 0.5% and 4% (w/v) in concentration. Gels were made and run in either 0.5x TAE or 1x TAE buffers. Ethidium bromide was added to the gel and buffer at 0.5µg/ml for DNA gels to allow visualization of the DNA when illuminated by ultraviolet light (254nm wavelength). When a permanent record of a gel was required, the gel was photographed using a Polaroid MP4 land camera loaded with Polaroid type 667 black and white positive film (Polaroid, St. Albans). Markers used on agarose gels were: λ DNA cleaved with *Bam*HI, *Dra*I, *Eco*RI or *Eco*RV; plasmid pUC18 cleaved with *Hpa*II; biotinylated-φX174 DNA-*Hinf*I fragments (Gibco BRL) and digoxigenylated-DNA VIII markers (Boehringer) were used to aid size determination of nick-translated probes (see section 2.3.7).

Purification of DNA Fragments from Agarose Gels

The method used is essentially that of McDonnell *et al.* (1977). Briefly, the desired fragment was excised from an agarose gel, placed inside dialysis tubing containing a small amount of the appropriate TAE buffer and electrophoresed at 5 V/cm for between 30 minutes and two hours. The eluted DNA was then ethanol precipitated, dried and resuspended in a suitable volume of Q water.

2.3.5 Restriction Enzyme Digestions

Restriction endonucleases were used according to the manufacturers' instructions, except $\frac{1}{10}$ volume 40mM spermidine was added after 30 minutes incubation to stabilize the DNA when digests of human genomic DNAs were carried out. All digests were incubated for sufficient time to attain complete digestion. $\frac{1}{10}$ volume of loading buffer (20mM EDTA, 50% (v/v) glycerol, 0.05% (w/v) bromophenol blue) was added and the sample loaded on an agarose gel together with a suitable size marker.

2.3.6 Southern Hybridization

Southern (DNA) Blotting

After electrophoresis and photography, if appropriate, capillary transfer of the DNA to a nylon membrane (Hybond-N, Amersham) was performed overnight essentially as described by Southern (1975) using 20x SSC as a transfer buffer. The filter was dried and the DNA cross-linked to the membrane by irradiation with ultraviolet light (254nm).

Preparation of ^{32}P Radiolabelled Probes

^{32}P radiolabelled probes were generated by the method of random priming (Feinberg and Vogelstein, 1983) using ^{32}P - α dCTP. Labelling reactions were performed at 37°C for 1-8 hours with 4U of Klenow polymerase (Amersham). New preparations of DNA were checked for efficiency of radionucleotide incorporation as follows: 1 μ l (approximately 1/200) of the stopped probe mix was pipetted onto a 2cm circle of DE81 paper (Whatman), dried and Cerenkov-counted in a Tri-Carb Minaxi-b 4000 series liquid scintillation counter (Packard Instruments, Downers Grove, Illinois). After washing off the unincorporated nucleotide with 0.5M Na_2HPO_4 , the filter was dried and counted again. Typically 70-95% incorporation was seen, and probes with over 60% incorporation were used without further purification.

Hybridization of Membrane-Immobilized Deoxyribonucleic Acids

Prehybridization was performed for a minimum of two hours at 65°C in 3x SSC, 5x Denhardt's solution, 0.1% (w/v) SDS, 6% (w/v) PEG 6000 and 200 μ g/ml sheared, denatured salmon testis DNA as described by Varley *et al.* (1987). Hybridization was performed in 3x SSC, 2x Denhardt's solution, 0.1% (w/v) SDS, 6% (w/v) PEG 6000 and 200 μ g/ml sheared, denatured salmon testis DNA with 0.5ng/ml of ^{32}P radiolabelled probe overnight (minimum of 12 hours) at 65°C as described by Varley *et al.* (1987). Prehybridization and hybridization were performed in a volume of 40ml in sealed perspex chambers. Probes used in the project are listed in table 2.1.

After hybridization the filters were typically washed three times at 2x SSC, 0.1% (w/v) SDS at 65°C, once at 0.5x SSC, 0.1% (w/v) SDS at 65°C and finally twice at

Table 2.1: Details of the probes used for Southern analysis of the tumour-lymphocyte panel.

a Fragment quoted was used as the probe in Southern hybridization protocols and did not always constitute the entire length of the insert in the plasmid

i) Probes detecting loci on chromosome 1

Probe	Locus	Vector	Fragment size ^a	Restriction enzyme to excise fragment ^a	Polymorphism	Heterozygosity	Reference
cYNA13	D1S74	pWE15	~20kb	<i>Bam</i> HI	VNTR: <i>Mbo</i> I	96%	Nakamura and White (1988)
pCMM12	D1S76	pUC18	4.0kb	<i>Eco</i> RI- <i>Hind</i> III	VNTR: <i>Taq</i> I	65%	Nakamura <i>et al.</i> (1988f)
pEFD53.2	D1S73	pUC18	4.7kb	<i>Taq</i> I	VNTR: <i>Taq</i> I	42%	O'Connell <i>et al.</i> (1989)
pEKH7.4	D1S65	pUC18	2.9kb	<i>Pst</i> I	RFLP: <i>Taq</i> I	54%	Kumlin-Wolff <i>et al.</i> (1987)
pHRnES1.9	REN	pUC18	1.9kb	<i>Eco</i> RI- <i>Pst</i> I	RFLP: <i>Mbo</i> I	41%	Chirgwin <i>et al.</i> (1984)
pMCT58	D1S77	pUC18	3.0kb	<i>Pst</i> I	VNTR: <i>Pst</i> I	66%	Nakamura <i>et al.</i> (1988c)
pMCT118	D1S80	pUC18	3.1kb	<i>Pst</i> I	VNTR: <i>Pst</i> I	90%	Nakamura <i>et al.</i> (1988b)
pMLAJ1	D1S61	pBR322	0.8kb	<i>Eco</i> RI- <i>Hind</i> III	VNTR: <i>Mbo</i> I	64%	O'Connell <i>et al.</i> (1989)
pMS1	D1S7	pUC13	4.6kb	<i>Sau</i> 3A1	VNTR: <i>Mbo</i> I	98%	Wong <i>et al.</i> (1987)
pMS32	D1S8	pUC13	5.9kb	<i>Sau</i> 3A1	VNTR: <i>Mbo</i> I	97%	Wong <i>et al.</i> (1987)
pMUC10	MUC1	pUC8	1.8kb	<i>Eco</i> RI	VNTR: <i>Mbo</i> I	86%	Gendler <i>et al.</i> (1987)
pTHH33	D1S81	pUC18	1.9kb	<i>Eco</i> RI- <i>Hae</i> III	VNTR: <i>Mbo</i> I	85%	Nakamura <i>et al.</i> (1988e)
pYNZ2	D1S57	pBR322	3.0kb	<i>Hind</i> III	VNTR: <i>Pst</i> I	65%	Nakamura <i>et al.</i> (1988d)
pYNZ23	D1S58	pBR322	5.0kb	<i>Msp</i> I	VNTR: <i>Mbo</i> I	38%	Nakamura <i>et al.</i> (1987a)
3021E1	SPTA1	pBR322	13.0kb	<i>Eco</i> RI	RFLP: <i>Msp</i> I	38%	Linnenbach <i>et al.</i> (1986)

ii) Probes detecting loci on the X chromosome

Probe	Locus	Vector	Fragment size ^a	Restriction enzyme to excise fragment ^a	Polymorphism	Heterozygosity	Reference
cpX289	DXS159	pUC12	1.5kb	<i>Pst</i> I	RFLP: <i>Pst</i> I	37%	Hofker <i>et al.</i> (1986)
CRI-pS232	DXS278	pUC8	6.7kb	<i>Eco</i> RI	Repetitive: <i>Mbo</i> I	90%	Donis-Keller <i>et al.</i> (1987)
cX52.5	DXS101	pAT153	4.0kb	<i>Eco</i> RI- <i>Hind</i> III	RFLP: <i>Msp</i> I	31%	Hofker <i>et al.</i> (1986)
L1.28	DXS7	pSP64	1.25kb	<i>Eco</i> RI	RFLP: <i>Taq</i> I	46%	Davies <i>et al.</i> (1983)
M27β	DXS255	pUC9	2.3kb	<i>Eco</i> RI	VNTR: <i>Pst</i> I	90%	Fraser <i>et al.</i> (1987)
pDP411a	DXYS28	pUC13	1.9kb	<i>Eco</i> RI	VNTR: <i>Taq</i> I, <i>Hinf</i> I	56%	Page <i>et al.</i> (1987)
pMS600	DXYS78	pBluescriptII KS ⁺	5.0kb	<i>Sau</i> 3AI	VNTR: <i>Hinf</i> I, <i>Mbo</i> I	91%	Armour <i>et al.</i> (1990)
pMS613	DXS438	pBluescriptII KS ⁺	5.5kb	<i>Sau</i> 3AI	VNTR: <i>Pst</i> I	35%	Armour <i>et al.</i> (1990)
pMS639	DXYS89	pBluescriptII KS ⁺	4.5kb	<i>Sau</i> 3AI	VNTR: <i>Mbo</i> I	34%	Armour <i>et al.</i> (1992)
p52A	DXS51	pBR322	5.15kb	<i>Eco</i> RI- <i>Hind</i> III	RFLP: <i>Taq</i> I	50%	Drayna <i>et al.</i> (1984)
RC8	DXS9	pAT153	6.1kb	<i>Eco</i> RI	RFLP: <i>Taq</i> I	28%	Murray <i>et al.</i> (1982)
St14-1	DXS52	pBR322	3.0kb	<i>Eco</i> RI	VNTR: <i>Taq</i> I, <i>Mbo</i> I	80%	Oberlé <i>et al.</i> (1985)
29C1	DXYS14	pUC9	1.8kb	<i>Pst</i> I	VNTR: <i>Hinf</i> I	97%	Cooke <i>et al.</i> (1985)
113D	DXYS15	pBR327	0.55kb	<i>Eco</i> RI- <i>Hind</i> III	VNTR: <i>Taq</i> I	60%	Simmmler <i>et al.</i> (1987)
602	DXYS17	pBS	1.0kb	<i>Eco</i> RI- <i>Hind</i> III	VNTR: <i>Mbo</i> I	80%	Simmmler <i>et al.</i> (1987)
754	DXS84	pSP64	2.2kb	<i>Hind</i> III	RFLP: <i>Pst</i> I	48%	Hofker <i>et al.</i> (1986)

iii) Probes detecting loci on other chromosomes

Probe	Locus	Vector	Fragment size ^a	Restriction enzyme to excise fragment ^a	Polymorphism	Heterozygosity	Reference
pMS31	D7S21	pUC13	5.7kb	<i>Sau3AI</i>	VNTR: <i>HinfI</i> , <i>MboI</i> , <i>TaqI</i>	98%	Wong <i>et al.</i> (1987)
pMS617	D20S26	pBluescriptII KS ⁺	5.5kb	<i>Sau3AI</i>	VNTR: <i>HinfI</i> , <i>MboI</i> , <i>TaqI</i>	79%	Armour <i>et al.</i> (1990)
pYNH24	D2S44	pUC18	2.0kb	<i>MspI</i>	VNTR: <i>AluI</i> , <i>HinfI</i> , <i>MboI</i> , <i>MspI</i> , <i>PstI</i> , <i>TaqI</i>	97%	Nakamura <i>et al.</i> (1987b)
pYNZ22.1	D17S5	pBR322	1.7kb	<i>BamHI</i>	VNTR: <i>MboI</i>	86%	Nakamura <i>et al.</i> (1987c)
VNTR	RB1	pUC18	2.3kb	<i>HindIII-SalI</i>	VNTR: <i>MboI</i>	80%	Varley <i>et al.</i> (1989)

0.2x SSC, 0.1% (w/v) SDS at 65°C, or until no further radioactivity could be detected in the washing solution. Filters were air-dried on Whatman 3MM paper, mounted on fresh 3MM paper and autoradiographed at -70°C using Kodak X-OMAT (Eastman Kodak Company, New York) with either Hi-Speed X or Smit Rapid intensifying screens for between 24 hours and eight weeks.

Removal of Probes and Re-use of DNA Blots

Probe sequences were removed from the filters by incubating the filter in 0.4M NaOH at 45°C for ten minutes followed by incubation in 0.1 x SSC, 0.1% (w/v) SDS, 0.2M Tris-HCl (pH 7.5) at 45°C for 25-30 minutes. The filter was immediately transferred to prehybridization solution and treated as normal for hybridization.

2.3.7 Laser Scanning Densitometry

A computing densitometer (Molecular Dynamics, Sevenoaks, Kent) with the ImageQuant software package was used to confirm the nature of allelic imbalances (see Appendix IV).

2.3.8 Fluorescence *In Situ* Hybridization

Preparation of Lymphocyte Splashes

Four to six drops of blood were incubated in 5ml culture medium (4ml McCoy's 5A medium, 0.1ml 5000U/ml penicillin/streptomycin, 0.5ml foetal bovine serum, 0.2ml phytohaemagglutinin) within a Nunc universal tube for 72 hours at 37°C. Tubes were gently inverted every 12 hours and during the final two hours, 0.3ml colcemid (10µg/ml Gibco BRL) were added with a final inversion. The culture was transferred to a 10ml conical-bottomed tube (Falcon) and the lymphocytes harvested at 750rpm for six minutes in a Heraeus minifuge RF. The supernatant was discarded except for 0.5ml in which the cells were resuspended by vortexing. 4ml 0.075M KCl (freshly-made and pre-heated to 37°C) were added while vortexing drop-wise for the first 0.5ml and then in a steady stream. The cells incubated at 37°C for six to ten minutes and then pelleted and resuspended as before. 5ml fixative (3:1 methanol:acetic acid) was added while vortexing, the first 1ml drop-wise and then in a steady stream. After a 20 minute-incubation on

ice, cells were re-pelleted and resuspended in 5ml fresh fixative. Several rounds of centrifugation and resuspension were carried out until the supernatant turned from brown to clear. The small, cleaned white pellet was resuspended in 100-200 μ l fresh fixative and then refrigerated for up to one month. Before being splashed onto microscope slides, the cells were pelleted and resuspended in an appropriate volume of fresh 3:1 fixative.

Microscope slides (Goldstar, Chance Propger, Warley) were cleaned with Q water, followed by ethanol and stored in 70% ethanol until ready for use when they were dried off. Using a siliconized glass pipette, a single drop of the resuspended cells was dropped onto each slide from a height of about 30cm. Depending on the relative humidity within the laboratory, splashing was carried out on the bench (55-65% humidity), over a 37°C water bath (<55% humidity), or on a hot plate (>65% humidity). Once the fixative was dried, the slide was examined on a Nikon Labophot-2 light microscope (fitted with the CF Plan objectives 10x, 20x, 40x) under phase contrast to determine the concentration of cells over the surface of the slide and the extent of spreading of chromosomes from burst cells. The area of each spread was marked on the under surface of the slide with a diamond-tipped marker pen and stored at 4°C in boxes containing desiccant. If the slides were required for hybridization less than one week after preparation, they were first artificially 'aged' by a ten minute-incubation on a metal sheet in an air-incubator set at 65°C. Before hybridization, slides were briefly rinsed in Q water and then for one minute in each of 30% IMS, 60% IMS, 80% IMS and 100% IMS to dehydrate the cells and chromosomes.

Preparation of Tissue Sections

5 μ m sections were cut from formalin-fixed paraffin-embedded breast tumours and adhered to saline-treated microscope slides. The sections were de-waxed in two 15-minute incubations at room temperature of Histo-clear (Fisons) and then washed in 100% IMS for ten minutes at room temperature. The sections were rehydrated by sequential immersion in each of 100% IMS, 80% IMS, 60% IMS, 30% IMS and Q water for one minute at room temperature. Sections were rinsed for one minute at room temperature in P buffer and digested for 30 minutes at 37°C with a range of concentrations of proteinase K (typically 100, 200, 350, and 500 μ g/ml) in pre-warmed P buffer. Sections were then immersed in PBS-glycine for one minute at room temperature, rinsed for one minute in PBS at room temperature and post-fixed in 4% paraformaldehyde for 20 minutes at room temperature. After rinsing in PBS for one minute at room temperature, sections

were dehydrated by sequential immersion in Q water for a few seconds, and for one minute in each of 30% IMS, 60% IMS, 80% IMS and 100% IMS. Sections were air-dried and stored during the day until used for hybridization in air-tight jars with 1-2ml 100% ethanol at the bottom of the container.

Preparation of Probes

Unlike probes prepared for Southern hybridization, probes for FISH were not excised from their vector. All probes were prepared with biotin-16-dUTP (Boehringer) or digoxigenin-11-dUTP (Boehringer) to label the DNA using the Nick translation kit from Gibco BRL according to manufacturer's instructions, generating modified fragments 400-600bp in size for hybridization to metaphase chromosomes. For hybridization to tissue sections, the DNaseI concentration during the reaction was increased for each probe to generate modified fragments 100-300bp in size. Size determination was carried out using the BLUGENE nonradioactive nucleic acid detection kit (Gibco BRL) according to manufacturer's instructions. Probes used are listed in table 2.2.

Hybridization

25µl of hybridization mix (50% formamide (Fluka, Gillingham), 10% dextran sulphate, 2x SSC, 0.5% Tween-20) in which each labelled probe was at a concentration of 1ng/µl, competitor sheared salmon sperm DNA was at 500ng/µl and competitor pUC18 plasmid DNA digested with *Hpa*II restriction endonuclease was at 250ng/µl was applied to the appropriate area of each slide. The hybridization solution was covered with a 22mm x 22mm siliconized Corning coverglass (Bibby Sterilin, Newport) and sealed with rubber cement. Probe and target were always denatured together on a metal sheet in an air-incubator: when using metaphase chromosomes, denaturation was at either 70°C for ten minutes or (preferentially) 80°C for three minutes; when sections were used, denaturation took place at 95°C for ten minutes. All slides were transferred to a sealed moisture chamber at 37°C for overnight incubation, containing tissues soaked with 3x SSC.

Table 2.2: Details of the probes used for FISH to metaphase chromosomes and tissue sections.

Locus	Probe	Vector	Insert size	Reference
D1Z1	pUC1.77	pUC18	1.77kb	Cooke and Hindley (1979)
D2Z1	pBS4D	pBluescript	1.3kb	Rocchi <i>et al.</i> (1990)

Washing Protocol

The rubber cement was removed with a pair of fine forceps and the coverglass flushed off in a beaker of 50% formamide, 2x SSC at 45°C before transfer to Coplin jars for three five-minute washes in 50% formamide, 2x SSC at 45°C, followed by two five-minute washes in 2x SSC at 45°C and three five-minute washes in 0.5x SSC at 45°C.

One of three different detection protocols was then implemented depending on the hybridization carried out: single hybridizations with a biotinylated probe, single hybridizations with a digoxigenylated probe, and dual hybridizations with both kinds of modified probe.

Detection Protocol for a Single Biotinylated Probe

All steps were performed at room temperature and in darkness. After a two-minute wash in 4x SSC, 0.05% Tween-20, slides were left for ten minutes in preincubation buffer at room temperature. 100µl 2µg/ml FITC-avidin DN in preincubation buffer was applied to each slide for 20 minutes, overlaid with a 22mm x 40mm coverglass (Chance Propper) and the excess washed off with three five-minute washes in 4x SSC. 100µl 2µg/ml biotinylated-anti-avidin D in preincubation buffer was applied to each slide for 20 minutes, under a coverglass and the excess washed off with three five-minute washes in 4x SSC. 100µl 2µg/ml FITC-avidin DN in preincubation buffer was applied to each slide for 20 minutes, under a coverglass and the excess washed off with three five-minute washes in 4x SSC. Slides were stained with either 10ng/ml propidium iodide in PBS or 10ng/ml DAPI in PBS for ten minutes and rinsed in PBS for five minutes.

Detection Protocol for a Single Digoxigenylated Probe

All steps were performed in darkness. After a 20-minute incubation in pre-warmed TNB at 37°C, slides were washed for two minutes in TNT at room temperature. 100µl 0.5µg/ml mouse anti-digoxigenin antibody in TNB was applied to each slide for 30 minutes at 37°C, under a coverglass and the excess washed off with three five-minute washes in TNT at room temperature. 100µl of rabbit anti-mouse antibody-TRITC conjugate diluted 1/1000 in TNB was applied to each slide for 30 minutes at 37°C, under a coverglass and the excess washed off with three five-minute washes in TNT at room temperature. 100µl of goat anti-

rabbit antibody-TRITC conjugate diluted in $1/1000$ TNB was applied to each slide for 30 minutes at 37°C , under a coverglass and the excess washed off with three five-minute washes in TNT at room temperature. Slides were stained with 10ng/ml DAPI in PBS for ten minutes at room temperature and rinsed in PBS for five minutes.

Dual-Detection Protocol for Biotinylated and Digoxigenylated Probes

All subsequent steps were performed in darkness. After a 20-minute incubation in pre-warmed TNB at 37°C , slides were washed for two minutes in TNT at room temperature. 100 μl 2 $\mu\text{g}/\text{ml}$ FITC-avidin DN in preincubation buffer was applied to each slide for 20 minutes at room temperature, under a coverglass and the excess washed off with three five-minute washes in TNT. 100 μl 2 $\mu\text{g}/\text{ml}$ biotinylated-anti-avidin D, 0.5 $\mu\text{g}/\text{ml}$ mouse anti-digoxigenin antibody in TNB was applied to each slide for 30 minutes at 37°C , under a coverglass and the excess washed off with three five-minute washes in TNT at 37°C . 100 μl 2 $\mu\text{g}/\text{ml}$ biotinylated-anti-avidin D, rabbit anti-mouse antibody-TRITC conjugate diluted $1/1000$ in TNB was applied to each slide for 30 minutes at 37°C , under a coverglass and the excess washed off with three five-minute washes in TNT at 37°C . 100 μl of goat anti-rabbit antibody-TRITC conjugate diluted in $1/1000$ TNB was applied to each slide for 30 minutes at 37°C , under a coverglass and the excess washed off with three five-minute washes in TNT at 37°C . Slides were stained with 10ng/ml DAPI in PBS for ten minutes at room temperature and rinsed in PBS for five minutes.

Mounting of Hybridized Slides

After counterstaining with either propidium bromide or DAPI, slides were briefly blotted dry and mounted with Vectorshield mounting medium (Vector). Coverglasses (22mm x 50mm, Chance Proper, previously washed and sterilized) were applied and sealed down with nail varnish.

Fluorescence Microscopy

The slides were examined with an Olympus fluorescence microscope, model BH2-RFL (reflected light fluorescence attachment) with two interchangeable dichroic mirror units with exciter filters, 20UV-W-2 and 20BG-W-2; a

supplementary exciter filter EY-455 was used in conjunction with the Blue filter of 20BG-W-2. The microscope was fitted with the fluorescence-free objectives UVFL 10x, UVFL 20x, UVFL 40x (oil) and UVFL 100x (oil) and mounted with a 35mm camera linked to an automatic exposure unit for photomicrography. Fujichrome 400 colour (slide) film was used to record the results, developed by the Central Photographics Unit, University of Leicester.

Latterly, the fluorescence attachments on the microscope were substituted with an updated system of filter units. A BH2-RFCA fluorescent illuminator was incorporated into the microscope and three filter blocks were mounted, BH2-TFC2 cube (DAPI/FITC/TRITC), BH2-DFC7 cube (FITC/PI), BH2- bright field cube. The DFC7 cube was also appropriate for illumination of FITC/TRITC only.

2.3.9 Statistical Analyses

Associations between the specific chromosomal rearrangements determined by Southern analysis and the clinico-pathological variables available were investigated using the chi-square test and in some cases the Fisher-Unwin exact test was also employed. Where appropriate, corrections to the chi-square test were incorporated. All tests were done by hand after contingency tables were drawn up, with a Casio (Japan) fx-100 scientific calculator for assistance.

CHAPTER 3

Allelic Imbalance Studies of Other Chromosomes

3.1 Introduction

From the beginning of the project, it was clear that controls would have to be implemented to determine the nature of the imbalances observed on chromosomes of interest. Whilst loss of heterozygosity was the predominant form of AI occurring, amplifications were also suspected. However, no control for loss of heterozygosity can be truly negative, especially in breast tumours, as the genome displays a background level of instability and thus imbalances will occur randomly at any site in the genome. Similarly, no control can be truly positive, as no region of the genome is guaranteed to undergo dosage changes (either amplification or deletion) in every tumour studied. Therefore, regions of the genome rarely found to undergo alteration can only be used to control for the amounts of DNA between tracks on an autoradiograph. By using a highly-polymorphic locus in such a region, it should be possible to identify the rare and random incidence of imbalance; monomorphic loci would be simpler to score, except that the rare AI event would go undetected. The only positive controls that can be used in such a study are loci targeted for imbalance at frequencies higher than background (random) events. Therefore, polymorphic loci previously shown to undergo imbalance (in possibly un-controlled studies) can only be used in this instance.

Initially, a hypervariable minisatellite locus on chromosome 2, D2S44, was chosen as a control to establish the amounts of DNA in the tracks of the autoradiographs, since no significant levels of LOH have been recorded for chromosome 2 sequences (Seizinger *et al.*, 1991). Rearrangements of this chromosome are not associated with breast tumours, nor indeed with any of the common solid tumours (Mitelman *et al.*, 1991, 1993). Additional control loci were also used for some filter sets. These mapped to chromosomes 7 and 20, also not implicated as targets for significant levels of LOH (Seizinger *et al.*, 1991), neither of which proved as satisfactory as D2S44.

To try to provide positive controls, loci on chromosomes 13 and 17 were analyzed, since there is considerable data available on LOH for these chromosomes in the literature (Seizinger *et al.*, 1991). These would help to determine if there were any large inconsistencies in the data obtained for chromosomes 1 and X, such as consistent over-estimation of allelic imbalances and later could be used to test for association with the rearrangements mapped to chromosomes 1 and X.

3.2 Results

3.2.1 Construction of the Panel of Patients and Probe Bank

Over a period of three years, paired tumour and blood specimens were obtained from female breast cancer patients. DNA was successfully extracted from 36 pairs of these specimens. After purification, testing and concentration estimation, the 36 pairs of DNA samples were then ready for restriction endonuclease cleavage and Southern blotting. The 35 prepared tumour-lymphocyte DNA samples from Drs. John Armour and Anthony Howell were used directly for restriction enzyme digestion and Southern blotting. Thus, a total of 71 paired DNA samples were used for the Southern hybridization analysis.

Sets of filters were made from the entire working panel of 71 DNA pairs digested with different restriction endonucleases as required to give the polymorphisms detected by all the probes that came under study (see table 2.1 for details on the probes). A total of three sets of *Mbo*I filters, three of *Taq*I filters, two of *Pst*I filters and one of *Hin*fI filters were assembled over three years. An incomplete set of *Msp*I filters was made initially but not expanded as the DNA proved difficult to digest to completion with this enzyme despite a number of different attempts. Therefore, only a fraction of the panel of patients proved amenable to analysis with those loci recognizing polymorphisms in *Msp*I-restricted DNA only, namely SPTA1 on chromosome 1 and DXS101 on the X chromosome. Finally, a small set of *Alu*I filters was made from those patients' DNA in which somatic mutations were suspected (see chapter 5). These filter sets were subjected to multiple rounds of hybridization and stripping to analyze all loci. In addition to these filters, 'test' filters were made from DNA digested with different restriction enzymes; for each of the restriction enzymes *Hin*fI, *Mbo*I, *Msp*I, *Pst*I and *Taq*I, DNA from five unrelated normal individuals (placental DNA samples) were digested. These test

filters enabled confirmation of probe polymorphisms before hybridization of filters from the panel of patients.

Not all loci were tested in all patients; those DNA pairs obtained at the beginning of the project were the most comprehensively studied as re-testing those samples could be carried out if no result was obtained initially. These patients are from the series PB 5 to PB 141 and RW 628 to RW 690 as well as FW, FY, JG and MI. Although a lot of data has been generated with these samples, a number of them quickly ran out and are not represented on all filter sets (e.g. PB 5, PB 37, PB 78, PB 80, PB 96, PB113, PB 126, PB 128, PB 133, PB141, FY, RW 630, RW 633). Therefore, as increasing numbers of loci were studied, these patients were excluded and the gaps in the data cannot be remedied. Some of those patients obtained initially as tissue specimens were also finished during the work (e.g. RW 860, RW 862, RW 864, RW 874), but it will be possible for most to be re-tested at a later date to complete the data. There are also the paired tissue specimens as yet unprocessed which will serve to increase the size of the working panel, with the opportunity for additional paired samples to be collected by Dr. Rosemary Walker if required.

Examples of the hybridization patterns observed with probes recognizing the loci studied on chromosomes 2, 7, 13, 17 and 20 are given in figures 3.1 to 3.3. Summaries of the results obtained for these loci are presented in table 3.1 and a full list is given in Appendix I.

3.2.2 Chromosome 2

Allelic imbalance at D2S44 occurred in 16% of the panel. Due to this low level of AI observed, D2S44 was used as the principal control to determine the nature of imbalances on other chromosomes, by determining the amount of DNA in each track of the autoradiographs when not undergoing imbalance itself. Since the level of overall AI in informative patients at this locus was so low, it was probably still safe to use as a control when uninformative for the three patients in this panel. However, additional control loci, informative in the three cases, were used to ensure there is strict controlling for the amounts of DNA in the tracks of the autoradiographs in question.

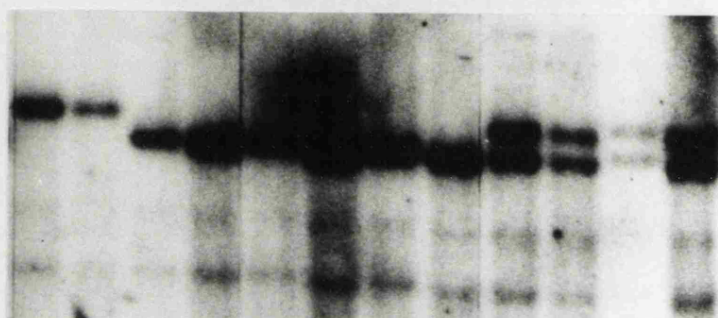
Deciding the level of background in LOH studies is fraught with difficulty since non-specific rearrangements occur with varying frequency in tumours of different type and origin. Therefore, in tumours with particularly extreme karyotype changes, the background is raised above those with very few alterations

Legend to figure 3.1: Autoradiographs of the VNTR from intron 16 of the RB1 gene on chromosome 13q14 from six patients, RW 660, RW 661, RW 663, RW 682, RW 686 and RW 690. Only RW 686 and RW 690 were informative and neither show allelic imbalance. The corresponding controls with D2S44 are also shown for all pairs of samples. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. Both sets of autoradiographs show invariant bands across all tracks. Allele sizes for the RB1 VNTR range between 1.3 and 1.2kb, with the invariant bands 1.0 and 0.8kb in size, while the allele range for the D2S44 locus varies from 3.9 to 1.0kb, with invariant bands 1.0 and 0.8kb in size.

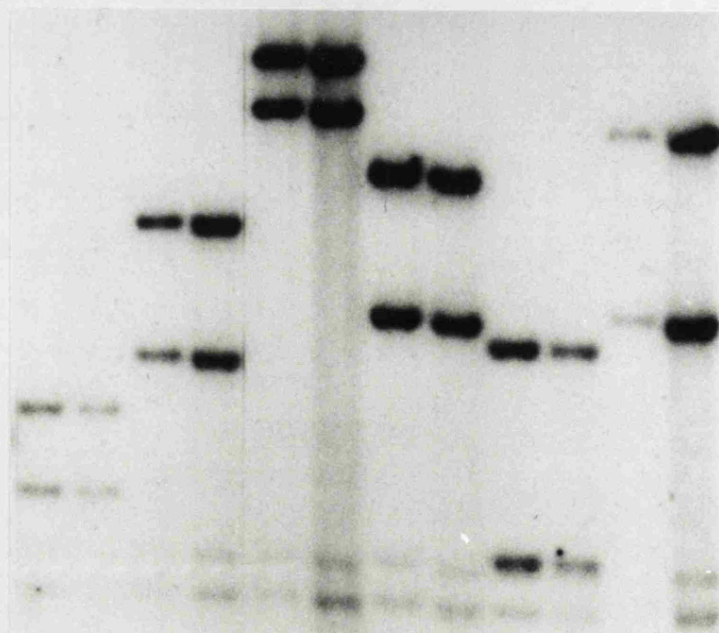
Figure 3.1: RB1

660 661 663 682 686 690
B T B T B T B T B T B T

RB1



D2S44



Legend to figure 3.2: Autoradiographs of D17S5 on chromosome 17p13 from five patients, RW 660, RW 661, RW 663, RW 682 and RW 686. All patients were informative, of which only RW 661 shows AI, as LOH (an arrow indicates the affected allele). The corresponding controls with D2S44 are also shown for all pairs of samples. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. Allele sizes for D17S5 range between 1.4 and 0.8kb, with a single invariant band 0.4kb in size, while the allele range for the D2S44 locus varies from 3.9 to 1.0kb, with invariant bands 1.0 and 0.8kb in size.

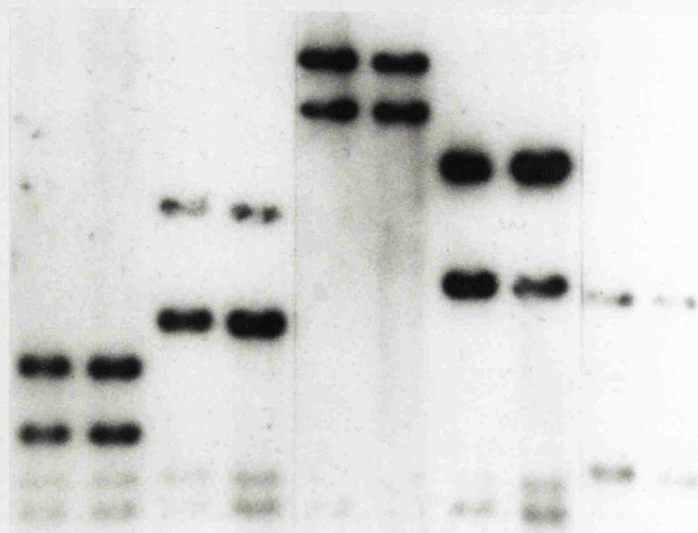
Figure 3.2: D17S5

660		661		663		682		686	
B	T	B	T	B	T	B	T	B	T

D17S5



D2S44



Legend to figure 3.3: Autoradiographs of the three controls, D2S44, D7S21 and D20S26 from five patients, RW 660, RW 661, RW 663, RW 682 and RW 686. Cross-referencing between the loci enables the nature of the imbalances at D2S44 in RW 682, D7S21 in RW 663 and RW 682, and D20S26 in RW 663 and RW 686 to be determined. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. Arrows indicate alleles of reduced intensity in the tumour lanes; the arrowhead indicates the allele of increased intensity (for GAL at D2S44 in RW 682). Allele sizes for the D2S44 locus vary from 3.9 to 1.0kb, with invariant bands 1.0 and 0.8kb in size, for D7S21 between 5.9 and 3.6kb, while the allele range for D20S26 varies from 7.0 to 4.6kb.

Figure 3.3: D2S44, D7S21 and D20S26

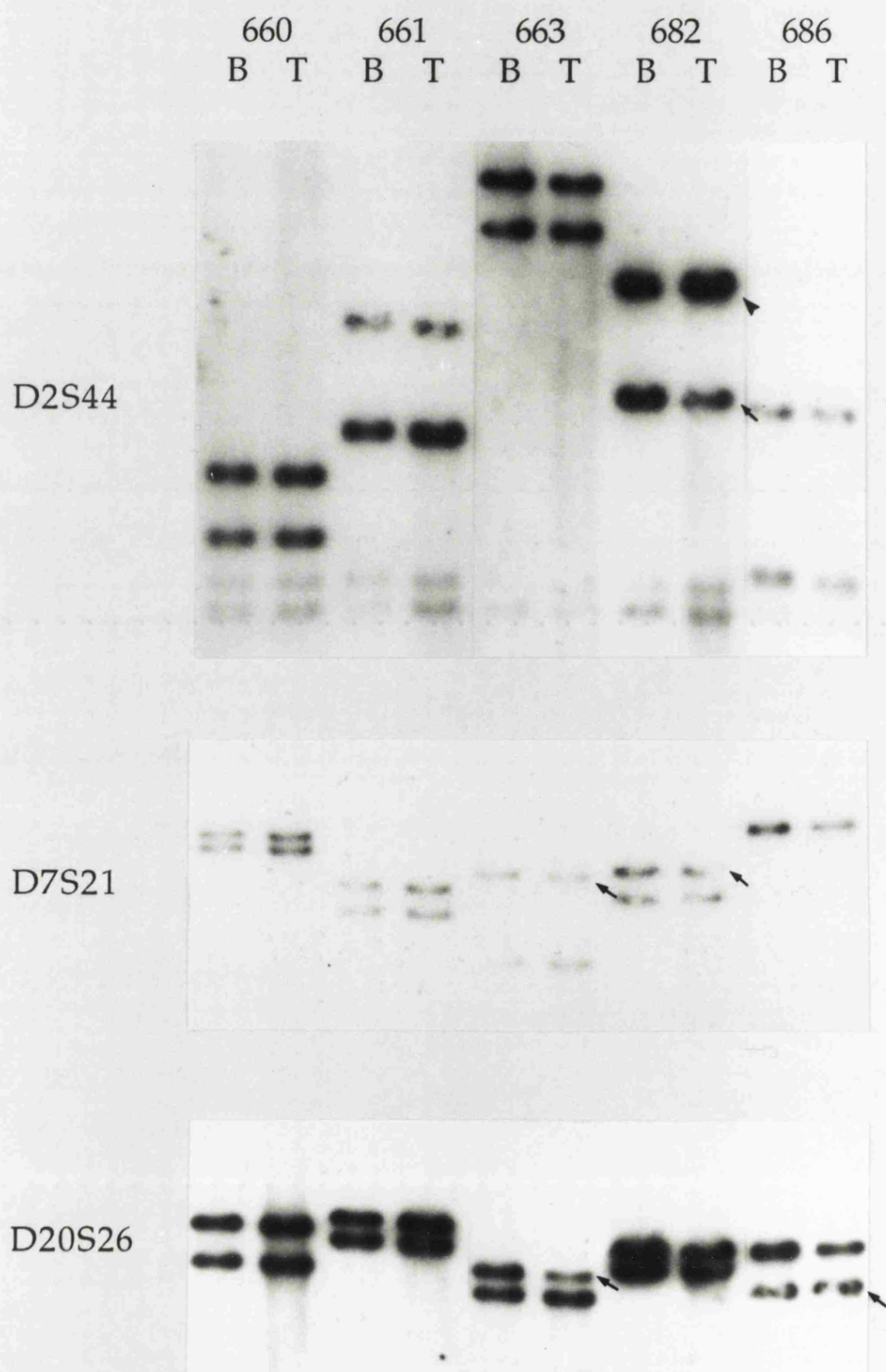


Table 3.1: Summary of probes and data on allelic imbalance (AI) on other chromosomes studied. The levels of heterozygosity of a locus varied between restriction enzymes, so that the same locus was not always informative in the same patient between restriction enzymes. A patient is designated informative when at least one of the different filter sets resolved the two alleles. pYNH24, pMS31 and pMS617 showed a polymorphism in *Hinf*I-, *Mbo*I- and *Taq*I-digested DNA; pYNH24 also showed a polymorphism in *Alu*I-, *Msp*I- and *Pst*I-digested DNA.

a Allelic loss (patients undergoing LOH or GAL - see text) shown as a fraction of the number of patients informative at the locus and expressed as a percentage in parentheses.

b Allelic gain (patients undergoing GCN - see text) shown as a fraction of the number of patients informative at the locus and expressed as a percentage in parentheses.

c The number of patients for which allelic imbalance had clearly occurred at each locus but it was impossible to establish the nature of the imbalance (i.e. whether LOH/GAL/GCN) due to AI at the control locus/loci in those patients.

d The total number of patients undergoing any form of imbalance as a fraction of the number of patients informative at the locus and expressed as a percentage in parentheses.

Locus	Probe	Position	Total no. of patients	Patients with allelic loss (%) ^a	Patients with allelic gain ^b	Unclear loss or gain ^c	Patients with imbalance (%) ^d
D2S44	pYNH24	2q	67	5/64 8%	1/64 2%	3/64	10/64 16%
D7S21	pMS31	7p	44	6/36 17%	2/36 6%	2/36	10/36 28%
RB1	RB VNTR	13q14	57	13/26 50%	2/26 8%	0/25	15/26 58%
D17S5	pYNZ22.1	17p13	40	8/25 32%	0/25	3/25	11/25 44%
D20S26	pMS617	20q	61	7/51 14%	4/51 8%	2/51	13/51 25%

as determined by karyotype analysis. Breast tumours are rarely found to have few chromosome structural changes, with a background noise level of ~8-12% LOH. Seizinger *et al.* (1991) quoted a 25% threshold as a minimum figure below which a locus was not considered to show targeted LOH. Loci with LOH (and even total AI) well below this threshold serve as good controls, but those with LOH levels approaching the cut-off point are poor controls. Similarly, loci under test on a chromosome of interest which have levels of LOH approaching the 25% threshold are highly unlikely to encode sequences targeted by LOH. The level of allelic loss at D2S44 is in accordance with the background noise and total AI is well below the 25% threshold. Importantly, this overall AI at D2S44 is not dominated by either allelic loss or allelic gain, as is to be expected with true background noise.

In addition to having a low level of AI, D2S44 is ideal as a control for loading between the lanes of an autoradiograph because it is so highly informative using a wide range of different restriction endonucleases. Therefore, D2S44 can be reliably used as a control for a diverse set of polymorphisms.

3.2.3 Chromosomes 7 and 20

In order to try to provide a control for those patients with AI at D2S44, additional loci were employed as controls. These mapped to different chromosomes to reduce the likelihood of AI at these loci since the AI affecting D2S44 could not extend to them also. However, the first locus chosen, D7S21, showed 28% AI and it was decided to find an alternative since this level of AI was above the 25% threshold. The locus D20S26 was used latterly, but AI occurred at a frequency of 25% here and so confidence in using D20S26 as a control when uninformative to establish AI at all other sites in the genome was not sufficient. Also, neither D7S21 nor D20S26 proved to be as highly informative as D2S44. Therefore, D2S44 (pYNH24) was used primarily as a control for the amounts of DNA in each track of the autoradiograph and analyzed on all filters prepared during the project. Probe pMS31 (D7S21) was hybridized to two-thirds of each of one set of *HinfI*, *MboI* and *TaqI* filters; pMS617 (D20S26) was hybridized to one entire set of each of *HinfI*, *MboI* and *TaqI* filters. However, it was possible to characterize some of the imbalances observed at the three control loci by cross-matching between the loci (see below and Appendix I).

3.2.4 Scoring the Autoradiographs for Allelic Imbalance

The autoradiographs for each probe for loci on chromosomes 1 and X, as well for RB1 and D17S5, were scored in conjunction with the corresponding autoradiograph from the control locus D2S44, and where available, with those from D20S26 and D7S21. Patients were designated uninformative when homozygous for the locus in question or informative when heterozygous. Those informative patients in which an imbalance between alleles in the tumour DNA track could be observed were then more closely analyzed. The loading of DNA between the lymphocyte and tumour DNA tracks was estimated from the control autoradiograph(s) first in order to determine which of the alleles was at the 'normal' intensity for the tumour lane when no imbalance was observed for these. In this way, the nature of the imbalance observed at the test locus was determined. Classic loss of heterozygosity was the most common form of AI observed: one allele in the tumour track is reduced in intensity with respect to the remaining allele and those in the lymphocyte track. Two additional types of imbalance were also identified. Rarely, a concomitant gain and loss of alleles (GAL) was established: both alleles are affected, where one allele is reduced in intensity in the tumour track and the remaining allele is increased in intensity with respect to the alleles in the lymphocyte track; and commonly, a gain in copy number (GCN) of one allele was determined: one allele in the tumour track is increased in intensity (i.e. amplified) with respect to the remaining allele and those in the lymphocyte track. The complete results for all patients and loci are listed in Appendices I (other chromosomes studied), II (X chromosome) and III (chromosome 1).

The different forms of imbalance detected 'by eye' were later examined by laser scanning densitometry for 39 test loci, with the corresponding control(s) (Appendix IV provides tables of the raw data obtained). While the principle of laser scanning densitometry is to provide a quantitative measurement of the intensity of the bands in each track of the autoradiograph, it became clear that the technique was incapable of this in some cases (table 3.2). Of the results in table 3.2, 18 were not as expected: the nature of the imbalance in 16 test loci could not be confirmed despite their controls and, for two other test loci, the AI could not be defined since their control locus (D2S44) could not be confirmed. These two cases (MUC1 and D1S61) occurred in the same patient, RW 942, on the same chromosome using a single set of *Mbo*I filters, so that in fact only one D2S44 control failed as it was used for both test loci. However, D2S44 also served as a control for a third locus, D1S81, also on chromosome 1, detected on another *Mbo*I filter set and the control was confirmed by densitometry here.

Table 3.2: Summary of results for all scanned loci, listed with the original results by eye. In total, 68 loci (test loci plus controls) were scanned, when shared controls are taken into consideration. The densitometry results are in disagreement with the visual analysis for 17/68 loci scanned, i.e. 25% inaccuracy with a further two undefinable, and appears to be due to the technique experiencing limitations (see text for details). For the test loci scanned, 16/39 (41%) produced different results, while for an additional two test loci, the control determined by eye could not be confirmed as such by densitometry: loci MUC1 and D1S61 in RW 942, which were hybridized to the same filter and the nature of imbalance at these test loci could not be established by densitometry. A third test locus from this patient, on another filter, was also scanned and its control was confirmed as such by densitometry.

Patient	Locus	Visual (eye)	Densitometry	
PB 5	D1S61	GAL	GCN	no
	D2S44	control	control	yes
	D1S58	GAL	GAL	yes
	D2S44	control	control	yes
	D1S8	GAL	GAL	yes
	D2S44	control	control	yes
PB 134	D1S80	LOH	GAL	no
	D2S44	control	control	yes
	D1S7	LOH	no AI	no
	D2S44	control	control	yes
	D1S57	LOH	LOH	yes
	D2S44	control	control	yes
	MUC1	GCN	GCN	yes
	D2S44	control	control	yes
RW 690	D1S81	GCN	GCN	yes
	D2S44	control	control	yes
	D1S8	GCN	GCN	yes
	D2S44	control	control	yes
RW 864	D1S7	LOH	LOH	yes
	D2S44	control	control	yes
RW 867	REN	i	no AI	yes
	MUC1	GCN	GCN	yes
	D2S44	control	control	yes
RW 869	D1S74	LOH	GAL	no
	D2S44	control	control	yes
	REN	LOH	GCN	no
	D2S44	control	control	yes
RW 869	D1S58	LOH	GCN	no
	D2S44	control	control	yes

RW 942	MUC1	LOH	AI	?
	D2S44	control	AI	no
	D1S61	LOH	AI	?
	D2S44	control	AI	no
PB 121	D1S81	LOH	LOH	yes
	D2S44	control	control	yes
	DXYS78	LOH	LOH	yes
	D2S44	control	control	yes
PB 124	DXYS89	LOH	LOH	yes
	D2S44	control	control	yes
	DXS278	LOH	LOH	yes
	D2S44	control	control	yes
RW 647	DXYS14	GAL	GAL	yes
	D2S44	control	control	yes
	DXYS28	GAL	GCN	no
	D2S44	control	control	yes
	DXYS17	GAL	GAL	yes
	D2S44	control	control	yes
RW 659	DXS84	i	no AI	yes
	DXS438	LOH	GCN	no
	D2S44	control	control	yes
	DXS278	LOH	GCN	no
RW 663	D2S44	control	control	yes
	DXS84	LOH	LOH	yes
	D2S44	control	control	yes
	DXS255	LOH	LOH	yes
RW 926	D2S44	control	control	yes
	DXYS78	LOH	LOH	yes
	D2S44	control	control	yes
	DXS438	LOH	GAL	no
RW 661	D2S44	control	control	yes
	DXYS89	LOH	LOH	yes
	D2S44	control	control	yes
	D17S5	LOH	GCN	no
RW 663	D2S44	control	control	yes
	D7S21	LOH	GCN	no
	D20S26	LOH	GCN	no
	D2S44	control	control	yes
RW 682	D7S21	LOH	GAL	no
	D20S26	control	control	yes
	D2S44	GAL	GCN	no
	D20S26	GCN	GCN	yes
RW 686	D2S44	control	control	yes
	D2S44	control	control	yes

Therefore, the densitometry results were in disagreement with the visual analysis for 17/68 loci scanned with two undefinable i.e. 25% inaccuracy (excluding the two undefinable test loci). This is probably mainly due to non-specific and irregular background on the autoradiographs, principally occurring during the long-exposures required for heavily-used filters. This background can cause difficulties for the densitometer when it is uneven over the area under analysis in general and cannot be easily removed from the calculation. Thus spotless films are required for densitometry, but scoring by eye can make allowances for such forms of background. All densitometry results are based on area integrations to establish the intensity of the bands/alleles. Although volume integrations can be carried out on the Molecular Dynamics densitometer, such calculations were not used as some autoradiographs had spots of background in close proximity to a band. Volumes can only be calculated from the selection on the scan of an entire single band and such spots can produce highly inaccurate results with volume integrations. However, area integrations can avoid this difficulty as selections of rectangles to be drawn down the track through all bands/alleles under analysis can be tailored to avoid the spots while enclosing the same proportion of each band. Therefore, in order to maintain uniformity in the mathematics used for all autoradiographs, only area integrations were carried out. However, by being able to select the size (especially the width across the track) of the superimposed rectangle, it is possible to introduce errors since the whole width of a band was not being taken into consideration for the calculation. By selection of different sections of the track, it is possible to vary the intensity produced from the calculation of the bands in these tracks (data not shown). The densitometer also developed difficulties in resolution of some of the allele bands that had clearly separated within a track; the peaks of intensity drawn by the software would become wider along the axis of the track and even merge when highly intense bands were scanned. Allelic imbalance, especially LOH and GAL, in such circumstances was very difficult to quantitate as one band would dominate the line graph, obscuring the minor band. Thus resolution was much reduced and the peak-recognition parameters used in calculation of intensities needed to be re-defined in the programme to try to generate separate bands.

The ability to manipulate the programme reduces the absolute quantitative properties of densitometry and the data generated must be interpreted with caution. Therefore this begins to negate the actual reason for carrying out the densitometric work, namely to produce a clearly-defined set of strictly quantitative values for the intensities of alleles, in order to be able to draw meaningful comparisons between alleles within a single track as well as between

the lymphocyte and tumour tracks. To generate results that were as consistent as possible and reduce the likelihood of variation in interpretation between individuals, only I carried out the visual and densitometric analyses of the autoradiographs. In addition, autoradiographs produced throughout the course of the project were re-scored at intervals to ensure that the interpretation was accurate and consistent.

3.2.5 Chromosomes 13 and 17

Single loci were studied on each chromosome to generate data for the panel on AI at sites in the genome previously associated with breast cancer. The VNTR situated in the sixteenth intron of the RB1 gene at 13q14 has been widely used in loss of heterozygosity studies in tumours, including breast tumours (Devilee *et al.*, 1989, 1991a, 1991d; Varley *et al.*, 1989; Andersen *et al.*, 1992; Borg *et al.*, 1992a; Chen, L.-C. *et al.*, 1992), where LOH of the RB1 gene occurs in 25% to 37% of cases (Devilee *et al.*, 1989; Varley *et al.*, 1989). Therefore the 50% level of allelic loss established in this project is higher than expected where the averaged percentage of LOH for all studies quoted above is 30% (n=289). However, when the figures from this project are incorporated into this average, it is raised only slightly to 32%, thereby not greatly disturbing the overall level of loss at RB1.

The locus D17S5 is a highly informative VNTR mapping to the short arm of chromosome 17 and was used as the second positive control for allelic imbalance in the project. Originally, D17S5 was used as an indicator of LOH for TP53 in tumours because it was included in deletions believed to be targeting the TP53 gene (Cropp *et al.*, 1990; Devilee *et al.*, 1990, 1991a; Thompson *et al.*, 1990; Varley *et al.*, 1991; Merlo *et al.*, 1992; Knyazev *et al.*, 1993), although Devilee *et al.* (1990) and Merlo *et al.* (1992) do state the caveat that another gene may be the target. Coles *et al.* (1990) provided the first evidence that the target of LOH at D17S5 was not in fact TP53. By including an intervening locus in the study, LOH of D17S5 was shown to occur independently of LOH specific to the TP53 locus in some breast tumours. Correlation of allele losses at D17S5 and over-expression of TP53 mRNA (Thompson *et al.*, 1990) therefore suggests that the target close to D17S5 participates in regulation of the TP53 gene (Coles *et al.*, 1990). Further work on 17p with additional markers has confirmed that allele loss distal to the TP53 gene does occur independently of allele loss and/or mutation at TP53 itself (Sato *et al.*, 1990, 1991a; Chen *et al.*, 1991; Andersen *et al.*, 1992; Lindblom *et al.*, 1993).

The high incidence of LOH at D17S5, in up to 69% of breast tumours, made it useful as a positive control for AI in the panel, although it is not targeting a gene characterized at this time. When applied to the panel of patients, LOH at D17S5 occurred at 32% (44% overall AI), which is lower than expected. In fact, there is considerable variation between studies and analysis of a panel of familial breast tumours showed LOH at D17S5 to be only 16% (Lindblom *et al.*, 1993), but the average from published levels is 50% (n=940). When the figures from this project are incorporated into this average, it remains unaffected.

3.3 Discussion

3.3.1 Allelic Imbalance Detection

The results obtained by scanning densitometry were judged to be inconsistent and unrepresentative of the imbalances taking place in the panel of tumours for the reasons detailed above. Therefore, the densitometry data were discarded and the AI results presented in the remainder of this work are derived from visual analysis of the autoradiographs only.

3.3.2 Chromosomes 2, 7 and 20

The locus D2S44 situated on chromosome 2 appears to represent the optimum controls for DNA content in loss of heterozygosity studies of breast tumours due to its relative low level of overall AI in the panel, its heterozygosity with a wide range of restriction endonucleases and its corresponding highly polymorphic nature with these enzymes. It may be that other similarly highly-polymorphic (hypervariable) markers on this chromosome would be also of more use than D7S21 and D20S26 from chromosomes 7 and 20 respectively, which proved to be inferior to D2S44 as a control for rare AI.

Whether, the usefulness of D2S44 (or other chromosome 2 loci) can be extrapolated to PCR analysis of polymorphic markers remains to be seen. A locus predisposing to hereditary non-polyposis colorectal cancer (HNPCC) has been mapped to chromosome 2p15-16 by genetic linkage (Peltomäki *et al.*, 1993). HNPCC is a familial form of colorectal cancer distinct from familial adenomatous polyposis, FAP (Lynch *et al.*, 1985a, 1985b; Lynch *et al.*, 1988). Whilst neither familial nor sporadic cases of colorectal cancer showed LOH at polymorphic

markers on chromosome 2, most of the familial HNPCC cases had widespread instability in microsatellites, principally as changes in repeat length (Aaltonen *et al.*, 1993; Thibodeau *et al.*, 1993). This suggests that numerous replication errors have occurred during tumour development and that the locus on chromosome 2 is not a classical tumour-suppressor gene, whose inactivation is not indicated by LOH. Therefore, PCR studies of breast tumours should be carefully controlled with an awareness of possible microsatellite instability, until a possible exclusion of such a mutator locus acting in breast cancer.

It is clear that chromosomes not previously identified as undergoing allelic imbalance can be subject to AI above the arbitrarily-assigned background threshold. This does not necessarily mean that they harbour genes important to progression of the tumour, but does suggest that AI on chromosomes of interest does actually have to be quite high to be readily identifiable and that the background threshold is never going to be accurately defined.

There has been some interest in LOH on the long arm of chromosome 7, specifically at the MET locus at 7q31 where LOH occurred in 41% of 121 informative breast tumours studied (Bièche *et al.*, 1992). This report was in disagreement with others (Ali *et al.*, 1987; Lundberg *et al.*, 1987; Larsson *et al.*, 1990; Sato *et al.*, 1990; Devilee *et al.*, 1991d; Cooke *et al.*, 1993). The total combined LOH in this region of the chromosome from these other studies is 5% (n=138). When the results of Bièche *et al.* (1992) are also incorporated, the level of LOH increases to 22% (n=259). Although only 36 patients in this project were informative at D7S31 which is situated on the short arm of chromosome 7, the 17% LOH seen here on the short arm is in accordance with the averaged figure on the long arm. Whilst the numbers of informative individuals is vastly different, these figures appear to indicate that chromosome 7 is subject to rearrangements in breast tumours, but that these probably do not confer a growth advantage on the tumours. Bièche *et al.* (1992) claimed correlation of both shorter metastasis-free survival and overall survival with the LOH at a single locus on 7q. Analysis of additional loci on the chromosome would help to clarify the possible significance of these sets of results.

Recently, LOH at the topoisomerase I locus on chromosome 20 in breast cancer has been described (Keith *et al.*, 1993). However, the rate of LOH was 12% (n=17), in accordance with levels determined by allelotype studies of breast tumours (Devilee *et al.*, 1991d), which are also within background. Haematologic tumours do show deletion of chromosome 20q sequences suggesting a tumour-suppressor gene may be located on this chromosome arm (Davis *et al.*, 1984; Le Beau *et al.*,

1985; Morris *et al.*, 1989), and mutations in topoisomerase I have been found that confer resistance to camptothecin, a plant alkaloid with anti-tumour activity, on a human leukaemia cell line (Tamura *et al.*, 1991). However, there is no apparent evidence for the involvement of chromosome 20 sequences in breast cancer, as determined by loss of heterozygosity studies.

3.3.3 Chromosomes 13 and 17

The predominance of allelic loss at the RB1 VNTR and D17S5, in accordance with the literature (Seizinger *et al.*, 1991), suggests that determination of the nature of allelic imbalances was consistently accurate in the project, maintaining confidence in the high incidence of GCN on the long arm of chromosome 1, as well as the lack of this event on the X chromosome in tumours. Although 50% allelic loss at the VNTR in RB1 could suggest over-zealous identification of allelic imbalance in the project as a whole, the 32% level of LOH at D17S5 seems to negate this, as does the fact that all imbalances were fully-controlled for with at least one control locus. The possibility of bias in patient sampling does exist, but would have been outside the deliberate control of this study as all tumours for which there was a corresponding blood sample were employed in the series of patients available. Therefore, these inconsistencies could be attributable to local variation in the samples used, being unlikely to reflect a new trend towards breast cancers developing via a new pathway incorporating RB1-13q14 alteration more frequently than a distal chromosome 17p/TP53 route.

3.3.4 Future Work

From the results obtained with loci on chromosomes 7 and 20, the project may have been better served by a second control locus situated on the short arm of chromosome 2. As D2S44 is mapped to the long arm of chromosome 2, it is less likely that rearrangements of this arm involving D2S44 would affect sequences on the short arm also, unless changes in copy number are responsible for the AI at D2S44 where an entire homologue is involved. This second locus would have to be also highly polymorphic to confirm that there is no imbalance before being used to control for the amounts of DNA between tracks.

Thus, it remains to be decided whether the loci used in this study proved good controls. Given the subjective nature of alleic imbalance studies, it will never be possible to completely control for the imbalances observed on a chromosome of

interest; levels of AI can only be compared to those presented in the literature for a particular locus, or in the case of LOH on the X chromosome, with other chromosomes to see if the levels are above background. The final test of the work is unfortunately, not in the controls but in the isolation of a candidate locus and the testing for mutation and expression patterns between normal and tumour cells. Loss of heterozygosity does not confirm that a tumour-suppressor gene is present and being targeted for deletion; loss of heterozygosity provides regions of interest for gene-hunters to sift through on their way to completing the Human Genome Project. The very complex histology and pathology of breast cancer suggests that the disease does not arise from alterations to only a few genes common to all patients, but to many genes, different subsets of which are altered in each patient. A number of confirmed and candidate tumour-suppressor genes have now been isolated as a result of studies that included deletion mapping, by cytogenetic and/or molecular analyses, namely RB1, DCC, WT1 and APC, thus highlighting the application of deletion mapping in the continuing hunt for further candidates. Even if candidate genes are later disproved, their isolation and characterization is still of relevance to the understanding of the biology of the human body in general.

CHAPTER 4

Allelic Imbalance Studies of the X Chromosome

4.1 Introduction

The X chromosome has been implicated in many genetic diseases and disorders, except cancer. Significant levels of loss of heterozygosity have not been detected on the X chromosome in human tumours (Seizinger *et al.*, 1991) and cytogenetic studies of breast tumours have not focused attention on the X chromosome either. Two LOH studies of breast cancer have used some probes mapping to the X chromosome in a general attempt to obtain an allelotype, finding 25% (n=20) LOH on Xq (Larsson *et al.*, 1990) and 16% on Xp and 6% on Xq (n=44) (Devilee *et al.*, 1991d) respectively.

Using a panel of paired tumour and lymphocyte DNA samples from 71 patients diagnosed with primary breast cancer, loss of heterozygosity for polymorphic markers in the human genome was assessed by Southern hybridization. One probe, pMS639 (DXYS89), a newly isolated minisatellite, was subsequently mapped to the pseudoautosomal region of the X chromosome (Armour *et al.*, 1992). Significant levels of LOH were observed at this locus, suggesting that sequences on the X chromosome are associated with a subset of breast tumours. This prompted a more detailed study of the region that was later extended to the whole of the chromosome, the results of which confirm that the X chromosome is subject to repeated deletion in breast tumours with a number of independent regions along the chromosome targeted for deletion.

4.2 Results

4.2.1 Panel of Breast Cancer Patients and the Probe Bank

The sets of filters and criteria for scoring imbalances used to study allelic imbalance on the X chromosome are detailed in chapter 3, section 3.2.1. The full

results for each of the 71 patients analyzed are listed in Appendix II for the X chromosome.

On average, ten out of a total of 16 loci from the X chromosome were successfully scored per patient, of which (also on average) five per patient were informative. This lower level of informative loci than on chromosome 1 is due to the use of more RFLPs on the X chromosome. The remaining stocks of paired DNA samples, especially from the series of patients RW 860 to RW 942, will allow many of the gaps in the patients' data to be filled in when re-testing can be carried out. However, the higher proportion of RFLPs used means that much of the missing data on the X chromosome will be uninformative in nature.

Examples of the hybridization patterns observed with each probe are given in figures 4.1 to 4.7. Summaries of the results obtained for each locus on the X chromosome are presented in table 4.1 and figure 4.8. Simple LOH was the primary form of allelic imbalance observed for X chromosome probes, with only two cases showing GAL. No GCN has been established on this chromosome, a distinct contrast to the chromosome 1 study. Five patients show indeterminate AI events on the X chromosome as the control(s) used were found to also undergo imbalance in these tumours.

4.2.2 Loss of Heterozygosity in the Pseudoautosomal Region: Evidence for Two Discrete Regions of Loss

DXYS89 is situated near to the boundary between the pseudoautosomal region and the sex-specific region of the X chromosome, closely linked to the DXYS17 locus, although their precise order is presently unclear. Loss of heterozygosity occurs at DXYS89 in seven patients (table 4.1 and figure 4.8), although only 27% of the panel tested are informative at this locus. Therefore, a closer investigation of the X chromosome was merited. Initially, additional loci mapping to the pseudoautosomal region were assessed and the extent of LOH on the X chromosome in the panel confirmed the results of DXYS89.

Overall, 43% of patients informative at one or more pseudoautosomal loci show LOH in the pseudoautosomal region (table 4.1). There are a number of tumours with small deletions within the pseudoautosomal region (figure 4.9), defining two discrete areas of loss. PB 137, RW 894 and RW 898 each have a solitary LOH event at DXYS78 immediately bordered on either side by DXYS14 and DXYS28 which are informative and retained. JG and RW 869 also display LOH at DXYS78

Note to figures 4.1 to 4.7: Each of the figures presents photographs of autoradiographs for the loci studied on chromosome 1. Overall, 15 of the total 16 loci assessed on the chromosome are represented (DXS101 was uninformative in all of the nine patients successfully tested with the probe). The allele sizes given in each of the legends are estimations. Two invariant bands were produced by the pYNH24 probe in all tracks, 1.0kb and 0.8kb in size, but are not always shown in the photographs. Invariant bands were also observed with other probes, as indicated in the appropriate figure legends.

u, uninformative

i, informative and unaffected by any imbalance

LOH, informative and showing loss of heterozygosity

GAL, informative and undergoing a concomitant gain and loss of alleles

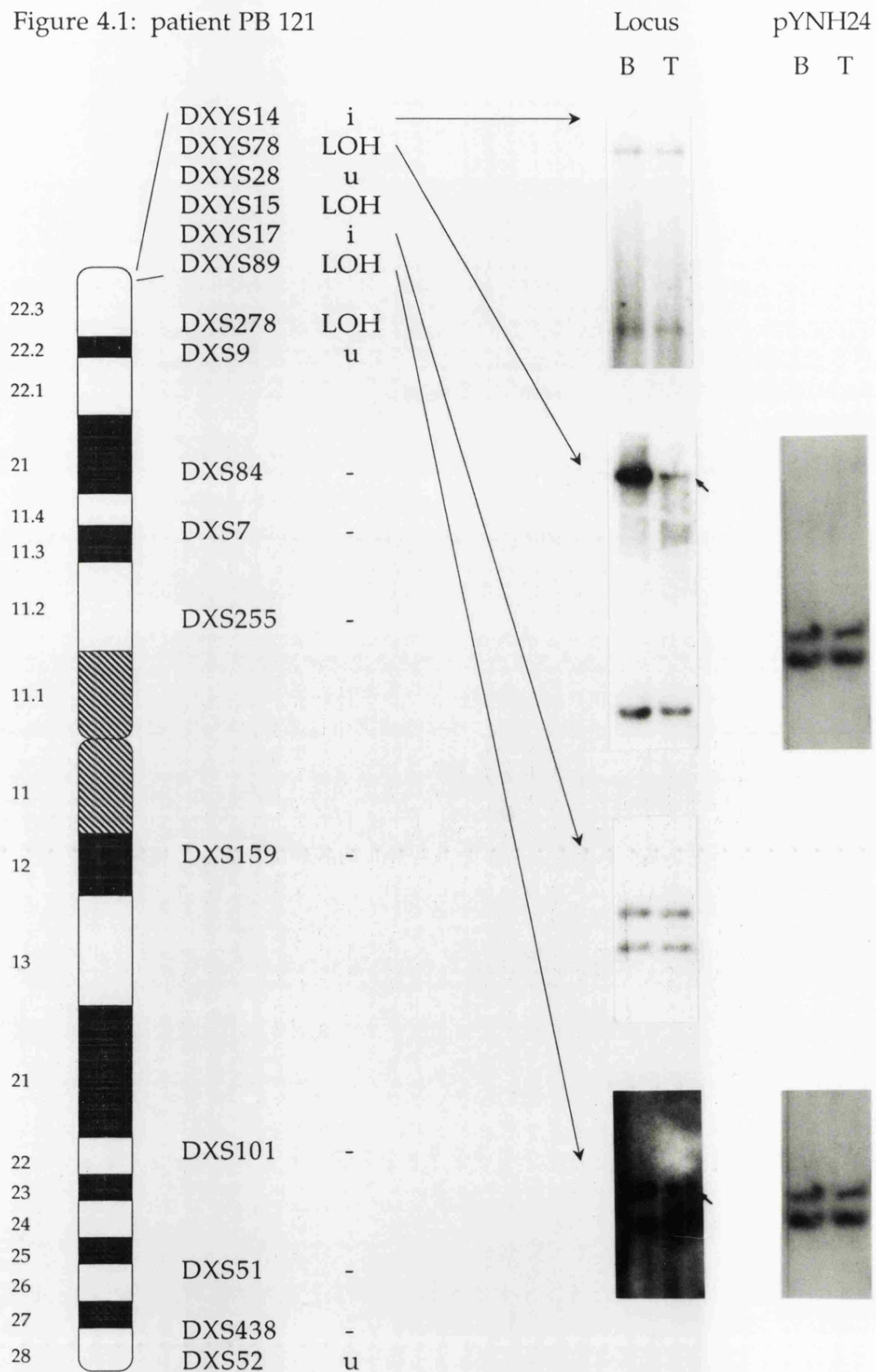
GCN, informative and showing a gain in copy number of one allele

AI, informative and undergoing allelic imbalance, the nature of which has not been determined

Legend to figure 4.1: Autoradiographs of four loci on the X chromosome from PB 121. Each was informative (Locus column), of which two show LOH. For those showing imbalance, the corresponding control with D2S44 is shown in the pYNH24 column. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. Arrows indicate the allele of reduced intensity in the tumour lanes for DXYS78 and DXYS89.

	Locus		Control (pYNH24)	
	Upper allele	Lower allele	Upper allele	Lower allele
DXYS14	2.5kb	1.5kb	n/a	n/a
DXYS78	12.0kb	2.7kb	0.6kb	0.4kb
DXYS17	1.4kb	1.2kb	n/a	n/a
DXYS89	4.5kb	4kb	0.6kb	0.4kb

Figure 4.1: patient PB 121

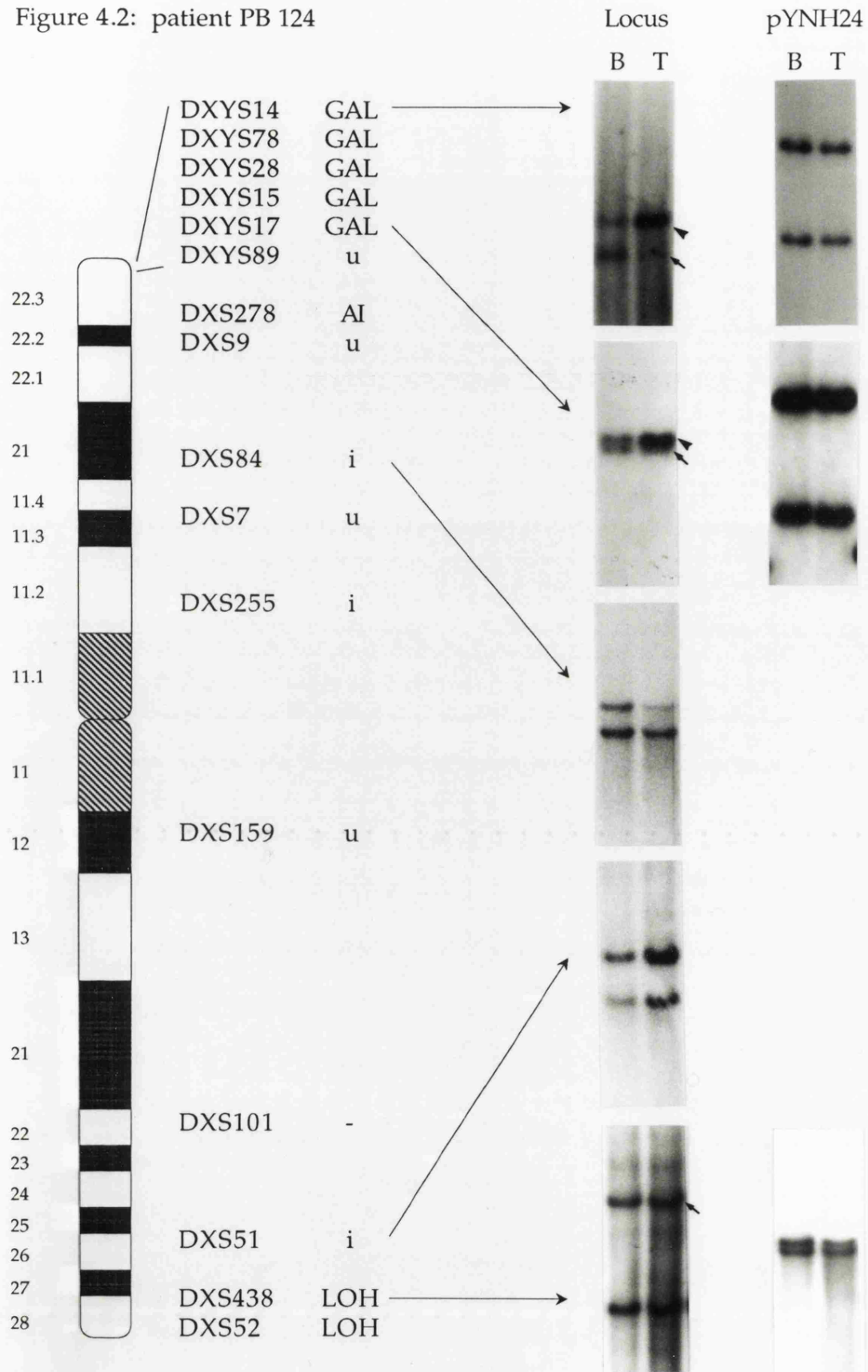


Legend to figure 4.2: Autoradiographs of five loci on the X chromosome from PB 124. Each was informative (Locus column), of which two show GAL and a third shows LOH. For those showing imbalance, the corresponding control with D2S44 is shown in the pYNH24 column. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. Arrows indicate the allele of reduced intensity in the tumour lanes for DXYS14, DXYS17 and DXS438; arrowheads indicate the allele gained in the tumour lanes for DXYS14 and DXYS17. Locus DXS84 is designated as i (informative and unaffected) since the ratio between the alleles in the T lane is very similar to that in the B lane, as confirmed by the laser scanning densitometry for this locus presented in Appendix IV.

	Locus		Control (pYNH24)	
	Upper allele	Lower allele	Upper allele	Lower allele
DXYS14	1.7kb	1.4kb	5.0kb	2.3kb
DXYS17	1.2kb	1.15kb	2.4kb	1.9kb
DXYS84	12.0kb	9.0kb	n/a	n/a
DXS51	1.5kb	1.2kb	n/a	n/a
DXS438 ^a	5.0kb	2.7kb	9.5kb	9.0kb

^a DXS438 has two invariant bands in all tracks, 1.7 and 0.8kb (not shown).

Figure 4.2: patient PB 124

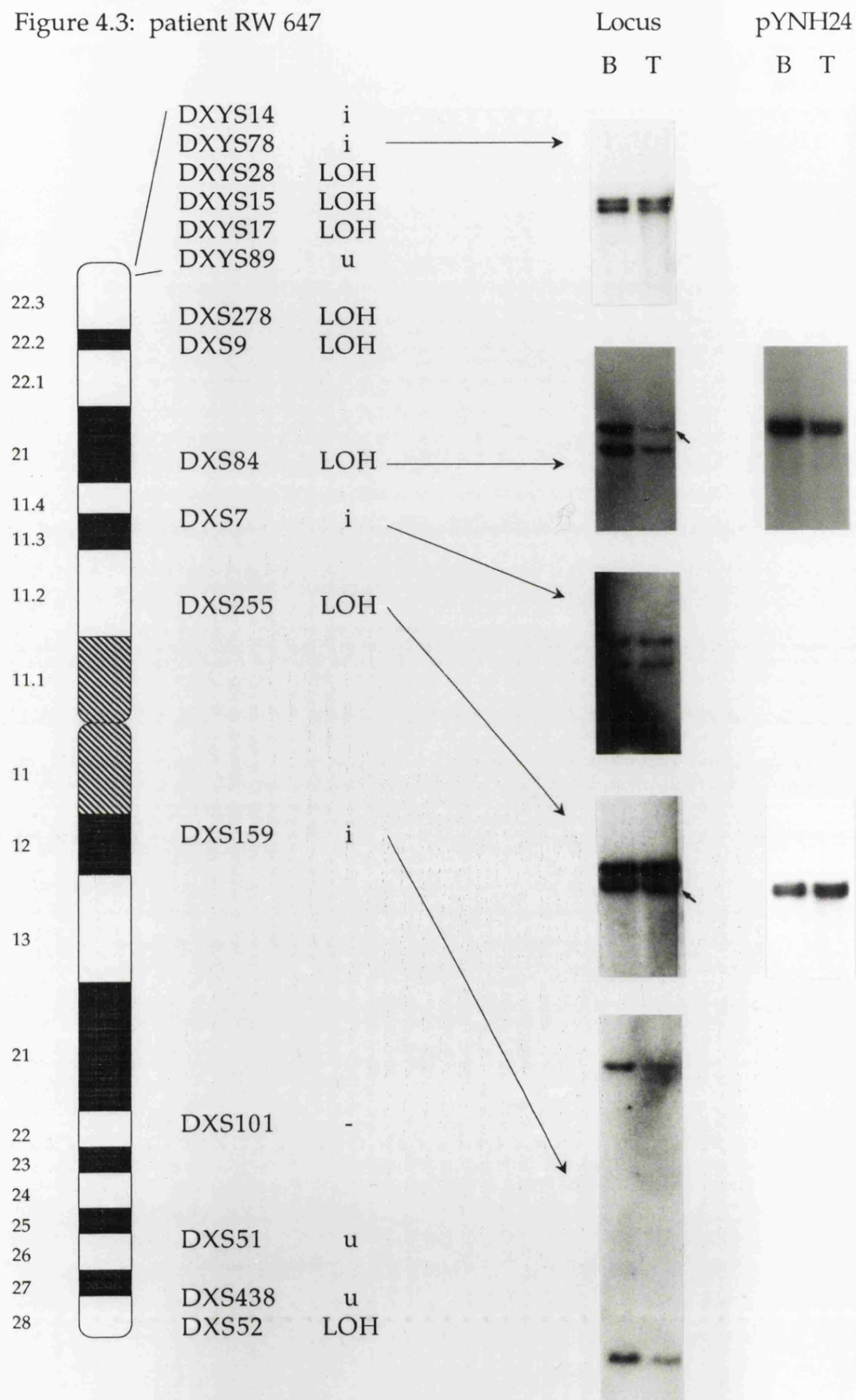


Legend to figure 4.3: Autoradiographs of five loci on the X chromosome from RW 647. Each was informative (Locus column), of which two show LOH. For those showing imbalance, the corresponding control with D2S44 is shown in the pYNH24 column. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. Arrows indicate the allele of reduced intensity in the tumour lanes for DXS84 and DXS255.

	Locus		Control (pYNH24)	
	Upper allele	Lower allele	Upper allele	Lower allele
DXYS78	21.0kb	20.0kb	n/a	n/a
DXS84	12.0kb	9.0kb	15.0kb	14.5kb
DXS7	12.0kb	9.0kb	n/a	n/a
DXS255	7.4kb	6.2kb	8.5kb ^a	n/a
DXS159	5.5kb	1.6kb	n/a	n/a

^a considered as a single band in each track as resolution of the alleles was not sufficient and is thus regarded as uninformative on this particular filter.

Figure 4.3: patient RW 647



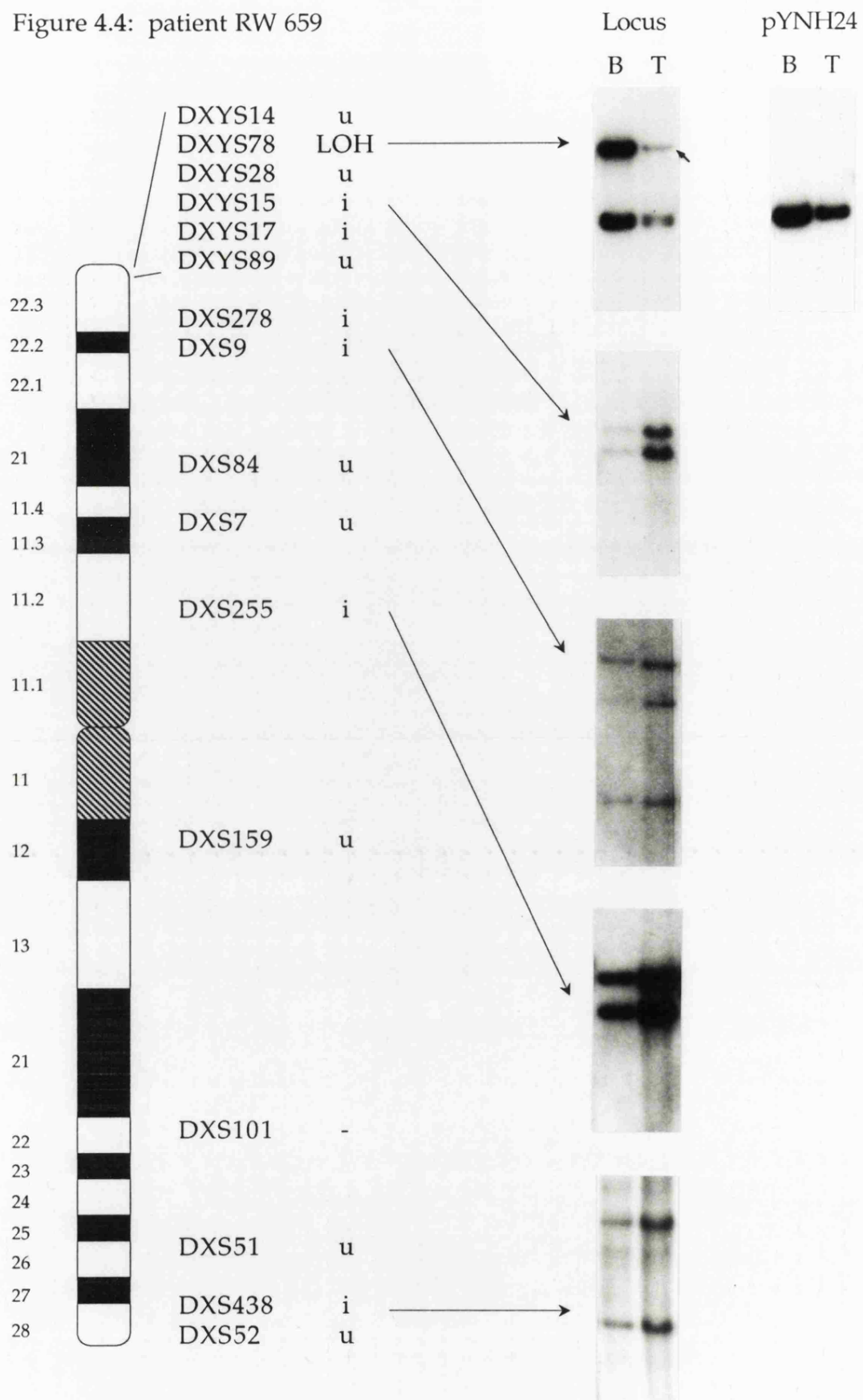
Legend to figure 4.4: Autoradiographs of five loci on the X chromosome from RW 659. Each was informative (Locus column), of which only one shows LOH. For the locus showing imbalance, the corresponding control with D2S44 is shown in the pYNH24 column. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. An arrow indicates the allele of reduced intensity in the tumour lane for DXYS78.

	Locus		Control (pYNH24)	
	Upper allele	Lower allele	Upper allele	Lower allele
DXYS78	21.0kb	12.0kb	2.2kb	n/a
DXYS15	2.5kb	2.3kb	n/a	n/a
DXS9 ^a	5.3kb	3.2kb	n/a	n/a
DXS255	4.8kb	4.4kb	n/a	n/a
DXS438 ^b	5.0kb	2.7kb	n/a	n/a

^a DXS9 has an invariant band in all tracks, 6.6kb (shown, largest band).

^b DXS438 has two invariant bands in all tracks, 1.7 and 0.8kb (not shown).

Figure 4.4: patient RW 659



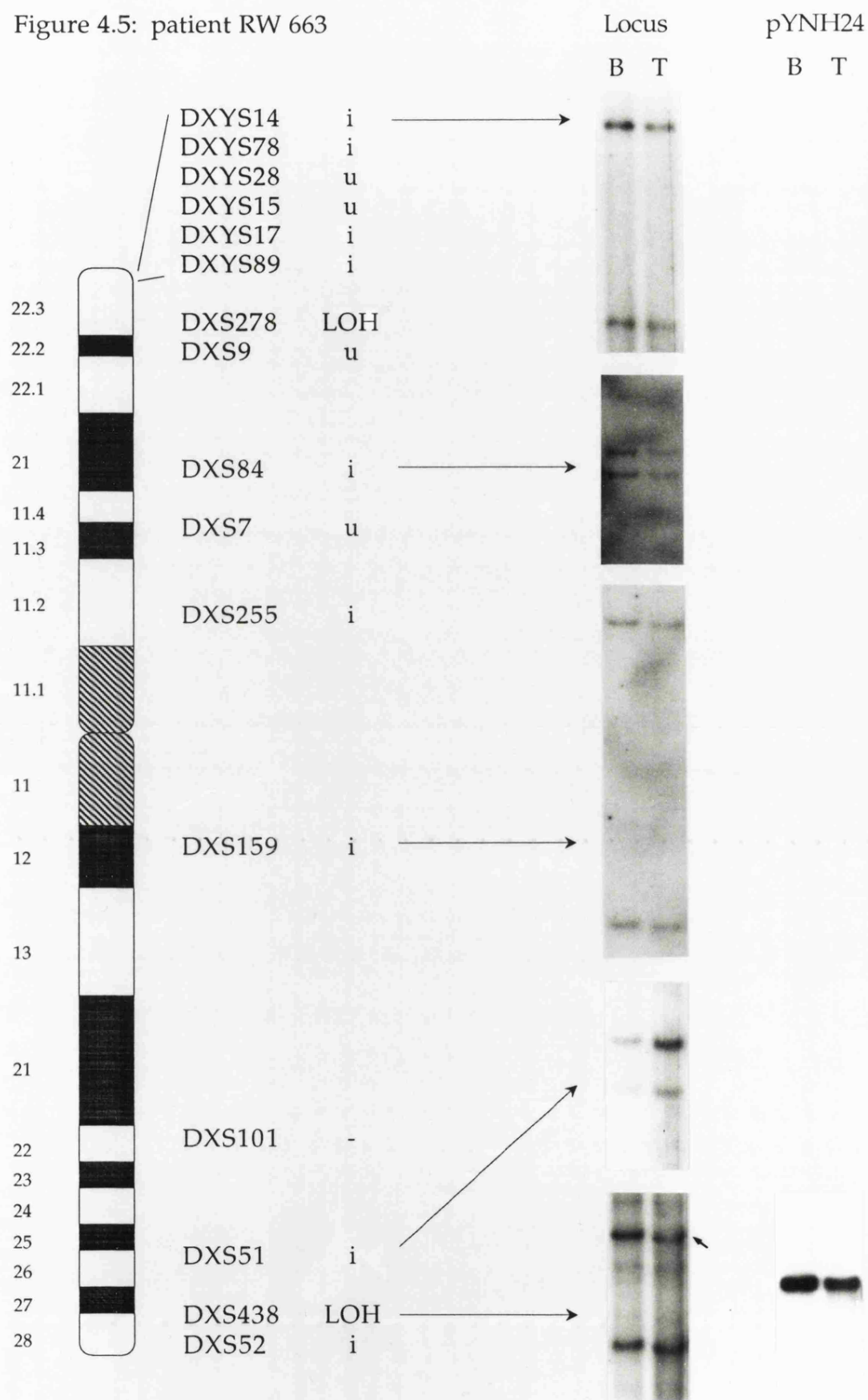
Legend to figure 4.5: Autoradiographs of five loci on the X chromosome from RW 663. Each was informative (Locus column), of which one shows LOH. For the locus showing imbalance, the corresponding control with D2S44 is shown in the pYNH24 column. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. An arrow indicates the allele of reduced intensity in the tumour lane for DXS438.

	Locus		Control (pYNH24)	
	Upper allele	Lower allele	Upper allele	Lower allele
DXYS14	4.0kb	1.0kb	n/a	n/a
DXS84	12.0kb	9.0kb	n/a	n/a
DXS159	5.5kb	1.6kb	n/a	n/a
DXS51 ^a	1.5kb	1.2kb	n/a	n/a
DXS438 ^b	5.0kb	2.7kb	13.0kb	n/a

^a DXS51 has one invariant band in all tracks, 6.0kb (not shown).

^b DXS438 has two invariant bands in all tracks, 1.7 and 0.8kb (not shown)

Figure 4.5: patient RW 663

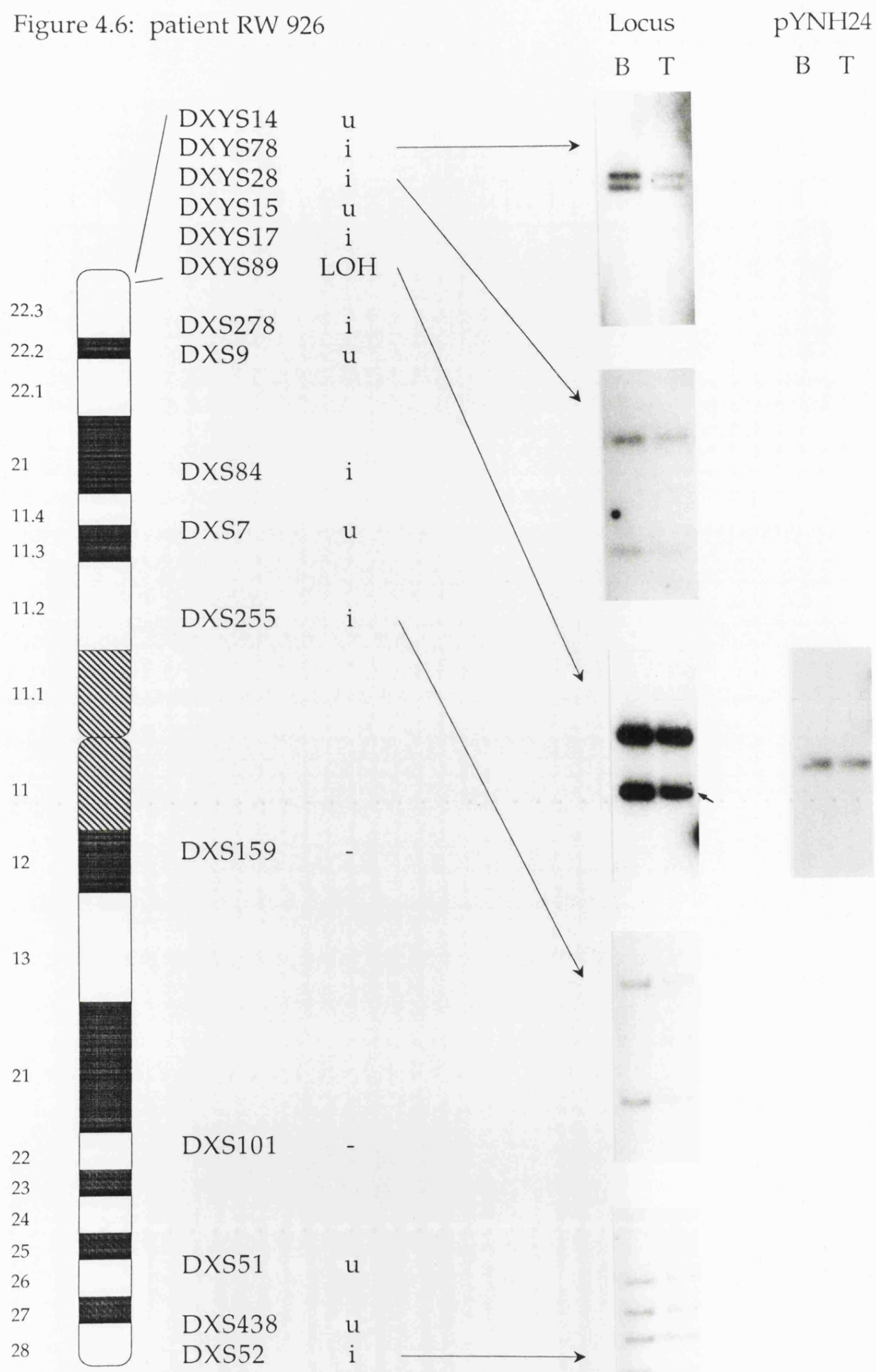


Legend to figure 4.6: Autoradiographs of five loci on the X chromosome from RW 926. Each was informative (Locus column), of which one shows LOH. For the locus showing imbalance, the corresponding control with D2S44 is shown in the pYNH24 column. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. An arrow indicates the allele of reduced intensity in the tumour lane for DXYS89.

	Locus		Control (pYNH24)	
	Upper allele	Lower allele	Upper allele	Lower allele
DXYS78	6.0kb	5.7kb	n/a	n/a
DXYS28	2.0kb	1.3kb	n/a	n/a
DXYS89	6.1kb	4.6kb	1.1kb	n/a
DXS255	6.6kb	3.8kb	n/a	n/a
DXS52 ^a	3.6kb	2.8kb	n/a	n/a

^a DXS52 has four invariant bands in all tracks, 4.1, 2.6 (both shown, largest and smallest bands), 1.9 and 1.5kb (neither shown).

Figure 4.6: patient RW 926



Legend to figure 4.7: Autoradiographs of the DXS278 locus on the X chromosome from three patients PB 121, RW 647 and RW 659. Each individual displayed variant multiple bands and are thus regarded as informative (Locus columns), of which two patients show an apparent reduction in intensity of some bands in the tumour lanes, designated as LOH. The corresponding control with D2S44 is shown in the pYNH24 column for each of the three patients. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. Arrows indicate the principal bands of reduced intensity in the tumour lanes for PB 121 and RW 647.

	DXS278	Control (pYNH24)	
	Alleles indeterminate	Upper allele	Lower allele
PB 121	Band sizes	0.6kb	0.4kb
RW 647	range between	2.7kb	1.3kb
RW 659	20.0 and 0.4kb	1.35kb	n/a

Figure 4.7: DXS278

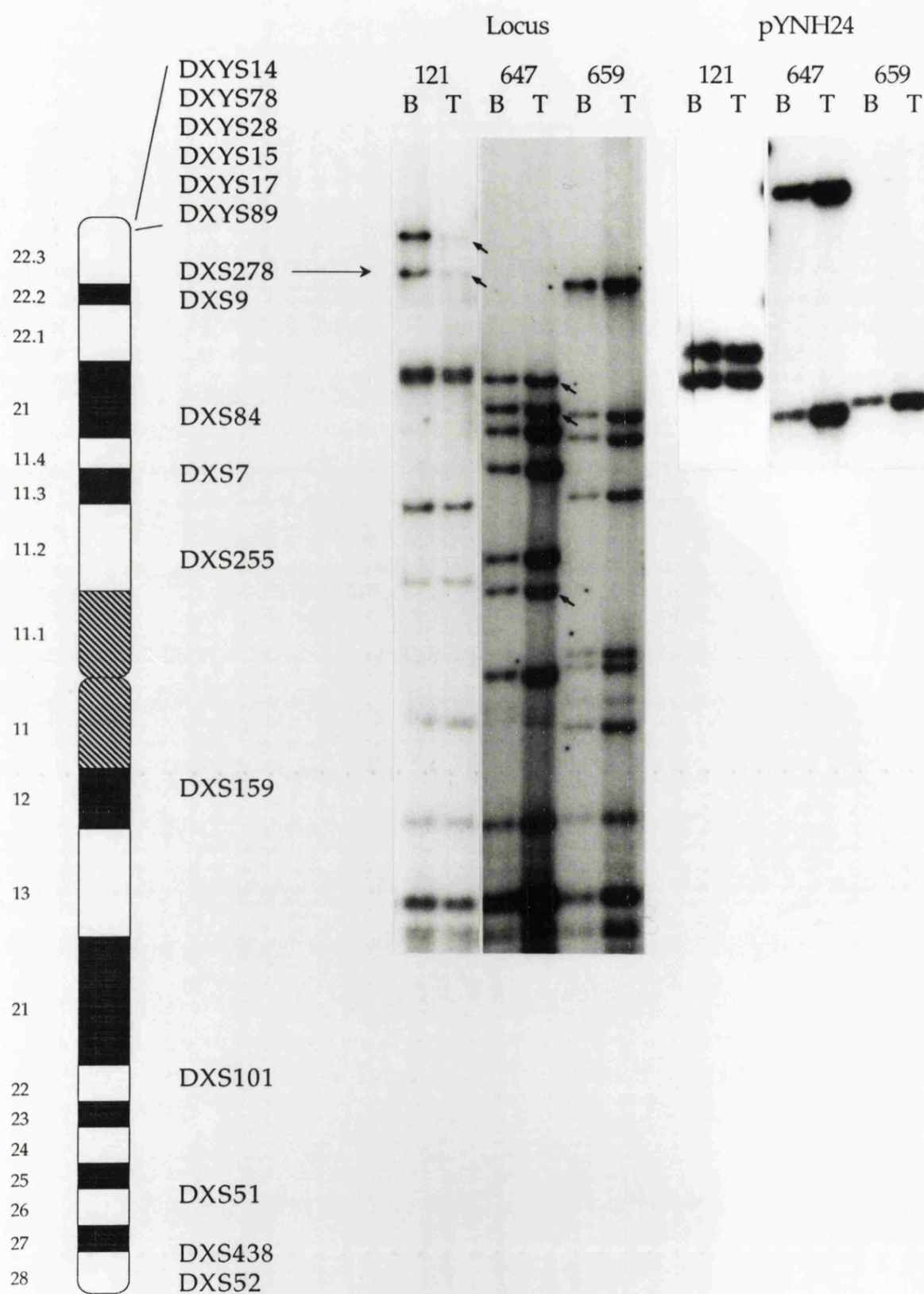


Table 4.1: Summary of probes used in the study and data on allelic imbalance (AI) on the X chromosome for each locus with overall totals for the pseudoautosomal region, Xp, Xq and the whole chromosome. To determine the nature of the imbalance, the locus D2S44 (pYNH24) was used primarily as a control for the amounts of DNA in each track of the autoradiograph. In some instances, two other control loci, D7S21 (pMS31) and D20S26 (pMS617) were used. pYNH24, pMS31 and pMS617 showed a polymorphism in *Hinf*I-, *Mbo*I- and *Taq*I-digested DNA; pYNH24 also showed a polymorphism in *Msp*I- and *Pst*I-digested DNA.

a Allelic loss (patients undergoing LOH or GAL - see text) shown as a fraction of the number of patients informative at the locus and expressed as a percentage in parentheses.

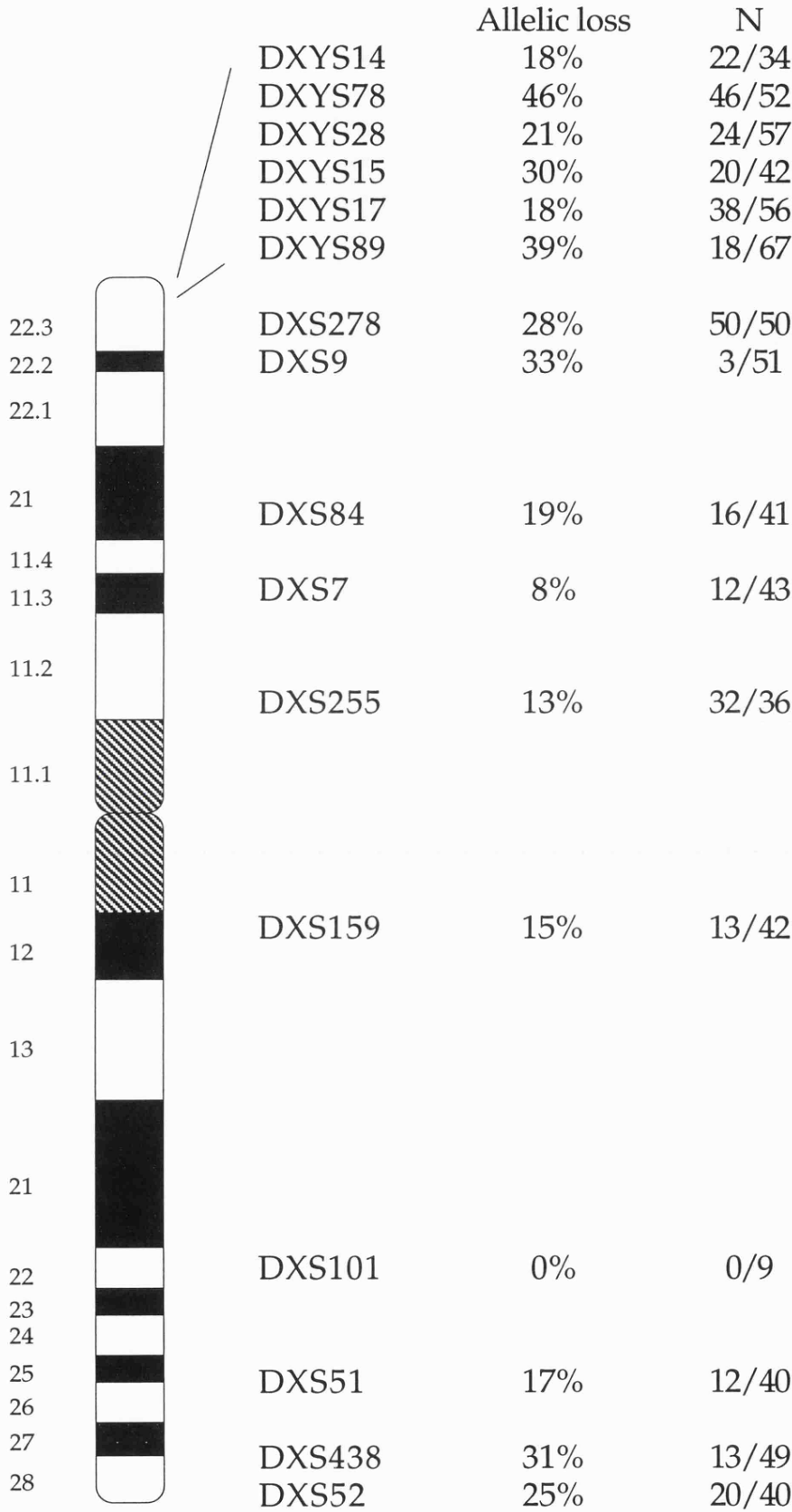
b The number of patients for which allelic imbalance had clearly occurred at each locus but it was impossible to establish the nature of the imbalance (i.e. whether LOH/GAL/GCN) due to AI at the control locus/loci in those patients.

c The total number of patients undergoing any form of imbalance as a fraction of the number of patients informative at the locus and expressed as a percentage in parentheses. No patients showed GCN - see text.

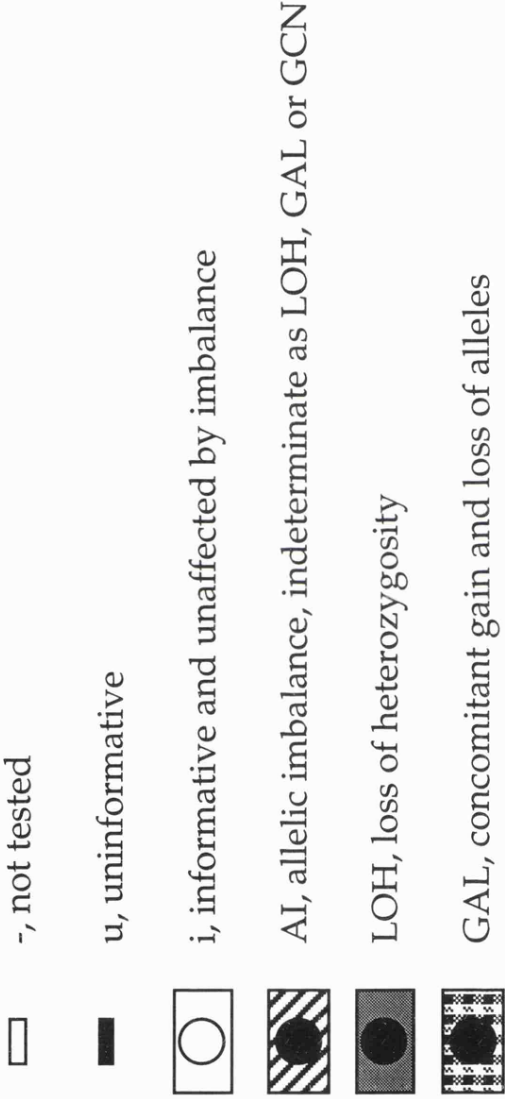
Locus	Probe	Position	Total number of patients	Patients with allelic loss (%) ^a	Unclear loss or gain ^b	Patients with imbalance (%) ^c
DXYS14	29C1	Xp22.3	34	4/22 18%	0/22	4/22 18%
DXYS78	pMS600	Xp22.3	52	21/46 46%	1/46	22/46 48%
DXYS28	pDP411a	Xp22.3	57	5/24 21%	1/24	6/24 25%
DXYS15	113D	Xp22.3	42	6/20 30%	1/20	7/20 35%
DXYS17	602	Xp22.3	56	7/38 18%	1/38	8/38 21%
DXYS89	pMS639	Xp22.3	67	7/18 39%	0/18	7/18 39%
DXS278	CRI-pS232	Xp22.3	50	14/50 28%	1/50	15/50 30%
DXS9	RC8	Xp22.3-p22.2	51	1/3 33%	0/3	1/3 33%
DXS84	754	Xp21.1	41	3/16 19%	0/16	3/16 19%
DXS7	L1.28	Xp11.4-p11.3	43	1/12 8%	1/12	2/12 17%
DXS255	M27b	Xp11.3-cen	36	4/32 13%	0/32	4/32 13%
DXS159	cpX289	Xq12	42	2/13 15%	0/13	2/13 15%
DXS101	cX52.5	Xq22	9	0/0	0/0	0/0
DXS51	p52A	Xq26.2-q26.3	40	2/12 17%	0/12	2/12 17%
DXS438	pMS613	Xq	49	4/13 31%	0/13	5/15 38%
DXS52	St14-1	Xq27-q28	40	5/20 25%	3/20	7/20 35%
<hr/>						
Pseudoautosomal region (PAR)				26/61 43%		26/61 43%
Chromosome Xp excluding PAR				18/54 33%		19/54 35%
Chromosome Xp including PAR				29/63 46%		31/63 49%
Chromosome Xq				10/34 29%		13/34 38%
X chromosome				30/63 48%		35/63 56%

Legend to figure 4.8: Loci studied on the X chromosome and a summary of the allelic imbalances observed. Allelic loss includes LOH and GAL since both are regarded as indicators of tumour-suppressor gene inactivation. No GCN (allelic gain) was observed at any locus on the X chromosome. N = number of informative patients / total number screened.

Figure 4.8



Note to figures 4.9 to 4.19: Each of the figures includes all patients that are compatible with the alteration presented in the title of the figure. A number of patients conform to more than one of the different alterations and are therefore shown in more than one figure. In figures 4.9 to 4.14, which present data on the smallest common regions of overlapping deletion, the area in question is indicated by the shading on the general plan of the X chromosome for each.



bordered immediately proximal by DXYS28, but as DXYS14 was unsuccessfully tested in the former and uninformative in the latter, strictly the deletion extends to the telomere in these cases, as in RW 659. Tumours RW 659 (figure 4.4) and PB 103 appear to have slightly larger deletions encompassing the telomere and strictly extending to include DXYS28 which was uninformative in both cases. The smallest common region of overlap between these tumours is therefore firmly centred on the DXYS78 locus. The retention of the most proximal locus, DXYS14, suggests that the LOH occurring so near to the tip of Xp is not a result of telomeric instability (telomere erosion) extending proximally into possible coding sequences on the chromosome arm.

RW 926 and RW 875 (figures 4.6 and 4.9) define a second distinct region of deletion probably also within the PAR, where the LOH event at DXYS89 is flanked immediately by loci that are informative and retained (DXYS17 and DXS278) due to the order of loci being

pter- DXYS15 - DXYS17 - DXYS89 - DXS278 - cen.

DXYS17 and DXYS89 are situated close to the pseudoautosomal boundary and DXS278, the next proximal locus studied, maps outside the PAR in the sex-specific sequences. Therefore it is possible that the target of this second deletion is not actually pseudoautosomal, being situated in the sex-specific sequences of Xp22.3. However, the exact orientation of DXYS17 and DXYS89 has not yet been established and the order of loci could be reversed to be

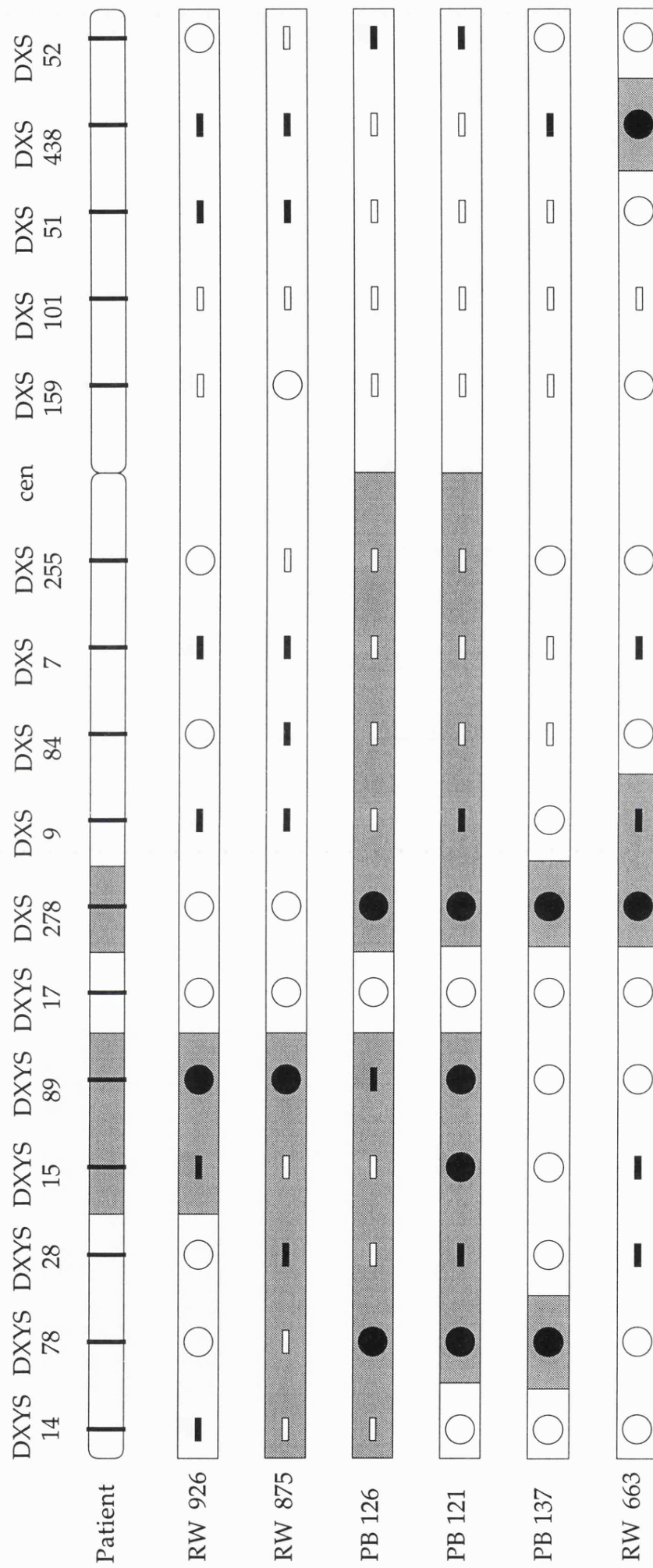
pter- DXYS15 - DXYS89 - DXYS17 - DXS278 - cen.

If this is the case then the second deletion is restricted to the pseudoautosomal region by DXYS17 and is only defined by RW 926 as shown in figure 4.10.

Additional tumours have much larger deletions spanning most of the 2.6Mb of the pseudoautosomal region (figure 4.11), frequently retained within the PAR by DXYS17. Such tumours include PB 121, PB 5, PB 40, PB 126 and RW 860. In two cases, PB 78 and PB 121 (figure 4.1), telomeric sequences are specifically excluded from the deletions, again confirming that at least some of the deletions involving the PAR are not the result of telomere erosion, but are likely to be significant events. The common region of overlap in these patients encompasses the distal PAR deletion at DXYS78.

From table 4.1, there are two definable peaks of allelic loss in the pseudoautosomal region flanked by reduced levels of loss which correspond to those loci identified by deletion mapping. DXYS78 has a peak of 46% allelic loss (48% total AI) flanked by levels of 18% allelic loss (18% total AI) and 21% loss (25% total AI) at DXYS14 and DXYS28, respectively. Towards the

Figure 4.10: Tumours for which the order of DXYS17 and DXYS89 is important in defining two independent regions of overlap at DXYS89 and DXS278, separated by DXYS17. Reversal of DXYS17 and DXYS89 to the order pter - DXYS89 - DXYS17 - DXS278 - cen.



pseudoautosomal boundary, the second peak is at DXYS89 with 39% allelic loss (39% total AI) also flanked by reduced levels whichever the orientation of DXYS17 and DXYS89 with respect to other loci on the X chromosome. The 28% allelic loss (30% total AI) at DXYS15 does not correspond to any defined smallest common region of deletion and is due to the larger deletions occurring within the PAR.

Because of the indefinite order of DXYS89 and DXYS17 and the possibility of deletions extending into the sex-specific sequences of Xp, such as in PB 78 and PB 121, it was decided to analyze sex-specific loci on the X chromosome to try to map the limits of these deletions.

4.2.3 Loss of Heterozygosity in the Sex-Specific Region of the X Chromosome: Additional Small Discrete Regions of Loss Identified

Using well-mapped polymorphic markers sited on Xp and Xq, the panel was examined for LOH in the sex-specific DNA. The probe CRI-pS232 recognizes a complex locus (DXS278) on Xp and autoradiographs show multiple bands in each track. In the tumour DNA tracks of 15 patients, there appeared to be a change in intensity of a number of bands with respect to those in the lymphocyte lane (figure 4.7). Although the imbalance observed at this locus is difficult to score because haplotypes cannot be determined, it indicates that there is a region of deletion outside the pseudoautosomal region, with tumours RW 663 and PB 137 mapping the limits of the area to this locus. The orientation of DXYS17 and DXYS89 is important in determining whether this site of loss has the same target as the loss identified at DXYS89. If the loci are ordered

pter- DXYS15 - DXYS89 - DXYS17 - DXS278 - cen,

then they are independent of each other because DXYS17 intervenes in a number of tumours (figure 4.10). However, if the locus order is reversed,

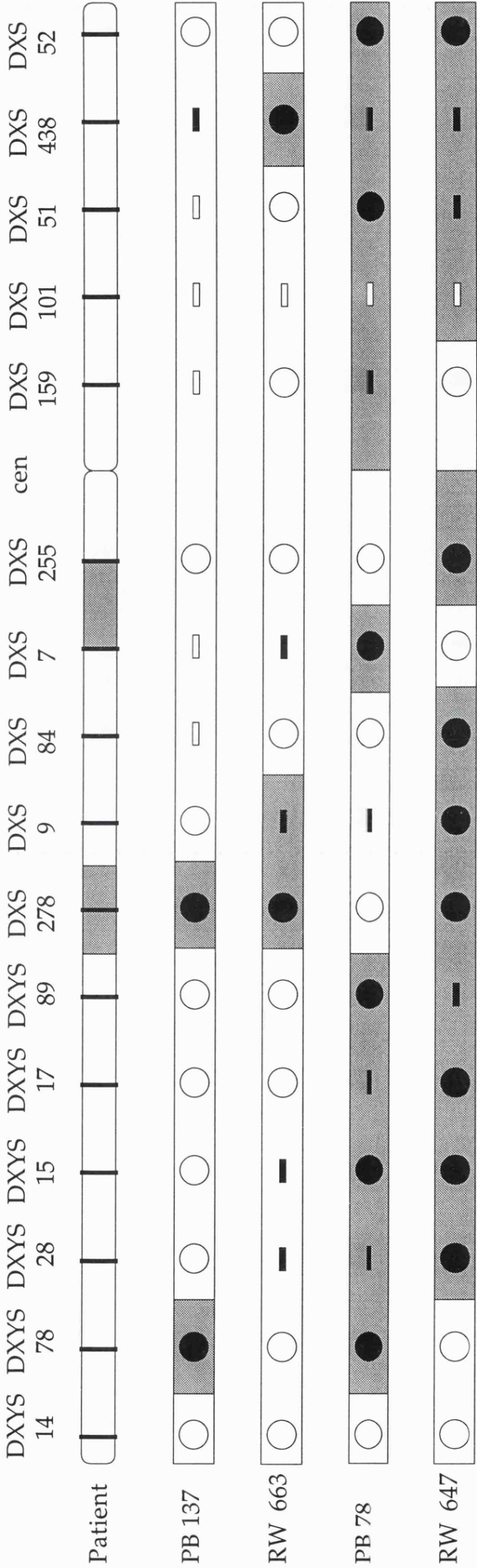
pter- DXYS15 - DXYS17 - DXYS89 - DXS278 - cen,

then this site of deletion appears to overlap with the second pseudoautosomal common region of deletion, so that the sequence targeted maps between DXYS89 and DXS278 (figures 4.9, 4.11 and 4.12). It is still possible that this sequence is situated within the PAR close to the boundary and this is the most parsimonious analysis of the data available. Hence, the results are listed in Appendix II under the order

pter - DXYS15 - DXYS17 - DXYS89 - DXS278 - cen,

as are the majority of the figures in this chapter.

Figure 4.12: Small deletions on the short arm of the X chromosome in the sex-specific sequences.



PB 78 also has a distinct region of LOH at DXS7 bounded immediately by loci that are informative and retained which could overlap with the small deletion of RW 647 at DXS255 (figures 4.3 and 4.12). However, as the X chromosome appears to be badly fragmented in both these tumours, the site may not be of significance.

Loci mapping to the long arm of the X chromosome also define small independent areas of deletion (figure 4.13). The loss at DXS159 in RW 870 is bordered by the centromere (though strictly, it is the DXS255 locus that forms the boundary) and DXS51. The second area of discrete loss on Xq is defined by three tumours, RW 663 (figure 4.5), PB 106 and PB 124. Although the order of DXS438 and DXS52 is unknown, they map close to one another. Their precise order is not currently essential as the deletions are all bounded proximally by DXS51, as shown by those tumours re-drawn in the alternative orientation (figure 4.14) and do not overlap with any other area of LOH. However, any further study in this area of the chromosome will require a precise orientation of DXS52 and DXS438 with respect to other loci.

4.2.4 Loss of Heterozygosity on the X Chromosome: Gross Deletions

Many of the large-scale deletions identified on Xp are known to encompass the pseudoautosomal region, but their proximal limits are still unknown due to loci that are unsuccessfully tested or are uninformative (figure 4.15). The centromere must therefore serve as the borderline in PB 80, RW 628 and PB 134, suggesting that whole arm losses have occurred in some cases. Both PB 80 and PB 96 appear to retain the most terminal short arm locus, DXYS14, despite gross deletions involving sequences further proximal.

RW 872 may have lost most of the X chromosome from the tumour cells (figure 4.16) and the region of the short arm that is retained would be translocated onto another chromosome. It is possible that the two loci with LOH define independent areas of loss which would correspond to two discrete small regions identified: DXS255 loss near to the proximal losses of PB 78 and RW 647, and DXS438 loss correlating to the distal Xq loss identified in RW 663, PB 106 and PB 124 (figure 4.2).

Three tumours appear to have highly fragmented X chromosomes: PB 40, RW 647 and PB 78 (figure 4.17). The precise limits of the majority of their deletions have not been determined and so maximal borders have been drawn. This highlights a major disadvantage of using RFLPs for deletion mapping, as many of

Figure 4.15: Tumours with large-scale deletions of the short arm of the X chromosome.

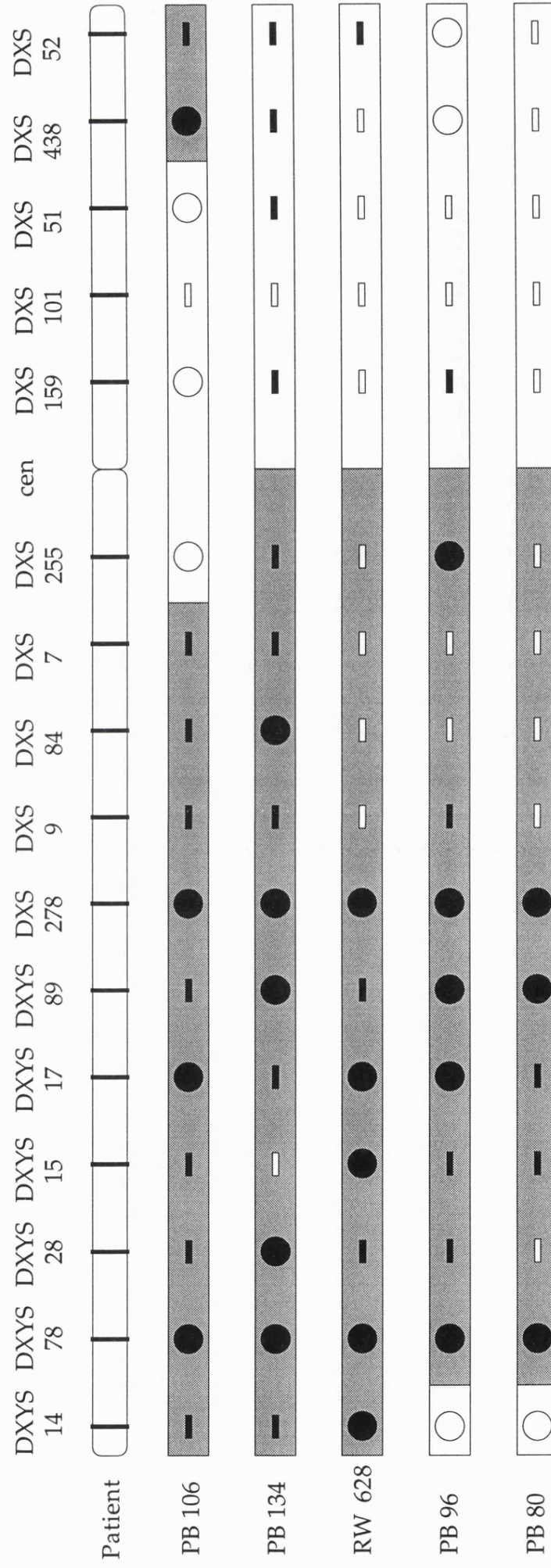
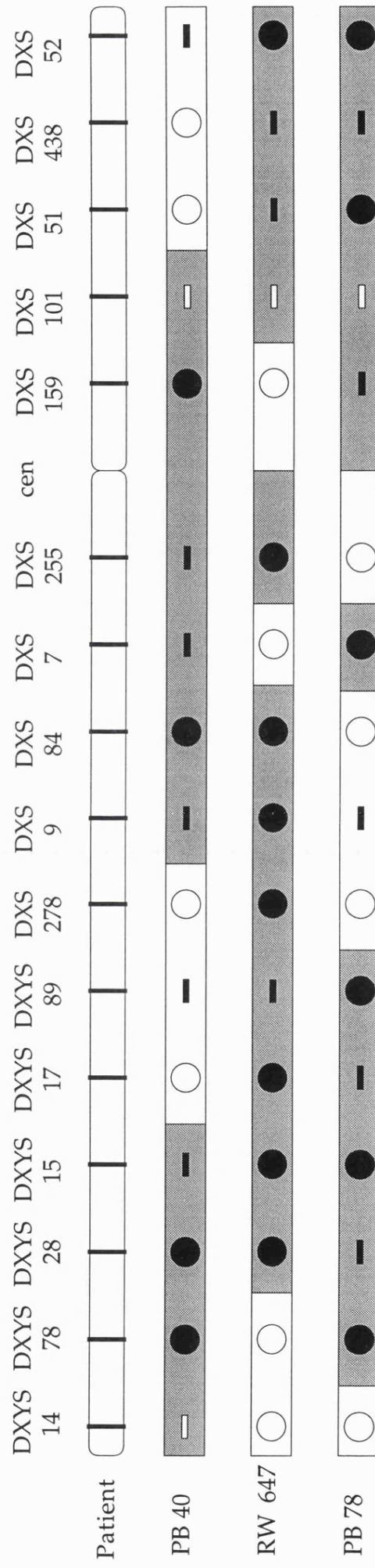


Figure 4.17: Tumours with highly-fragmented X chromosomes.



the critical loci are uninformative. PB 40 could well retain the centromeric sequences so that three small discrete deletions have occurred: one within the PAR, a second on proximal Xp near to the area defined in PB 78 at DXS7, and a third on proximal Xq corroborating this region in RW 870. RW 647 is a similar case, although the tumour has a large deletion precisely mapped that straddles the pseudoautosomal boundary for some distance in either direction (figure 4.3). The LOH on Xq in RW 647 could well be localized to the tip of the arm, as could be the case for PB 78.

Whole chromosome loss is suspected in a single patient only, RW 690 (figure 4.18), although the uninformative loci in this patient could be masking breakpoints in the DNA where the chromosome is fragmented and areas are retained by the tumour genome.

4.2.5 Concomitant Gain and Loss of Alleles on the X Chromosome: Two Patients Identified

GAL occurs in two patients on the X chromosome, PB 124 and RW 920 (figures 4.2 and 4.19). In PB 124, the event appears to involve most of the short arm and is bounded most proximally by DXS84 which is informative and retained. Surprisingly, a small area of LOH is discernable at the tip of the long arm which is bounded by DXS51. GAL is localized to the terminal cytogenetic band Xp22.3 in RW 920, and possibly restricted to the pseudoautosomal region. Both tumours therefore appear to have undergone somatic recombination on Xp to produce these specific imbalances.

4.3 Discussion

4.3.1 Loss of Heterozygosity on the X Chromosome

The establishment of common regions of deletion restricted to the pseudoautosomal region of the X chromosome in so many tumours suggests that there are specific sequences targeted for deletion in breast cancer here. The possibility that one or more tumour-suppressor genes do map to the X chromosome raises interesting questions about gene dosage. The extra copy of X-located genes in females is compensated for by the random inactivation of one X chromosome in female cells (Lyon, 1961). A tumour-suppressor gene located in

Figure 4.18: A single tumour with the whole loss of one copy of the X chromosome.

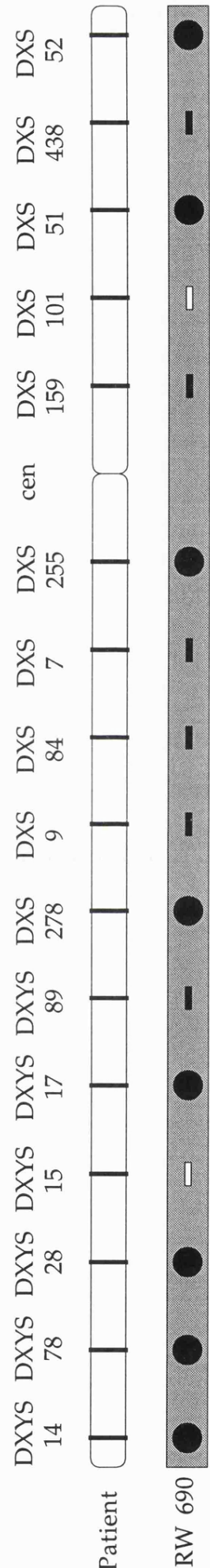
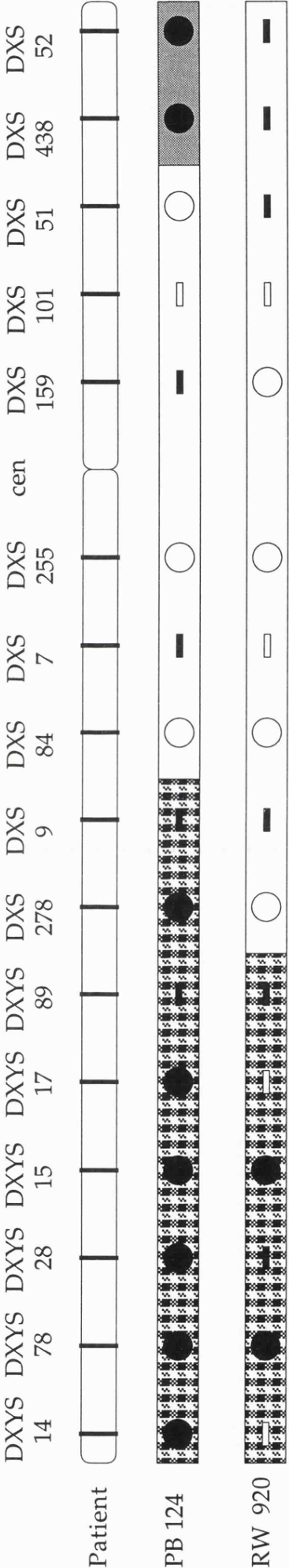


Figure 4.19: Concomitant gain and loss of alleles for loci on the X chromosome.



the sex-specific regions of the X chromosome would be an easy target for inactivation in tumour cells, requiring only a single 'hit', rather than the two 'hits' hypothesized by Knudson (1971), since one copy in the female would be already inactivated and there is only a single copy in the male. Therefore the deletion of sequences on the X chromosome would be a very frequent event in tumourigenesis as indicated by an extremely high rate of LOH in tumours. Yet only ~50% of the panel of tumours used in this study showed LOH anywhere on the X chromosome suggesting two possible scenarios. Firstly, the putative target of the deletions, a tumour-suppressor gene, may escape X-inactivation and has an active homologue on the Y chromosome; a number of such X-located genes, including STS, XG, ZFX, KALIG-1, GS1, A1S9T, RPS4X and XIST, have been found to escape X-inactivation and are interspersed among genes known to be X-inactivated (Ellis and Goodfellow, 1989; Brown and Willard, 1990; Wang *et al.*, 1992). Alternatively, the target gene may be situated in the pseudoautosomal region; the small localized deletions observed in some members of the panel would appear to confirm this is the case. The pseudoautosomal region is highly conserved between the X and Y chromosomes due to genetic recombination and the region escapes X-inactivation in the female, so there are no gene dosage effects. Any tumour-suppressor gene mapping to this region would be subject to the 'two-hit' hypothesis (Knudson, 1971). Therefore it remains possible that a tumour-suppressor gene(s) mapping to the X chromosome could conform to Knudson's hypothesis.

The most distal common region of smallest overlapping deletion at DXYS78 corresponds to an area found to be rich in CpG islands, whose presence is attributed to either a structural feature of mammalian telomeres or to a very high density of CpG island-containing genes in this locale (Rappold and Lehrach, 1988). The exclusion of the most telomeric locus, DXYS14, from a number of the small-scale deletions (figures 4.9 to 4.12) as well as from the gross deletions (figure 4.15), helps to confirm the specificity of deletions at the tip of Xp. It would appear that the very telomeric sequences of Xp are highly stable, being maintained in the tumour cell genome to generate interstitial deletions in this area. It is possible that as the DXYS14 locus maps 18kb from the telomere (Cooke *et al.*, 1985; Inglehearn and Cooke, 1990), sequences mapping close to this locus are retained as a 'cap' for broken ends of chromosomes to give some degree of chromosome stability. If this is indeed the case, then such a mechanism for healing the broken ends of chromosomes in tumour cells has consequences for the capping of other apparently terminal deletions on other chromosome arms, including Xq and 1p. Although reports of telomere healing and rescue of fragmented chromosomes are usually attributed to the addition of the basic telomeric repeat motif (T₂AG₃)_n

onto the broken end (Farr *et al.*, 1991; Lamb *et al.*, 1993; Murnane and Yu, 1993), it would appear that in the tumours from this panel, subtelomeric sequences can also be used in the healing process. This suggests that instead of an activated telomerase enzyme adding the telomere repeats to heal the end, a mechanism of telomere capture by genetic recombination is operating within tumour cells. If a broken end can be healed by whichever method, then the chromosome is stabilized and translocation onto another chromosome is less likely to occur. Experiments using telomere-associated chromosome fragmentation (TACF) to generate a panel of rodent-human somatic cell hybrids show that in the majority of hybrids there is no evidence of the usual occurrence of interstitial deletions or of the translocation of human DNA to rodent chromosomes, but that the truncations are healed by functional telomeres (Farr *et al.*, 1992). Meltzer *et al.* (1993) described the phenomenon of cryptic subtelomeric translocations in tumour cells as determined by microdissection, DOP-PCR (degenerate oligonucleotide primed-PCR) and FISH to normal human chromosomes; the existence of these subtle rearrangements was previously undetectable by conventional cytogenetics. Breakage of a chromosome end would yield a highly recombinogenic end which is stabilized by the acquisition of a telomere, including subtelomeric sequences, from the fragment lost or another chromosome. These newly-created raw ends may then go on to cause further rearrangements that do become visible by conventional karyotype analysis. Therefore, if the retention of DXYS14 is indicative of telomere healing of terminal Xp deletions, where its own telomere was repeatedly recaptured, it could help to explain why rearrangements to the X chromosome are rarely observed in breast tumours. If the deletions generated on the X chromosome are small and even restricted to the pseudoautosomal region, it is highly unlikely that they would be detectable because of the limitations of standard methods of karyotype analysis of solid tumours. These can only reliably detect those changes that affect regions greater than 30-50Mb (Sandberg *et al.*, 1988) and the total size of the PAR is far below this threshold (~2.6Mb). It would be expected that some, as well as the larger deletions in RW 647 and PB 78, would be detectable due to loss of a normal X chromosome from the karyotype and the appearance of translocated X material and/or derivative X chromosomes within the tumour if the telomere captured was not from the fragment broken off. Further work with TACF in immortalized and transformed cell lines may help to clarify the association between chromosome fragmentation and tumourigenesis.

The second common site of deletion in the pseudoautosomal region overlaps with that determined at DXS278 in the sex-specific sequences of Xp22.3, so that the target may be either pseudoautosomal in its location or be situated just proximal

to the pseudoautosomal boundary. The location of this site is dependent on the interpretation of results with the complex DXS278 locus and also on the order of DXYS17 and DXYS89. If DXYS17 does split the region into two targets, then the data for DXS278 does require corroboration by examination of intervening polymorphic markers. Whether the putative target(s) of LOH here are analogous to autosomal tumour-suppressor loci or conform to the alternative, a gene that escapes X-inactivation and has an active Y chromosome homologue, remains to be seen.

The additional common regions of deletion established further proximal on Xp and also found on Xq are less well defined than those above. The common region between DXS7 and DXS255 could correspond to that determined in ovarian tumours between DXS84 and DXS7 (Yang-Feng *et al.*, 1992; Yang-Feng *et al.*, 1993). The region between DXS7 and DXS255 was specifically excluded in that report, but as the definition of this region depends on RW 647, which appears grossly fragmented, it is possible that the two areas do overlap. Clearly, the panel of breast tumours needs to be better studied in this region of the chromosome as ovarian and breast cancer are widely regarded to have similar routes of evolution. Those areas on Xq are also tentative due to the low numbers of probes used and the high incidence of unsuccessfully-tested loci. The smallest common region of deletion distal to DXS51 appears to correspond to the site of the second proposed pseudoautosomal region on Xq28 (Freije *et al.*, 1992), close to DXS52. As the common region of overlap is centred on DXS438 and the precise order of DXS52 and DXS438 is unknown, it is possible that the target of deletion is pseudoautosomal in nature. Identification of expressed sequences within the second PAR will be of great interest and they will require examination within those tumours with allelic loss in this region.

Interest is increasing in the androgen receptor (AR) gene which has been mapped to Xq11-q12 (Brown *et al.*, 1989; Schlessinger *et al.*, 1993) and is responsible for X-linked spinal and bulbar atrophy (SBMA, Kennedy disease) due to an expansion of an expressed CAG trinucleotide repeat in affected individuals (La Spada *et al.*, 1991). Germline mutations in the AR gene were first reported in two brothers with androgen resistance both of whom had developed breast cancer (Wooster *et al.*, 1992). The mutation occurred as an arginine to glutamate substitution at codon 607, a highly conserved position in the second zinc-finger of the DNA-binding domain and was not detected in more than 100 alleles from unrelated controls. More recently, a mutation in the AR gene of one of 13 males with breast cancer was established, occurring at position 608 as an arginine to lysine substitution (Lobaccaro *et al.*, 1993a, 1993b). That these two mutations involve

adjacent amino acid substitutions suggests a non-accidental association between the AR gene and male breast cancer. Interestingly, the latter substitution has also been reported in an adult male with partial androgen insensitivity syndrome (Saunders *et al.*, 1992) who had undergone bilateral mastectomy at 18 years of age because of marked gynecomastia. This could explain the absence of breast tumour development (Lobaccaro *et al.*, 1993a). Lobaccaro *et al.* (1993a) suggested two possible links between AR mutations and breast cancer development. The mutated AR acquires the ability to bind to oestrogen responsive elements (EREs), thus activating oestrogen regulated genes. Alternatively, androgens confer a protective effect that is lost or dramatically reduced in individuals with AR mutations. This idea of a protective effect of androgen is supported by the androgenic inhibition of cell proliferation on the human breast cancer cell line, MFM-223 (Hackenberg *et al.*, 1991). Therefore the study by PCR of the trinucleotide repeat (which forms a polymorphic microsatellite) within the AR gene on Xq may be of value to see if the AR gene is subject to large intragenic perturbations in sporadic male breast tumours, but SSCP (single-strand conformation polymorphism) analysis would have to be implemented to try to identify single base changes in both familial and sporadic cases.

The incidence of uninformative and untested loci on the X chromosome has had a great effect on deletion mapping. There are a number of tumours in which the extent of the deletion has been drawn to its maximal limits despite only one or two loci showing loss. Many of these tumours in which maximal limits have been drawn, may turn out to have small-scale rearrangements and aid deletion mapping at a later date since there is so little cytogenetic data indicating that the X chromosome is subject to huge rearrangements.

4.3.2 Concomitant Gain and Loss of Alleles on the X Chromosome

As on chromosome 1, there were very few tumours with GAL on the X chromosome. The two tumours in question had both undergone a regionalized event consistent with somatic recombination on the X chromosome (Cavenee *et al.*, 1983). Whilst in PB 124, GAL extended from the telomere of the short arm through the pseudoautosomal sequences into the sex-specific sequences, the event is restricted to the PAR in RW 920. Therefore, the common region of overlap is the PAR which corresponds to the most frequent common area of LOH. The incidence of an independent region of LOH at Xq28 in PB 124 is unexpected and may have occurred on either homologue. The incidence of GAL

on the X chromosome in the PAR suggests that there is at least one sequence of significance to breast cancer in this region that may be a tumour-suppressor gene.

4.3.3 Future work

This part of the project is the most promising in terms of future studies. Re-testing of those loci unsuccessfully analyzed would be a priority to fill in the gaps on the current panel, as is the ordering of the two pairs of loci, DXYS17-DXYS89 and DXS52-DXS438. Two-colour FISH interphase mapping with a third locus, such as DXYS15 should provide the answer for DXYS17 and DXYS89. A locus closer to DXS52 and DXS438 than DXS51 would be required to order those two markers on the chromosome by similar methods, although this is of lower priority as only three tumours corresponded to a consensus deletion here. In the near future, Dr. Gudrun Rappold (Heidelberg, Germany) should be carrying out experiments to order DXYS17 and DXYS89 using restriction mapping by pulsed field gel electrophoresis (personal communication). Until these two pseudoautosomal loci can be ordered on the X chromosome linkage map, the data are not considered to be publishable. The high concentration of VNTRs in the pseudoautosomal region suggests it could be profitable to try to reduce the two smallest regions of overlapping deletion occurring here still further. In particular, there are at least five polymorphic loci between DXYS14 and DXYS28 that can be used to refine the DXYS78 common region of deletion further to a point where a cosmid library of the region can be screened. Candidate gene sequences can then be identified by a number of approaches including investigation of DNA sequences adjacent to the abundant undermethylated CpG sites in this region, exon amplification, cDNA screening and search for sequence conservation through analysis of zoo blots. Once a candidate sequence has been isolated, and mapped back to the consensus region of deletion, formal testing of its possible function can be initiated; mutation analysis in tumours would be the primary screen for discovering any involvement in tumourigenesis.

CHAPTER 5

Allelic Imbalance Studies of Chromosome 1

5.1 Introduction

Cytogenetic studies of human breast tumours show multiple regions on chromosome 1 involved in a variety of rearrangements. The short arm of chromosome 1 mostly experiences unbalanced translocations or deletions (Hainsworth *et al.*, 1991), resulting in net loss of sequences, while the long arm undergoes a complex series of rearrangements including unbalanced translocations and deletions (Gebhart *et al.*, 1986) but also is subject to increase in copy number, frequently in the form of isochromosomes (Jones Cruciger *et al.*, 1976). Loss of heterozygosity on chromosome 1 has been reported by a number of groups at varying levels and three smallest common regions of deletion identified: at 1q23-q32 (Chen *et al.*, 1989), distal 1p (Genuardi *et al.*, 1989b; Bièche *et al.*, 1993) and proximal 1p (Bièche *et al.*, 1993).

In order to characterize the role of chromosome 1 more fully in breast cancer, polymorphic markers mapping along the length of the whole chromosome were used, in accordance with appropriate control loci, to assess a panel of 71 tumour-lymphocyte pairs for allelic imbalance. Complex patterns of rearrangements were established that are highly consistent with cytogenetic data in the literature. Deletion mapping of individuals identifies five independent smallest common regions of deletion. There are also possibly three areas of the chromosome targeted for amplification which may harbour proto-oncogenes. The two arms of the chromosome appear to be subject to different events, such that the short arm primarily undergoes interstitial deletions whereas the long arm is subject to both whole arm events (gains and losses) and more localized deletions.

5.2 Results

5.2.1 Construction of the Panel of Patients and Probe Bank

The sets of filters and criteria for scoring imbalances used to study allelic imbalance on chromosome 1 are detailed in chapter 3, section 3.2.1. The full results for each of the 71 patients analyzed are listed in Appendix III for chromosome 1.

On average, ten out of a total of 15 loci from chromosome 1 were successfully scored per patient, of which (also on average) six per patient were informative. Those patients with less than ten loci successfully tested tend to hamper attempts at deletion mapping, since the imbalances observed are rarely flanked closely by informative loci that are retained but more often by unsuccessfully tested loci (see Appendix III). As there are still stocks of paired DNA samples remaining from the panel, especially the series RW 860 to RW 942, and as most of the loci on chromosome 1 used in this study are VNTRs, gaps in the patients' data should be filled in with largely informative results when re-testing can be carried out.

Examples of the hybridization patterns observed with each probe are given in figures 5.1 to 5.8. Summaries of the overall results obtained for each locus on chromosome 1 are presented in table 5.1 and figure 5.9. The level of imbalances remain above background throughout the chromosome, with imbalance on 1q notably higher than that on 1p (being roughly double). There are two peaks of allelic loss on the short arm: the first at D1S80, the most distal locus analyzed and the second less well-defined covering D1S7 to D1S73. Allelic loss remains high along the length of the long arm although there appears to be a trough at D1S61 and a second at D1S81. This second trough on 1q is comparative with respect to adjacent loci used in the study. Allelic gain is low throughout 1p but reaches more significant levels on 1q with a notable peak covering SPTA1 to D1S61. However, the low number of individuals informative at SPTA1 (only six patients) seriously questions the significance of the results. Until more patients can be successfully tested at this locus, the result can only be regarded as a preliminary indication. Those members of the panel with unclear imbalance have also undergone imbalance at the control locus/loci used. In order to determine the nature of these imbalances it will be necessary to identify another region of the genome with low levels of imbalance and choose a highly polymorphic marker from this region.

Note to figures 5.1 to 5.8: Each of the figures presents photographs of autoradiographs for the loci studied on chromosome 1. Overall, all 15 loci assessed on the chromosome are represented. The allele sizes given in each of the legends are estimations. Two invariant bands were produced by the pYNH24 probe in all tracks, 1.0kb and 0.8kb in size, but are not always shown in the photographs. Invariant bands were also observed with other probes, as indicated in the appropriate figure legends.

u, uninformative

i, informative and unaffected by any imbalance

LOH, informative and showing loss of heterozygosity

GAL, informative and undergoing a concomitant gain and loss of alleles

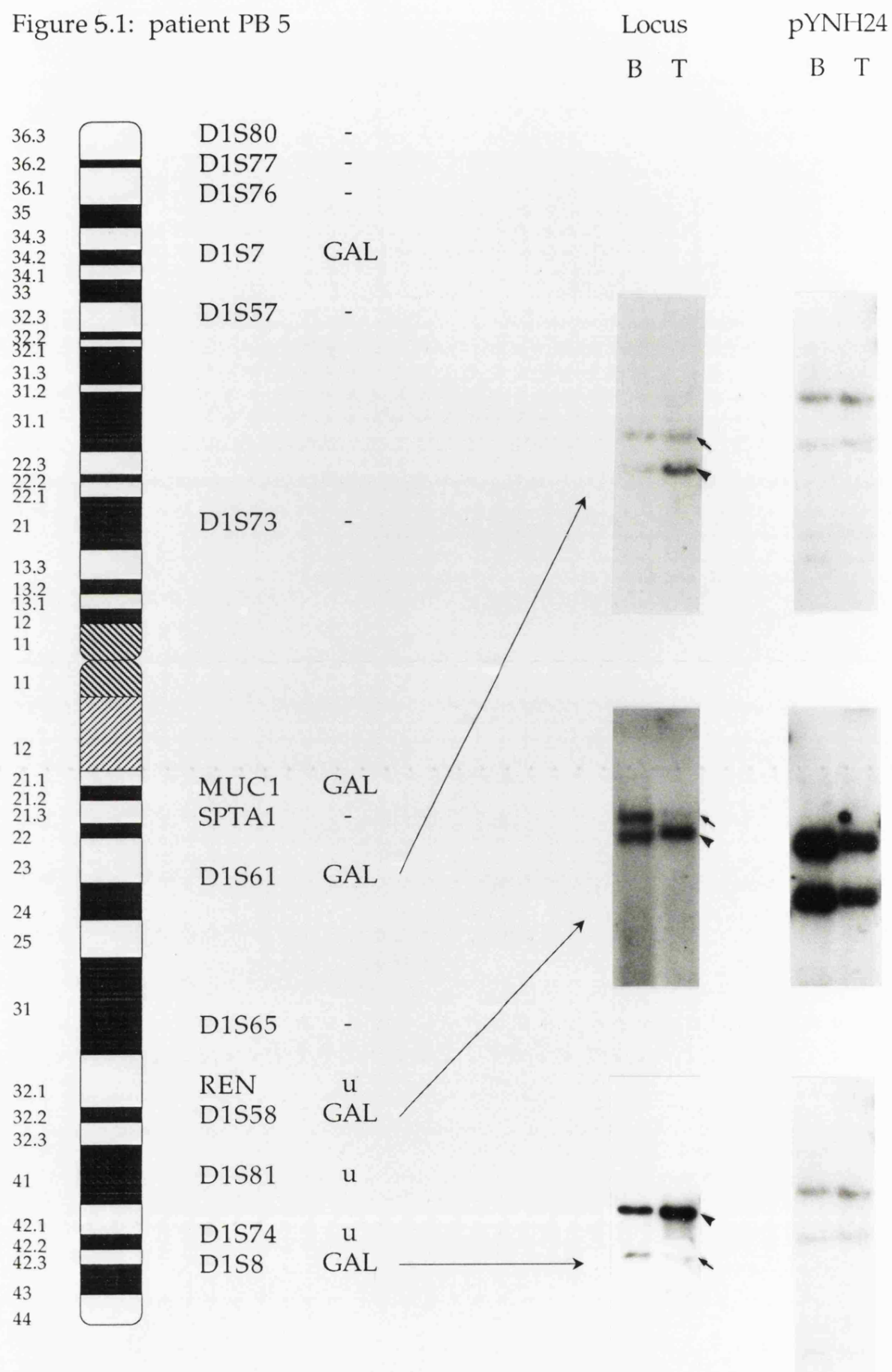
GCN, informative and showing a gain in copy number of one allele

AI, informative and undergoing allelic imbalance, the nature of which has not been determined

Legend to figure 5.1: Autoradiographs of three loci on chromosome 1 from PB 5. Each was informative (Locus column) and all show GAL. The corresponding controls with D2S44 are shown in the pYNH24 column. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. Arrows indicate the allele of reduced intensity and arrowheads indicate the allele with increased intensity in the tumour lanes for each locus.

	Locus		Control (pYNH24)	
	Upper allele	Lower allele	Upper allele	Lower allele
D1S61	1.7kb	1.5kb	1.9kb	1.6kb
D1S58	1.6kb	1.5kb	1.9kb	1.6kb
D1S8	6.6kb	5.3kb	1.9kb	1.6kb

Figure 5.1: patient PB 5

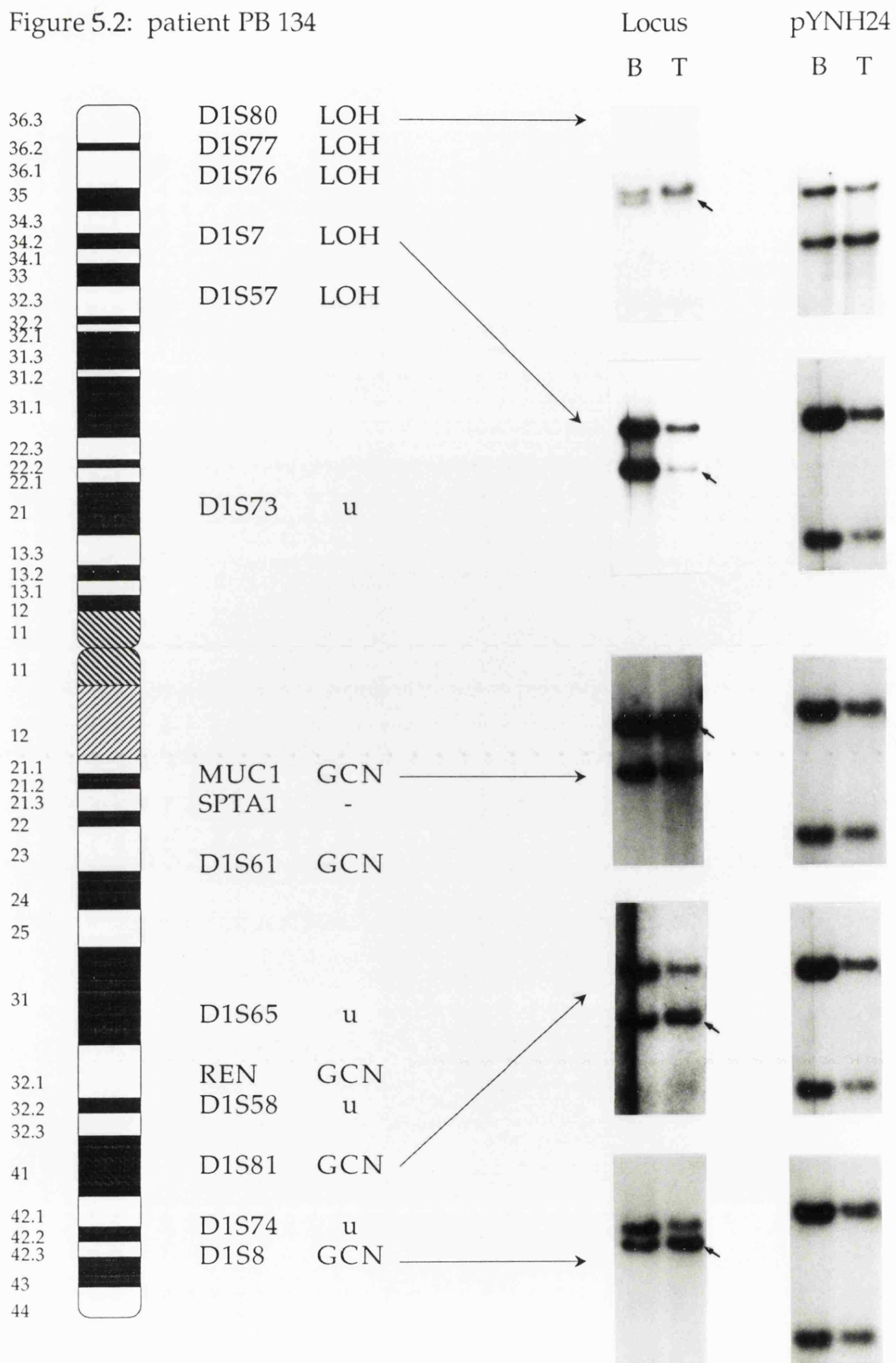


Legend to figure 5.2: Autoradiographs of five loci on chromosome 1 from PB 134. Each was informative (Locus column), of which two show LOH and the remaining three show GCN. The corresponding controls with D2S44 are shown in the pYNH24 column. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. Arrows indicate the allele affected by the imbalance at each locus.

	Locus		Control (pYNH24)	
	Upper allele	Lower allele	Upper allele	Lower allele
D1S80	2.1kb	2.05kb	8.5kb ^a	6.0kb
D1S7	12.0kb	8.0kb	1.4kb	1.1kb
MUC1	5.4kb	4.5kb	1.4kb	1.1kb
D1S81	2.1kb	1.8kb	1.4kb	1.1kb
D1S8	4.6kb	4.4kb	1.4kb	1.1kb

^a This allele of D2S44 shows a reduction in intensity. Although apparent with this filter (*Pst*I), it was not reproducible on further digests with *Pst*I in an attempt to confirm this loss. All other filter sets containing DNA from this patient digested with different restriction endonucleases (*Hinf*I, *Mbo*I and *Taq*I) showed no such loss with an allele of D2S44. Even with the apparent level of allelic imbalance with the control locus, there was still appreciable loss of one allele at D1S80.

Figure 5.2: patient PB 134

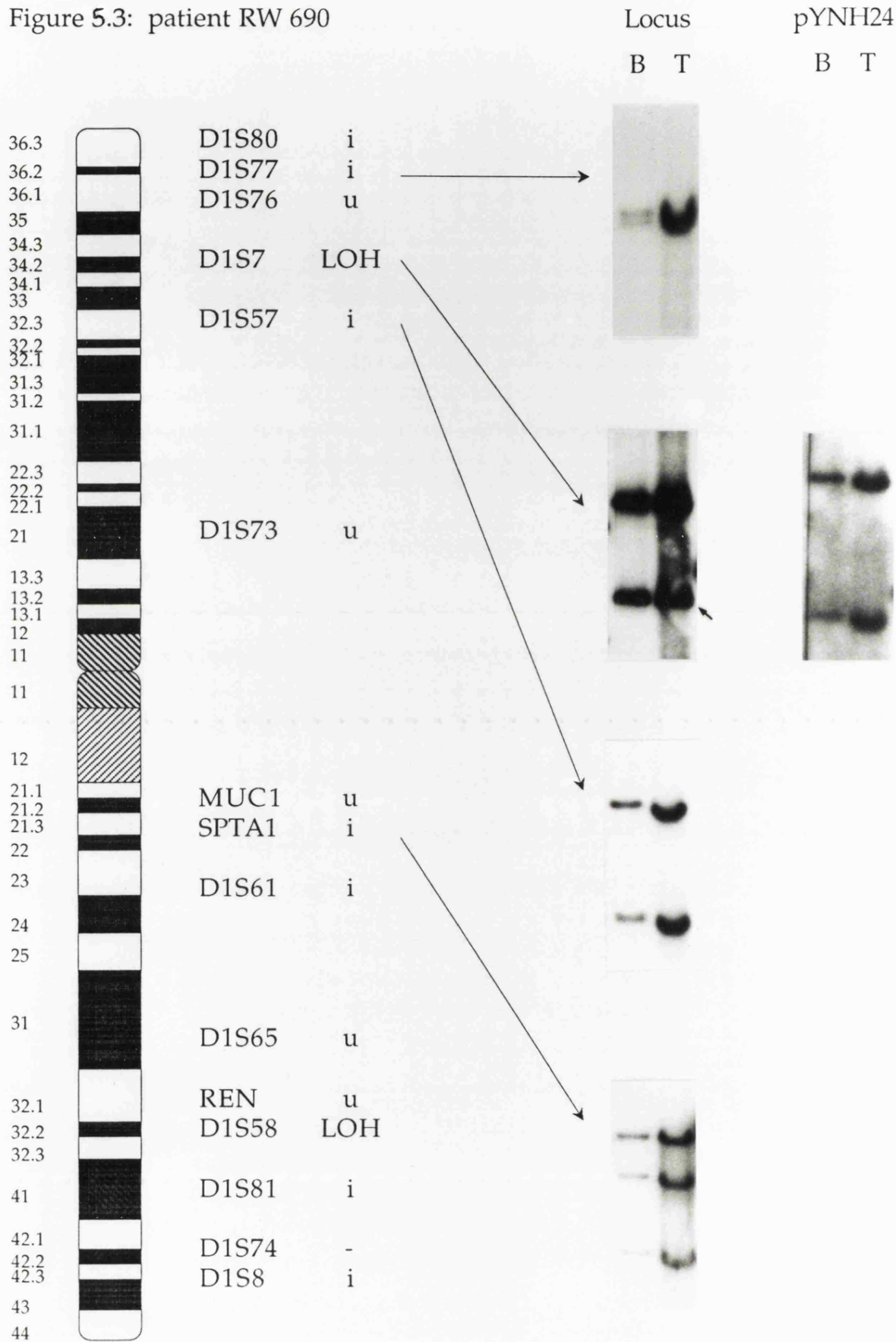


Legend to figure 5.3: Autoradiographs of four loci on chromosome 1 from RW 690. Each was informative (Locus column), of which only one shows LOH. For the locus showing imbalance, the corresponding control with D2S44 is shown in the pYNH24 column. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. An arrow indicates the allele of reduced intensity in the tumour lane for D1S7.

	Locus		Control (pYNH24)	
	Upper allele	Lower allele	Upper allele	Lower allele
D1S77	2.5kb	2.45kb	n/a	n/a
D1S7	9.0kb	5.7kb	3.4kb	1.9kb
D1S57	4.4kb	2.5kb	n/a	n/a
SPTA1	16.0kb	10.0kb, 6.2kb ^a	n/a	n/a

^a two bands constituted the second allele.

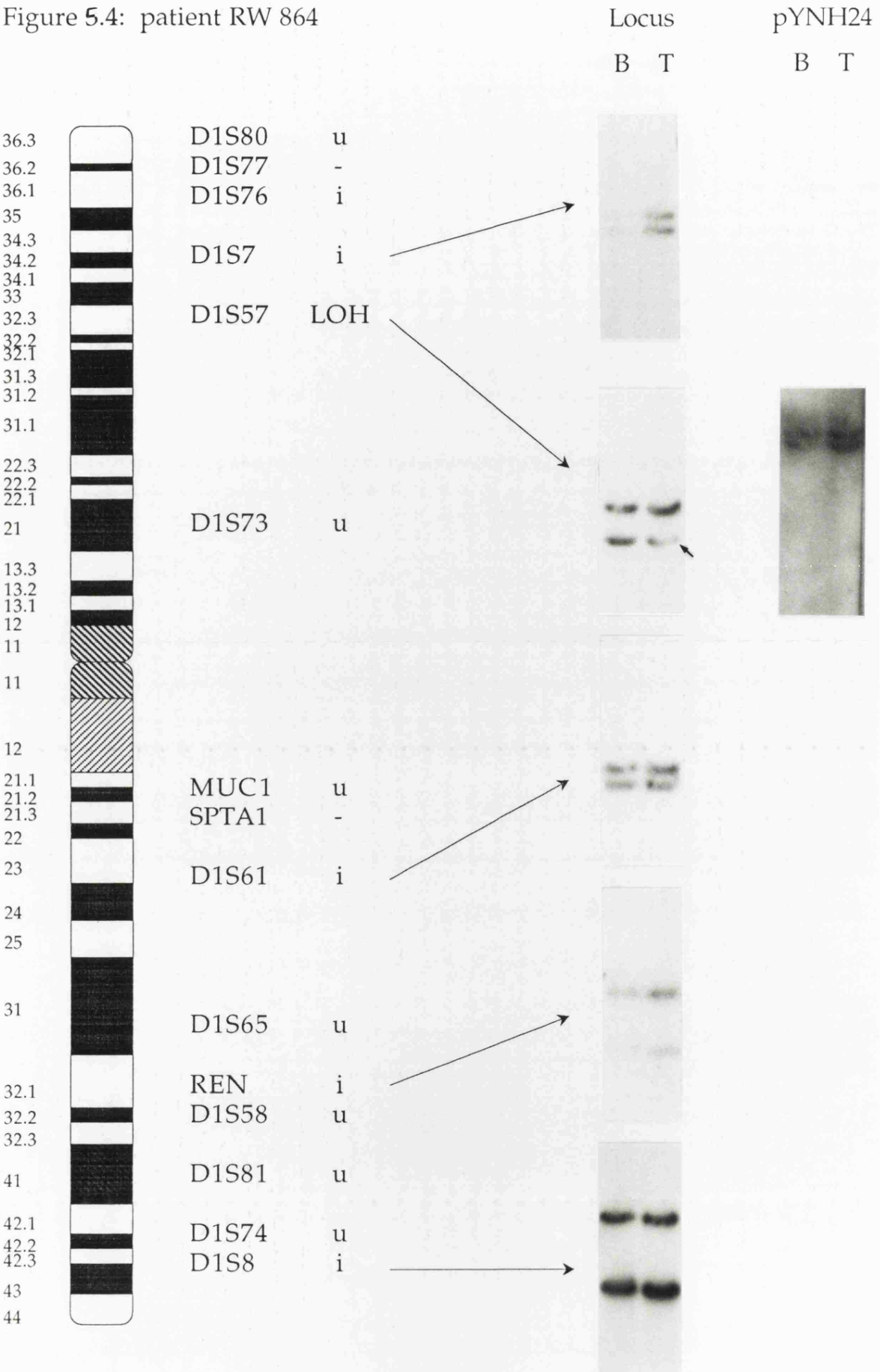
Figure 5.3: patient RW 690



Legend to figure 5.4: Autoradiographs of five loci on chromosome 1 from RW 864. Each was informative (Locus column), of which only one shows LOH. For the locus showing imbalance, the corresponding control with D2S44 is shown in the pYNH24 column. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. An arrow indicates the allele of reduced intensity in the tumour lane for D1S57.

	Locus		Control (pYNH24)	
	Upper allele	Lower allele	Upper allele	Lower allele
D1S7	5.1kb	4.9kb	n/a	n/a
D1S57	4.4kb	3.7kb	11.0kb	10.5kb
D1S61	1.15kb	1.1kb	n/a	n/a
REN	1.4kb	1.0kb	n/a	n/a
D1S8	6.9kb	5.7kb	n/a	n/a

Figure 5.4: patient RW 864

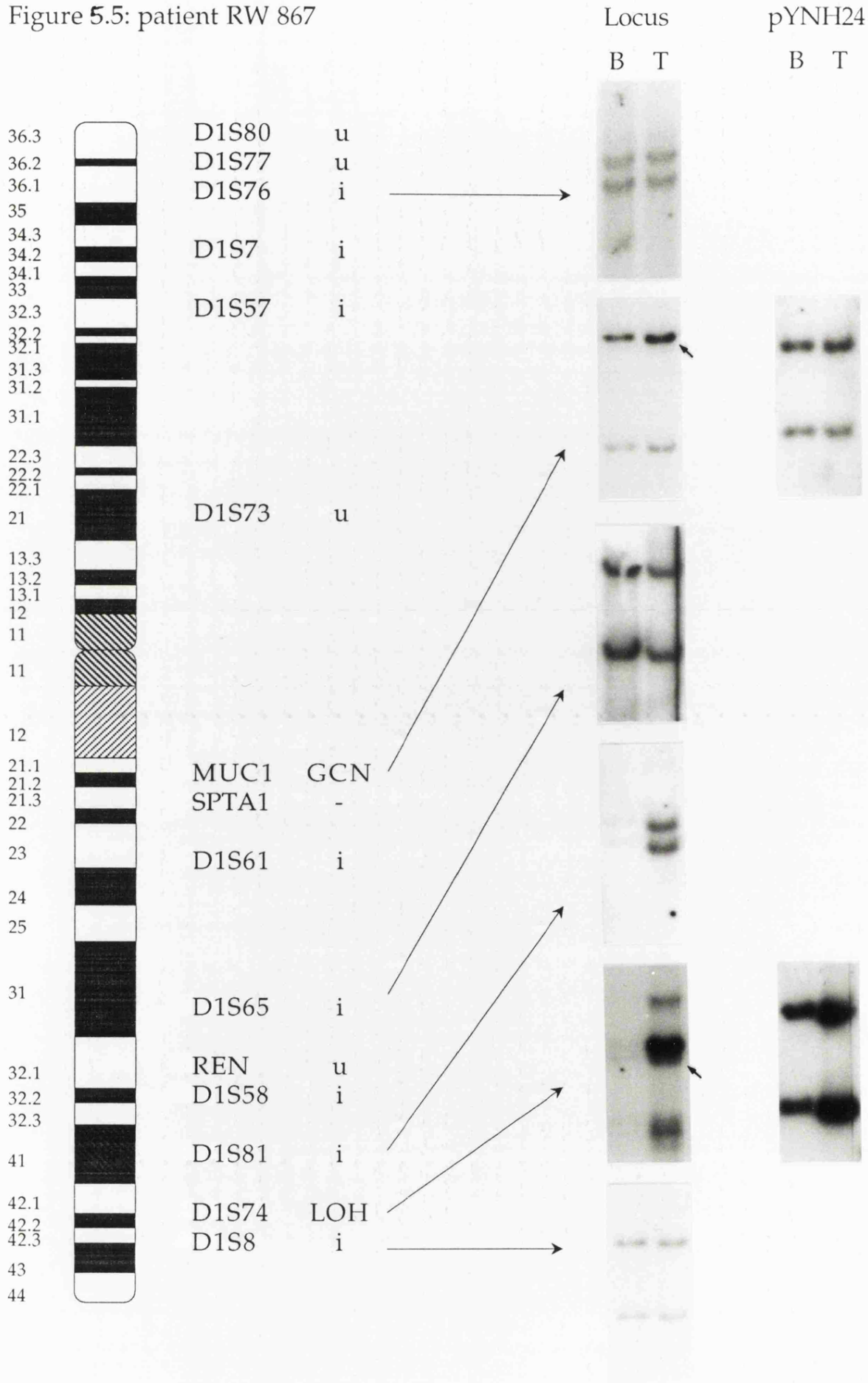


Legend to figure 5.5: Autoradiographs of six loci on chromosome 1 from RW 867. Each was informative (Locus column), of which two show LOH. For the loci showing imbalance, the corresponding controls with D2S44 are shown in the pYNH24 column. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. Arrows indicate the allele affected by imbalance in the tumour lanes of MUC1 and D1S74.

	Locus		Control (pYNH24)	
	Upper allele	Lower allele	Upper allele	Lower allele
D1S76	2.9kb	2.7kb	n/a	n/a
MUC1	7.4kb	3.9kb	2.2kb	1.6kb
D1S65	5.0kb	3.8kb	n/a	n/a
D1S81	2.4kb	2.3kb	n/a	n/a
D1S74 ^a	1.45kb	1.4kb	2.2kb	1.6kb
D1S8	7.1kb	4.9kb	13.0kb	n/a

^a D1S74 has a number of invariant bands in all tracks, those shown are 1.9 and 1.0kb in size, while additional invariant bands, one 2.2kb and another two 0.5-0.6kb in size, are not shown.

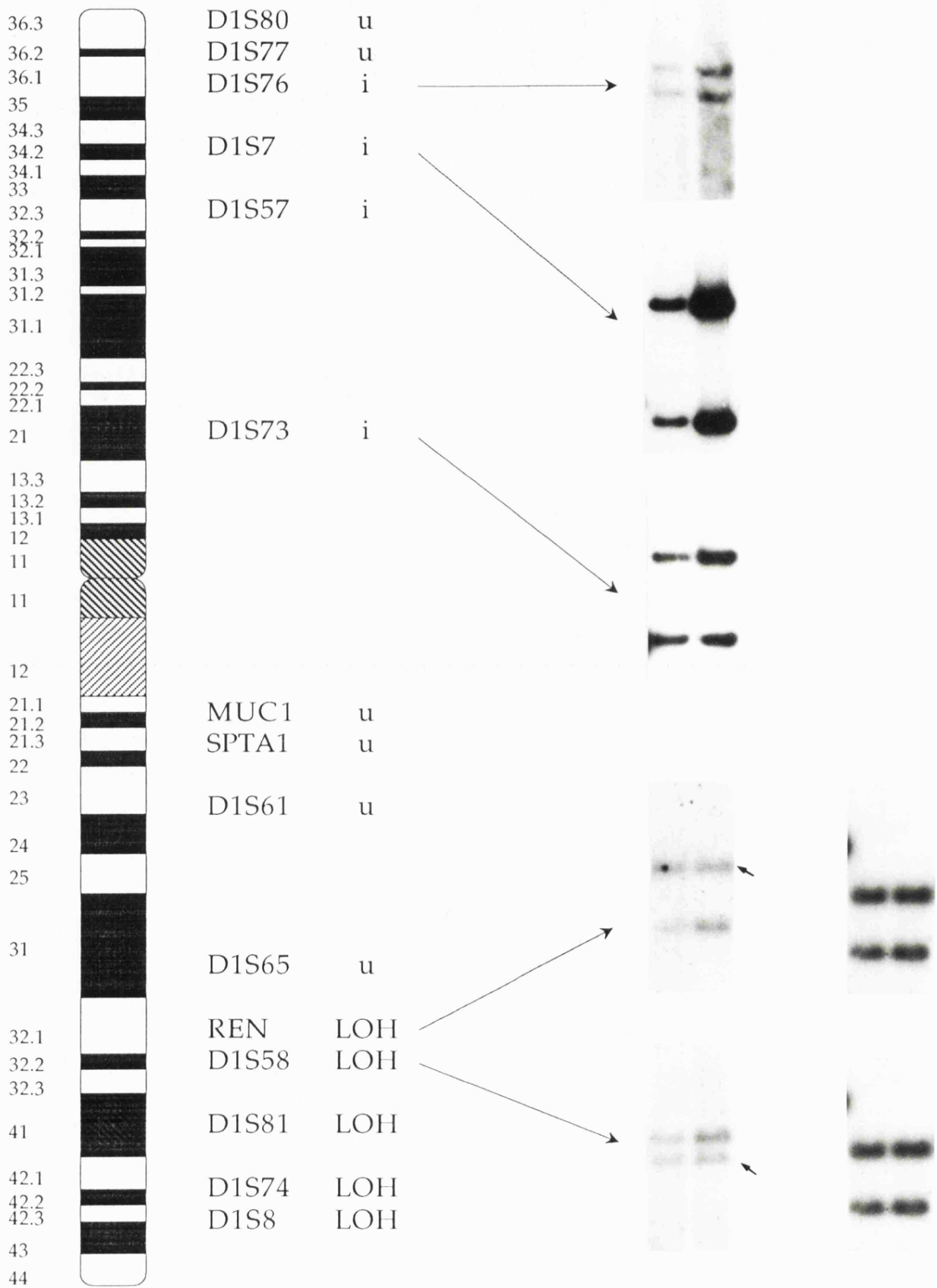
Figure 5.5: patient RW 867



Legend to figure 5.6: Autoradiographs of five loci on chromosome 1 from RW 869. Each was informative (Locus column), of which two show LOH. For the loci showing imbalance, the corresponding controls with D2S44 are shown in the pYNH24 column. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. Arrows indicate the alleles of reduced intensity in the tumour lanes for REN and D1S58.

	Locus		Control (pYNH24)	
	Upper allele	Lower allele	Upper allele	Lower allele
D1S76	3.7kb	3.5kb	n/a	n/a
D1S7	10.0kb	4.9kb	n/a	n/a
D1S73	7.6kb	5.6kb	n/a	n/a
REN	1.4kb	1.0kb	1.5kb	1.1kb
D1S58	1.9kb	1.8kb	1.5kb	1.1kb

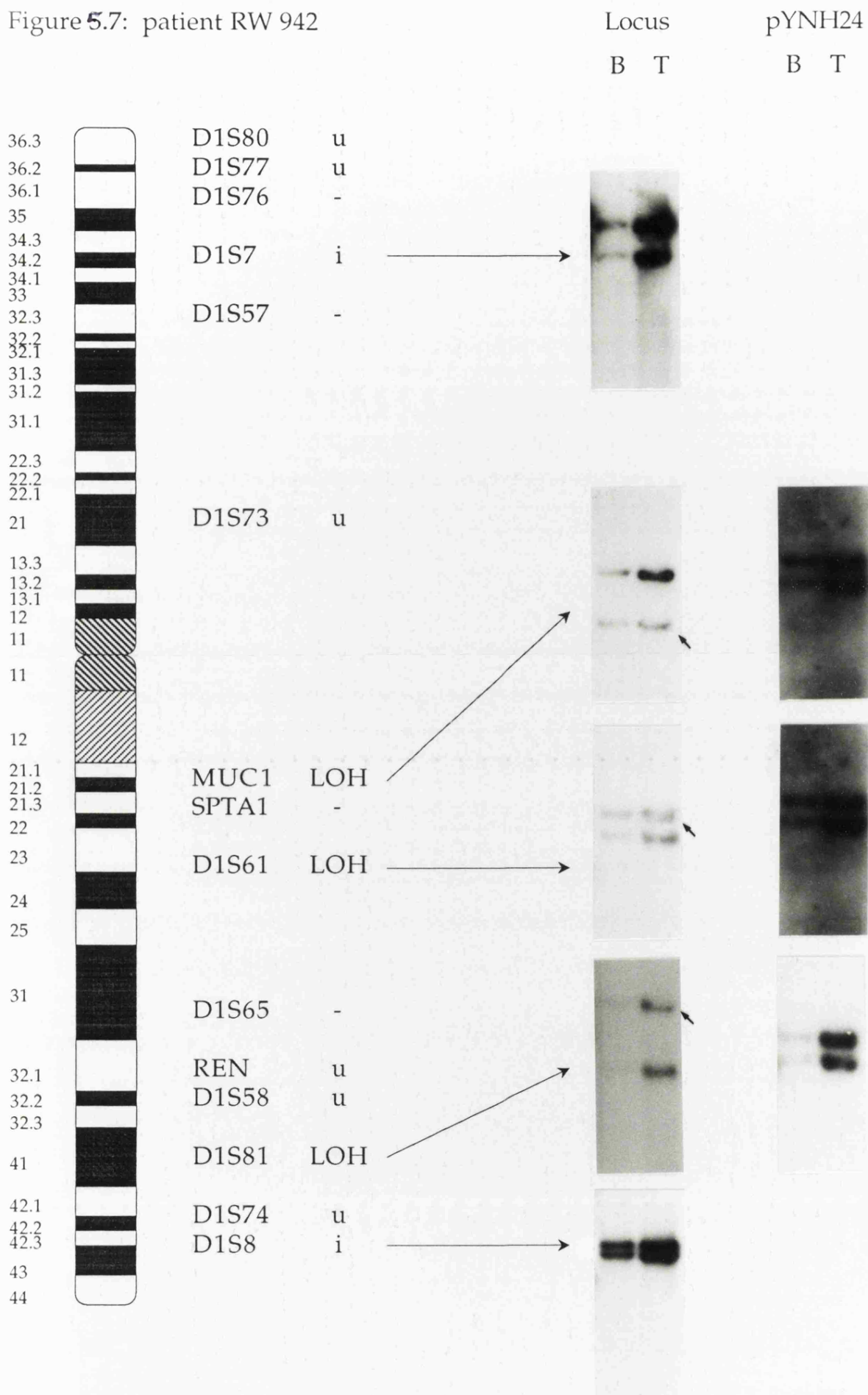
Figure 5.6: patient RW 869



Legend to figure 5.7: Autoradiographs of five loci on chromosome 1 from RW 942. Each was informative (Locus column) and three show LOH. For the loci with imbalance, the corresponding controls with D2S44 are shown in the pYNH24 column. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. Arrows indicate the alleles of reduced intensity in the tumour lanes for MUC1, D1S61 and D1S81.

	Locus		Control (pYNH24)	
	Upper allele	Lower allele	Upper allele	Lower allele
D1S7	7.5kb	6.5kb	n/a	n/a
MUC1	6.8kb	5.4kb	1.8kb	1.4kb
D1S61	1.4kb	1.3kb	1.8kb	1.4kb
D1S81	2.4kb	1.9kb	1.8kb	1.4kb
D1S8	10.0kb	9.0kb	n/a	n/a

Figure 5.7: patient RW 942



Legend to figure 5.8: Autoradiographs of the MUC1 locus on chromosome 1 from PB 64. The locus was informative and showed an additional band in the tumour DNA track when *Mbo*I-restricted DNA was probed. This probable somatic mutation was found again when *Alu*I-restricted DNA from this patient was probed. The letters B and T indicate the lymphocyte (blood) and tumour DNA lanes on each autoradiograph. The nature of the AI at this locus could not be determined as D2S44 also showed AI in PB 64 (data not shown).

PB 64	New allele	MUC1	
		Upper allele	Lower allele
<i>Mbo</i> I-restricted DNA	9.0kb	7.4kb	5.7kb
<i>Alu</i> I-restricted DNA	5.7kb	4.7kb	3.5kb

Figure 5.8: PB 64, allelic imbalance accompanied by a somatic mutation at the MUC1 locus.

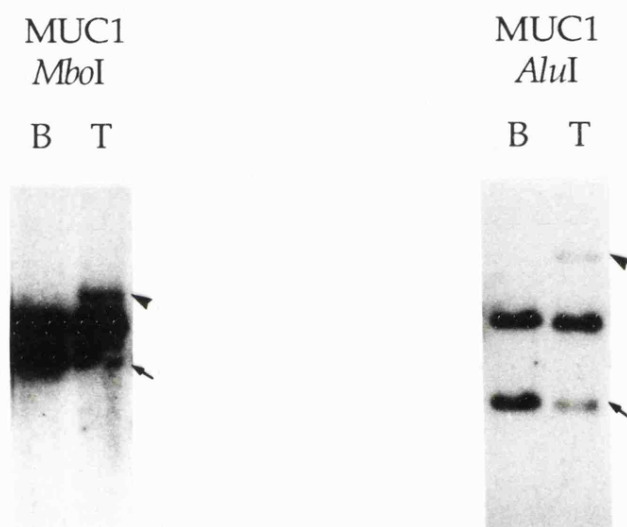


Table 5.1: Summary of probes used in the study and data on allelic imbalance (AI) on chromosome 1 for each locus and overall totals for 1p, 1q and the whole chromosome. To determine the nature of the imbalance, the locus D2S44 (pYNH24) was used primarily as a control for the amounts of DNA in each track of the autoradiograph. In some instances, two other control loci, D7S21 (pMS31) and D20S26 (pMS617) were used. pYNH24, pMS31 and pMS617 showed a polymorphism in *MboI*- and *TaqI*-digested DNA; pYNH24 also showed a polymorphism in *AluI*-, *MspI*- and *PstI*-digested DNA.

a Allelic loss (patients undergoing LOH or GAL - see text) shown as a fraction of the number of patients informative at the locus and expressed as a percentage in parentheses.

b Allelic gain (patients undergoing GCN - see text) shown as a fraction of the number of patients informative at the locus and expressed as a percentage in parentheses.

c The number of patients for which allelic imbalance had clearly occurred at each locus but it was impossible to establish the nature of the imbalance (i.e. whether LOH/GAL/GCN) due to AI at the control locus/loci in those patients.

d The total number of patients undergoing any form of imbalance as a fraction of the number of patients informative at the locus and expressed as a percentage in parentheses.

Locus	Probe	Position	Total no. of patients	Patients with allelic loss (%) ^a	Patients with allelic gain ^b	Unclear loss or gain ^c	Patients with imbalance (%) ^d
D1S80	pMCT118	1p	47	4/17 24%	2/17 12%	0/17	6/17 35%
D1S77	pMCT58	1p	46	3/19 16%	2/19 11%	0/19	5/19 26%
D1S76	pCMM12	1p36-p33	34	2/16 13%	1/16 6%	2/16	5/16 31%
D1S7	pMS1	1p34	49	13/46 28%	2/46 4%	2/46	17/46 37%
D1S57	pYNZ2	1pter-p31	42	5/23 22%	0/23	2/23	7/23 30%
D1S73	pEFD53.2	1p22-p13	51	6/22 27%	0/22	2/22	8/22 36%
MUC1	pMUC10	1q21	61	13/44 30%	7/44 16%	3/44	23/44 52%
SPTA1	3021E1	1q21	19	2/6 33%	2/6 33%	0/6	4/6 67%
D1S61	pMLAJ1	1q21-q32	62	8/39 21%	9/39 23%	2/39	19/39 49%
D1S65	pEKH7.4	1q31-q32	47	5/18 28%	3/18 17%	3/18	11/18 61%
REN	pHRnES1.9	1q32	62	7/21 33%	4/21 19%	2/21	13/21 62%
D1S58	pYNZ23	1q32	58	11/24 46%	5/24 21%	2/24	18/24 75%
D1S81	pTHH33	1q32-qter	49	12/39 31%	8/39 21%	4/39	24/39 62%
D1S74	cYNA13	1q	52	4/11 36%	1/11 9%	0/11	5/11 45%
D1S8	pMS32	1q42-q44	56	14/51 27%	6/51 12%	3/51	23/51 45%
<hr/>							
Chromosome 1p				18/51 (35%)	3/51 (6%)		26/51 (51%)
Chromosome 1q				29/61 (48%)	12/61 (20%)		44/61 (72%)
Chromosome 1				35/62 (56%)	13/62 (21%)		50/62 (81%)

Legend to figure 5.9: Loci studied on chromosome 1 and a summary of the allelic imbalances observed. Allelic loss includes LOH and GAL since both are regarded as indicators of tumour-suppressor gene inactivation. N = number of informative patients / total number screened in the project.

Figure 5.9

			Allelic loss	Allelic gain	N
36.3		D1S80 (pMCT118)	24%	12%	17/47
36.2		D1S77 (pMCT58)	16%	11%	19/46
36.1		D1S76 (pCMM12)	13%	6%	16/34
35					
34.3					
34.2		D1S7 (pMS1)	28%	4%	46/49
34.1					
33					
32.3		D1S57 (pYNZ2)	22%	0%	23/42
32.2					
32.1					
31.3					
31.2					
31.1					
22.3					
22.2					
22.1					
21		D1S73 (pEFD53.2)	27%	0%	22/51
13.3					
13.2					
13.1					
12					
11					
11					
12					
21.1		MUC1 (pMUC10)	30%	16%	44/61
21.2		SPTA1 (3021E1)	33%	33%	6/19
21.3					
22					
23		D1S61 (pMLAJ1)	21%	23%	39/62
24					
25					
31					
		D1S65 (pEKH7.4)	28%	17%	18/47
32.1		REN (pHRnES1.9)	33%	19%	21/62
32.2		D1S58 (pYNZ23)	46%	21%	24/58
32.3					
41		D1S81 (pTHH33)	31%	21%	39/49
42.1					
42.2		D1S74 (cYNA13)	36%	9%	11/52
42.3		D1S8 (pMS32)	27%	12%	51/56
43					
44					

5.2.2 Loss of Heterozygosity on Chromosome 1: Identification of Small Discrete Regions

By studying 15 loci mapping along the length of the chromosome, six on 1p and nine on 1q, a total of five discrete regions of loss of heterozygosity on chromosome 1 can be identified. These deletions appear to be independent of one another: distal 1p (distal to D1S77), mid-region 1p (centred on D1S7), proximal 1p (between D1S57 and cen), mid-region 1q (between D1S65 and REN) and distal 1q (between D1S81 and D1S8). However, the none of these smallest regions of overlap are derived from more than five patients and so none can be said to be consistently targeted for deletion in the panel of tumours.

LOH at the tip of the short arm occurs in a number of patients but in only one case, RW 647, is the deletion bounded immediately proximal by the D1S77 locus which is informative and retained (figure 5.10). The deletions on 1p in RW 880 and PB 80 may coincide with the distal 1p region. However, the distal boundaries in neither patient are clearly defined due to homozygous or untested loci and so the deletions in these two patients could actually not extend as far distal as D1S80. The distal 1p site of loss in RW 647 is highly consistent with that identified by other studies of chromosome 1 in breast tumours (Genuardi *et al.*, 1989a, 1989b; Bièche *et al.*, 1993) but cannot be regarded as confirmatory.

The LOH events on 1p in tumours PB 80 and RW 880 appear to coincide more closely with the site of the second independent deletion in the mid-region of the short arm as they actually take place at loci in this area of the chromosome (figure 5.11). This second site is centred on the locus D1S7 as defined by PB 40 and only two other tumours, PB 103 and RW690, have LOH at D1S7 that cannot extend to another independent SRO on this arm (figures 5.3 and 5.11). The data for remaining tumours, PB 80, RW 880, PB 124, RW 864 and RW 647, are ambiguous at this time.

The loss of proximal 1p sequences is well-defined in only two cases, RW 874 and PB40 (figure 5.12). The deletion in both of these tumours is restricted to D1S73 and is bounded on either side by retention of D1S57 and MUC1. Whether the centromere is intact in these tumours is unknown, but is assumed to be for the most parsimonious analysis of the data available. This assumption is made for RW 647 and PB 124, which also exhibit loss at D1S73, because of retention of MUC1 on 1q21. However, the status of the centromere in RW 864 is more ambiguous as MUC1 is uninformative and SPTA1 is untested in this patient, although D1S61 is informative and retained (figure 5.4). Strictly, the deletion

Note to figures 5.10 to 5.23: Each of the figures includes all patients that are compatible with the alteration presented in the title of the figure. A number of patients conform to more than one of the different alterations and are therefore shown in more than one figure. In figures 3.10 to 3.14, which present data on the smallest common regions of overlapping deletion, the area in question is indicated by the shading on the general plan of chromosome 1 for each. Similarly, figure 3.20, presenting the small regional GCN events, also has these areas marked along the general plan of chromosome 1.

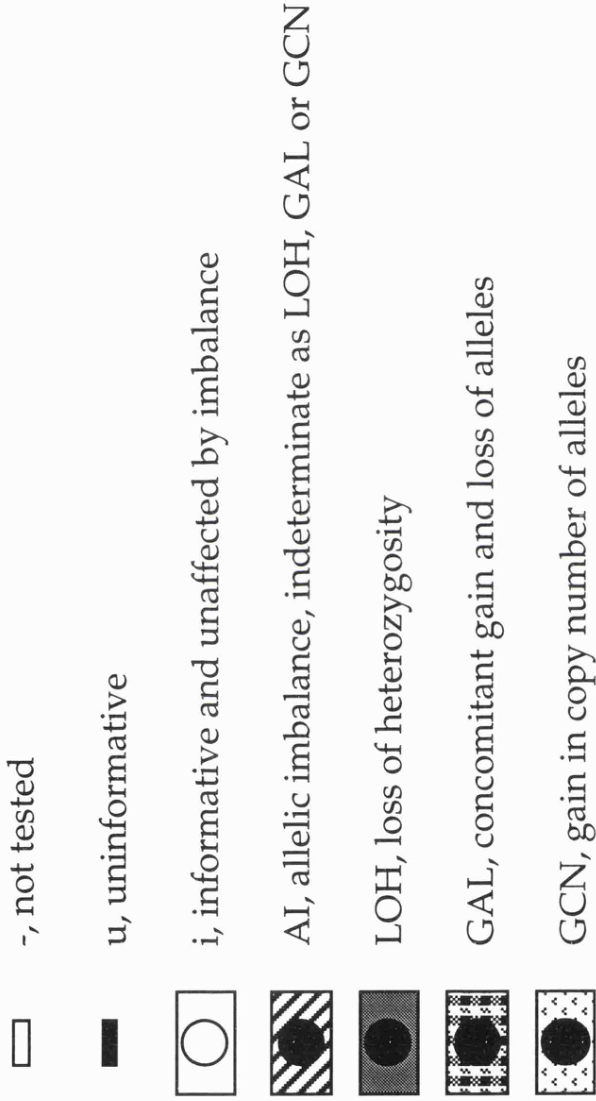
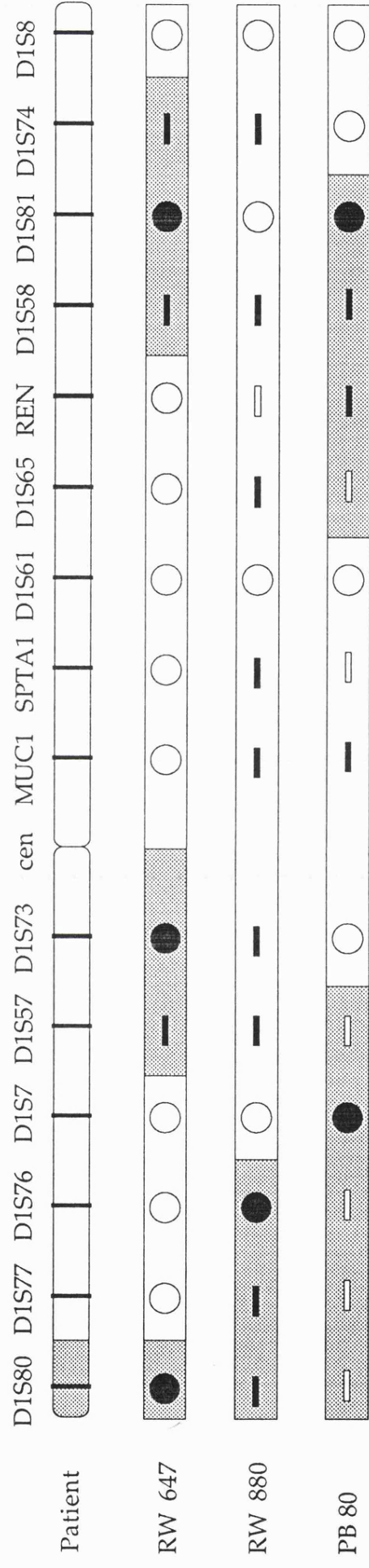


Figure 5.10: Patients defining a discrete region of LOH in the distal region of chromosome 1p.



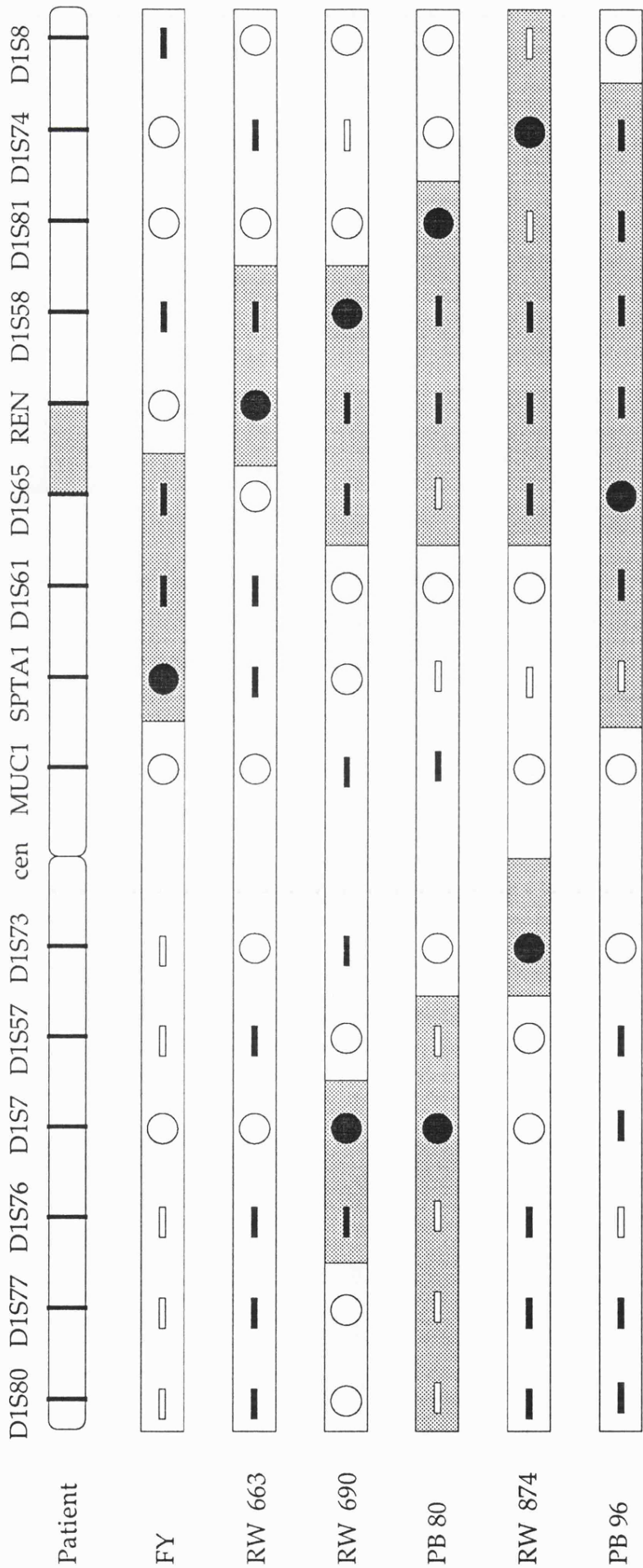
should be drawn between D1S7 and D1S61 in RW 864 as it is conceivable that the chromosome is fragmented with those pieces maintained in the genome translocated to other chromosomes due to the loss of the centromere. This third region of deletion could correspond to the second site identified by Bièche *et al.* (1993) between D1S9 and D1S73 and possibly also that determined by Devilee *et al.* (1991b) using the D1S33 locus which maps to 1p31-p21, although the distance between D1S57 and the centromere is considerable, ~110Mb.

The loss of proximal 1q sequences, usually MUC1, was frequently observed by a number of groups (Merlo *et al.*, 1989; Gendler *et al.*, 1990; Borg *et al.*, 1992b), but as the examination of more distal loci on 1q was carried out in only a few patients, it is unknown whether this region is specifically targeted for deletion in breast tumours. In the present study, only one patient, FY, shows a regionalized deletion where SPTA1 undergoes LOH flanked by MUC1 and more distantly the REN locus as both D1S61 and D1S65 are uninformative in this individual (figure 5.13). This area therefore could extend from 1q21 down to 1q32 and overlap with the mid-region of 1q deletion, so that the proximal 1q region cannot be regarded as a discrete region of deletion in breast tumours from the data currently available.

The mid-region 1q deletion is defined primarily by RW 663 and RW 690 where LOH events have occurred at REN in RW 663 and D1S58 in RW 690 (figure 5.13). PB 96 also has LOH at D1S65, but the limits of the deletion are very poorly-defined. The remaining tumours, FY, PB 80 and RW 874, have LOH events at loci some distance from REN, and only correspond to this consensus when maximal limits are drawn for the deletions, so that they cannot really be regarded as targeting this area. This mid-region loss on 1q corresponds to the smallest common region of overlap identified by Chen *et al.* (1989) only if FY is targeting the same sequences as RW 663 and RW 690, thereby restricting the common region of deletion to the area between D1S65 and REN (figure 5.13). Therefore, the data here cannot corroborate the SRO proposed by Chen *et al.* (1989).

The fifth discrete region of loss, situated around D1S74 is inferred from a number of tumours (figure 5.14), but specific LOH at D1S74 actually only occurs in RW 867. LOH events tend to occur at D1S81 (RW 661, RW 647, RW 921 and PB 80), but the boundaries of these are not as well-defined. PB 113 shows LOH at D1S8 only, but the telomeric sequences of 1q are excluded by five patients. Although this region is novel, the number of patients with an unambiguous deletion centred on D1S74 is only three.

Figure 5.13: Patients defining a discrete region of LOH in the mid-region of chromosome 1q.



5.2.3 Loss of Heterozygosity on Chromosome 1: Gross Deletions

Chromosome 1 is not just subject to small scale LOH events in breast tumours. Deletions of variable sizes occur in tumours within the panel. In some cases, the deletion straddles two of the independent regions impeding analysis: RW 880, PB 80, PB 124, RW 874 and PB 96 (figures 5.10 to 5.14). Additional cases show deletions involving vast tracts of DNA sequences up to and including whole chromosome arms (figures 5.7, 5.15 and 5.16), although cases such as RW 869 (figures 5.6 and 5.16) are ambiguous as the four proximal 1q loci studied in this patient are uninformative so that the proximal boundary of the deletion may occur anywhere between the centromere (as drawn) and the REN locus. Whether such large deletions are targeting specific tumour-suppressor genes for inactivation is debatable; instead it is possible that these are reflections of general genome instability within these tumours. The significance of whole chromosome losses is also questionable. Patient MI is the most convincing example of the loss of the whole of one copy of chromosome 1 in the panel (figure 5.17), as PB 121, RW 628 and PB 141 could have retained sequences that are uninformative or untested on 1p. PB 78 is an example of the possible alternative nature of these three tumours, where all informative sequences tested are showing LOH, except for D1S8 which is informative and clearly retained. Some of these grossly deleted chromosomes could be forming marker chromosome structures commonly found in cytogenetic karyotyping of breast tumours (Gebhart *et al.*, 1986). Similarly, those tumours with more than two independent imbalances could have become highly fragmented and retained sequences scattered throughout the genome, namely PB 40, PB 56 and RW 647. PB 40 and RW 647 have been especially useful in defining the smallest common regions of deletion on chromosome 1, but the possibility that these are fragmented chromosomes questions the suitability of their inclusion.

5.2.4 Concomitant Gain and Loss of Alleles on Chromosome 1: a Second Mechanism of Tumour-Suppressor Inactivation

In four patients, a concomitant gain and loss of alleles was determined for loci mapping to chromosome 1. In two patients, PB 5 and PB 106, the event appears to involve the entire chromosome (figures 5.1 and 5.18) closely reflecting one of the mechanisms of tumour-suppressor inactivation proposed by Cavenee *et al.* (1983) for the retinoblastoma susceptibility gene, RB1. Although there are untested loci in both patients as well as uninformative loci, the distribution of informative loci

Figure 5.15: Tumours with LOH possibly involving the whole of the short arm of chromosome 1.

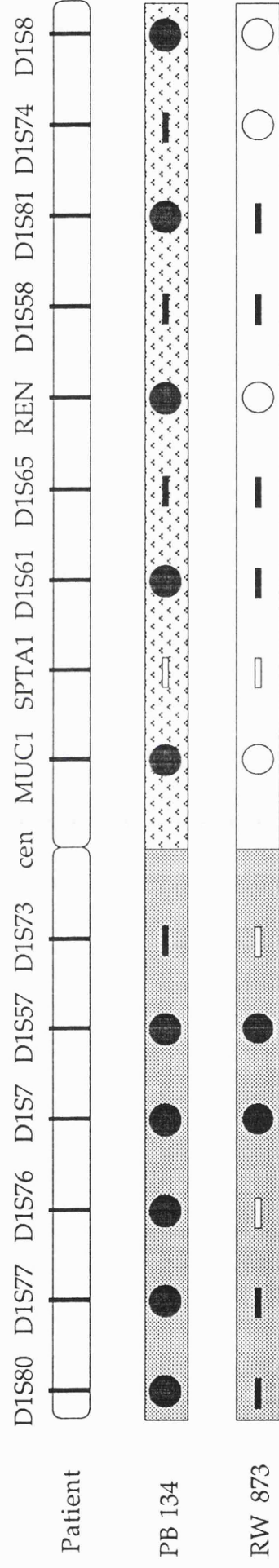


Figure 5.16: Tumours with LOH involving the whole or most of the long arm of chromosome 1.

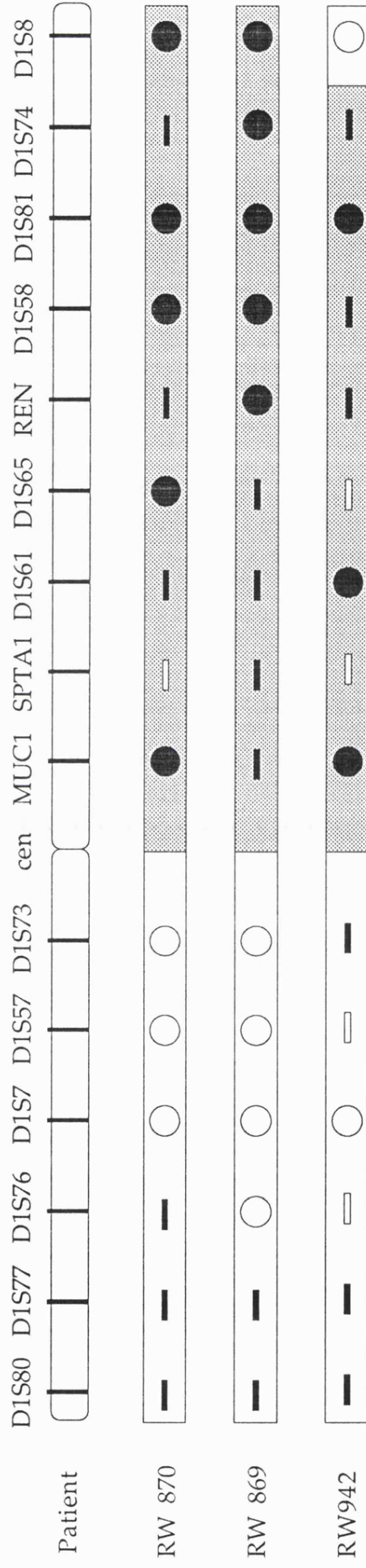


Figure 5.17: Tumours with loss of the whole or most of one copy of chromosome 1.

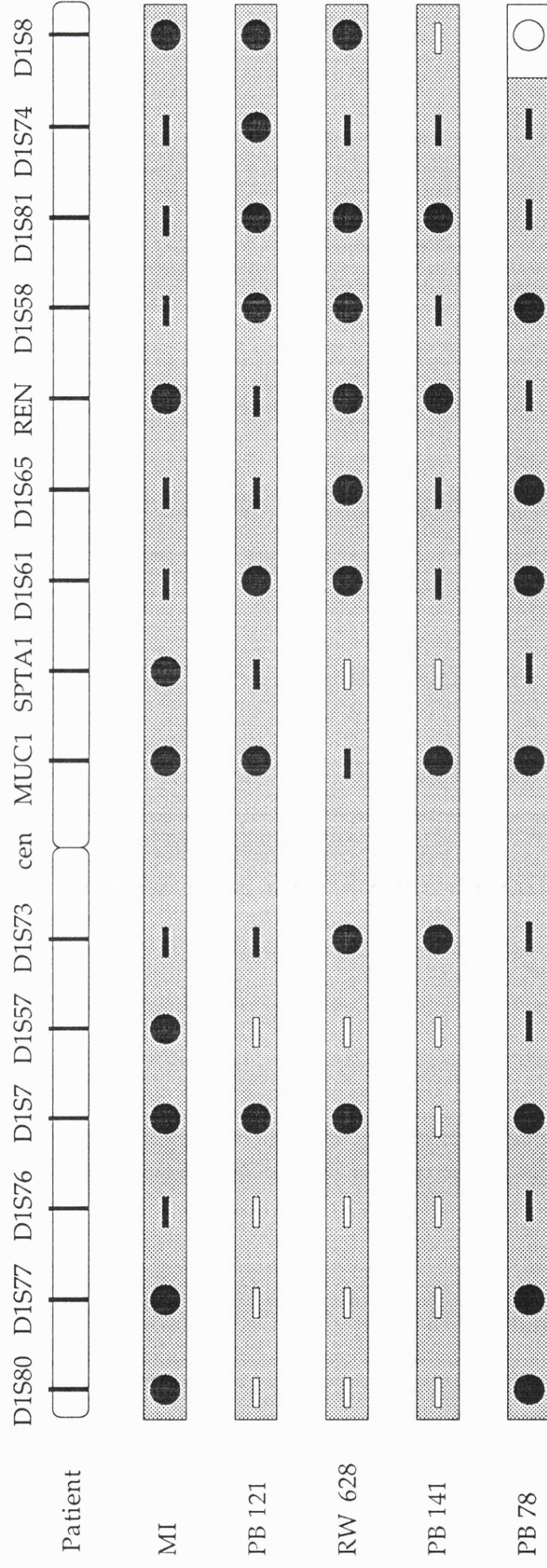
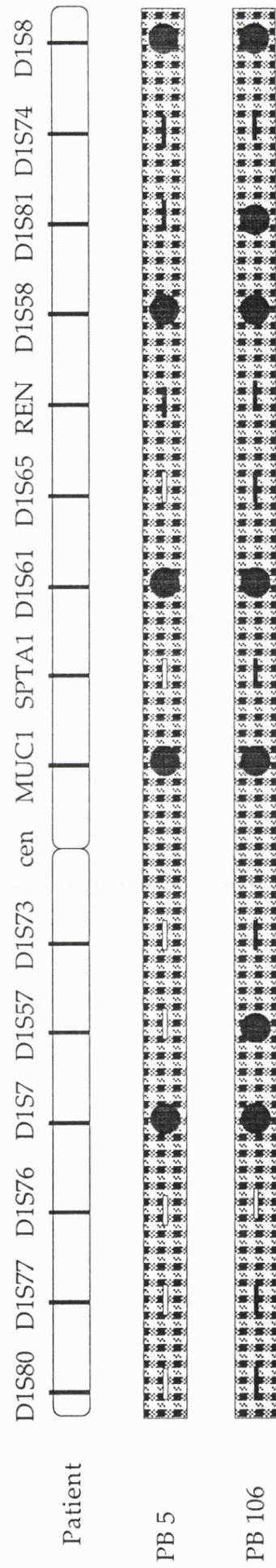


Figure 5.18: Patients with a concomitant gain and loss of alleles (GAL) at loci along the length of chromosome 1.



with this form of AI is widespread throughout the length of the chromosome in both cases.

In RW 686 and RW 895 (figure 5.19), GAL occurs as an interstitial event with flanking informative loci that are unaffected. The limits of the event in RW 686 are well-defined, mapping to the proximal half of the long arm of chromosome 1, unlike RW 895 which has unsuccessfully-tested loci flanking those undergoing GAL. Therefore the precise borders of the GAL event in RW 895 are unknown. However, the same region of chromosome 1 is targeted in both tumours from MUC1 distally to D1S58 in RW 686 and possibly extending further distally in RW 895. Notably, the common region of overlap of GAL in these two tumours encompasses the independent site of LOH in the mid-region of 1q determined in this project.

5.2.5 Gain in Copy Number of Loci on Chromosome 1

Gain in copy number (GCN) of sequences on chromosome 1 can occur as small localized events or large-scale, possibly non-specific, changes affecting some levels of the whole chromosome or the long arm within a tumour cell. There appear to be three distinct regional targets for increased copy number on chromosome 1 (figure 5.20): distal region of 1p identified in two tumours, RW 872 and PB 56; proximal 1q sequences at MUC1 (RW 660 and RW 867); and mid-region of 1q distal of D1S61 to D1S74 (PB 56, PB 40 and RW 920). Although the precise limits of some of these amplification events are unclear, they do appear to constitute independent and specific local disturbances.

The gain of whole arms is identifiable in several tumours, with multiple cases of gain of 1q (figure 5.21); gain of the whole of 1p was not observed in the panel, but extra copies of the whole chromosome occur in RW 866 (figure 5.22). This accurately reflects cytogenetic karyotyping of breast tumours and it is possible that some of these extra copies of 1q are forming isochromosomes of this arm. The principal candidate for such a structure is PB 134 (figure 5.2 and figure 5.23), which not only has additional copies of 1q, but appears to have lost the whole of 1p. Although the number of informative 1p loci identified in the patients shown in figure 5.21 is minimal, no imbalance of these loci has been determined, suggesting that there are additional copies of 1q present without detriment of the short arm.

Figure 5.19: Patients with an interstitial GAL event on chromosome 1.

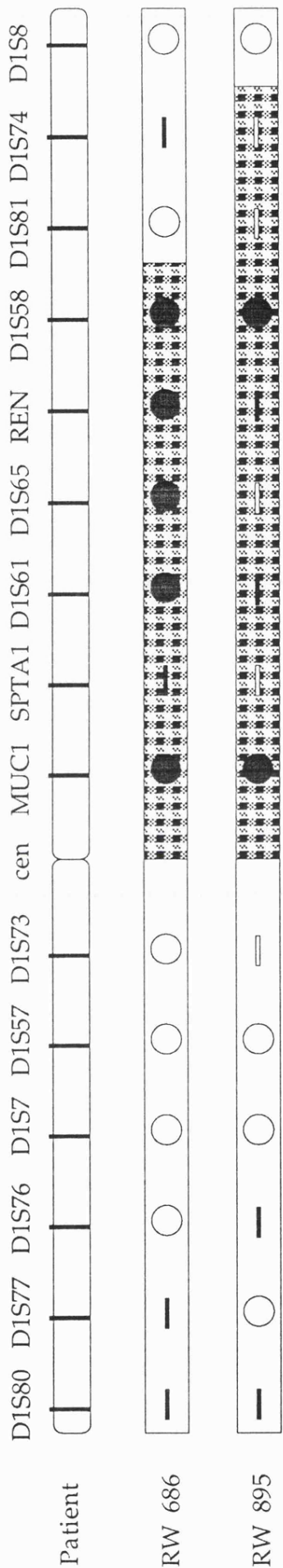


Figure 5.21: Patients with gain in copy number of the whole of the long arm of chromosome 1.

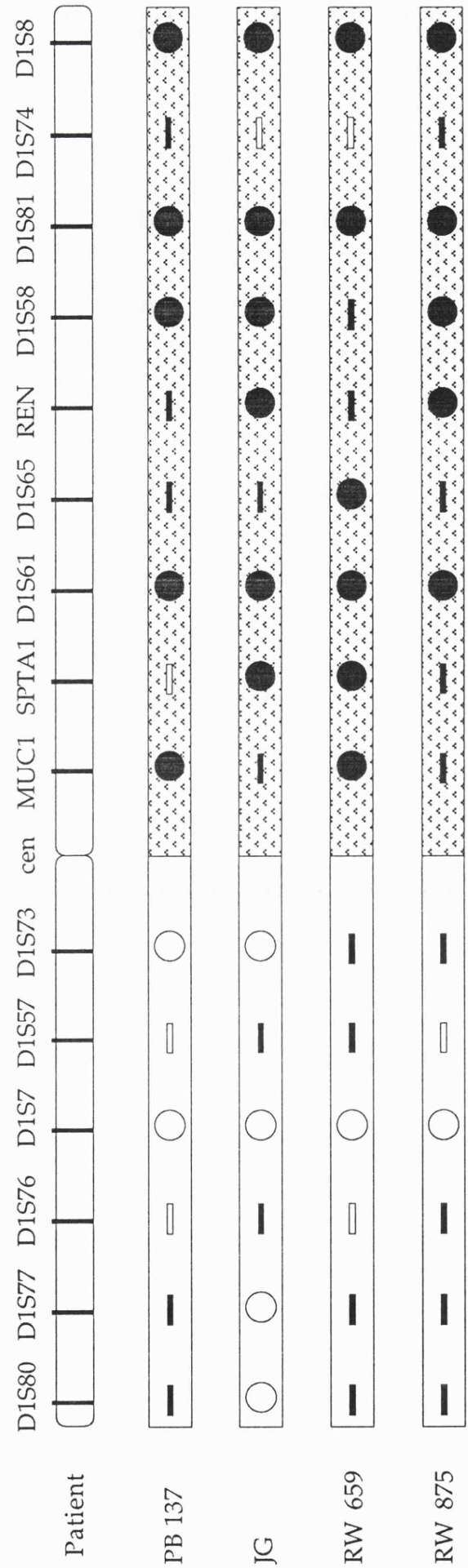


Figure 5.22: A single patient with gain in copy number involving the whole of chromosome 1.

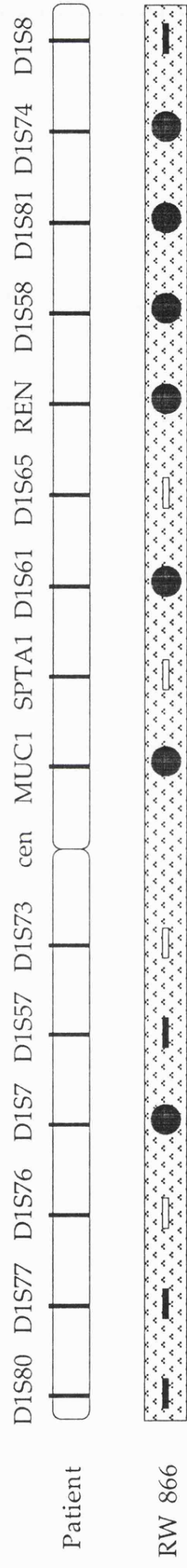
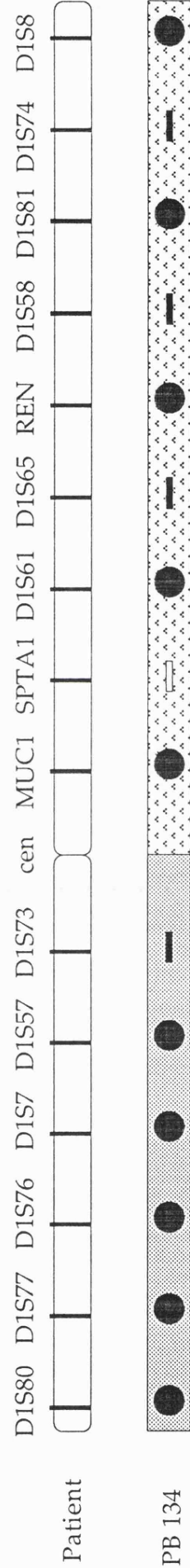


Figure 5.23: A single patient with LOH involving the whole of the short arm and GCN involving the whole of the long arm of chromosome 1, suggesting that an isochromosome of 1q has been formed.



5.2.6 Somatic Mutations: Generation of New Alleles

Somatic mutations are suspected to have occurred at single loci in four tumours: D1S8 in PB 40, MUC1 in PB 64, D1S8 in RW 898 and D1S7 in RW 921. These mutations take the form of an additional band in the tumour DNA track (figure 5.8) not observed again when other probes are used on the filters. When filters of these samples digested with other restriction endonucleases are also probed, the extra band is still observed only at the locus in question. In PB 40, RW 898 and RW 921, the additional band was larger than either of the constitutional alleles in each case and appears to be formed at the expense of one of these alleles, so that the reduction in intensity of the parental allele corresponds to the intensity of the new allele; the remaining original allele is unaffected. In contrast, there is allelic imbalance at MUC1 in PB 64 in addition to the somatic mutation which creates a new larger allele, but because the control locus, D2S44, also shows AI for patient PB 64, the precise nature of the imbalance cannot be determined.

5.3 Discussion

5.3.1 The Significance of Loss of Heterozygosity on Chromosome 1

The frequency of deletions in tumours from the panel resulting in the identification of five common regions of overlap is low and it is not clear whether any of these are of significance in the development and progression of breast cancer. Instead, the chromosome appears to be very unstable, undergoing many gross rearrangements in the breast tumours studied here. There is considerable evidence that as tumours progress they can develop genome instability with random rearrangements camouflaging others that occurred earlier in the development of the tumour. Small localized alterations defined by large numbers of tumours are more likely to be areas to which tumour-suppressor genes map than those defined by only one or two patients, since a tumour-suppressor gene would be repeatedly inactivated if its loss is important in malignant breast disease. Although none of the five independent regions are clear candidate sites for tumour-suppressor loci, three of these smallest common regions of deletion appear to correspond to areas identified in other studies, namely, distal 1p, proximal 1p and mid-region 1q (distal 1p is by far the weakest candidate).

The distal sequences on the short arm have been implicated as a site for a tumour-suppressor locus using the D1Z2 midisatellite (Genuardi *et al.*, 1989a, 1989b) and the D1S80 and D1S95/96 loci (Bièche *et al.*, 1993) in different panels of breast tumours. Although D1S95 and D1S96 have not been ordered with respect to each other on the chromosome, they map between D1S76 and D1S7 in the region 1p36.1-36.2 (Collins *et al.*, 1992). Therefore this smallest common region of overlap occurs in the most distal cytogenetic band, 1p36, and corresponds with the distal 1p regional deletion identified in RW 647 (figure 5.10). The chromosome in RW 647 has undergone three distinct losses and may not actually be intact with those fragments retained moved to other chromosomes. The lack of tumours from the panel with a specific deletion here appears at first glance to be in contrast with published work. However, after telomeric loci such as D1Z2 were examined, D1S57 was usually the nearest locus to be included (Genuardi *et al.*, 1989a, 1989b; Mars *et al.*, 1991; Borg *et al.*, 1992b) so that a considerable portion of 1p, between the tip of 1p and D1S57 was ignored. Bièche *et al.* (1993) covered the short arm with a series of 22 loci and the most distal region of overlap identified appears to depend on a single patient as a number of much larger deletions are shown that happen to extend to the telomere, overlapping with the single small deletion. It may be that there were additional tumours also found to have this very localized terminal deletion that were not shown. Although on close examination, the evidence for the loss of 1p36 sequences in breast cancer becomes sketchy, there is considerable evidence from the literature for this region to be important in neuroblastoma and possibly other solid tumours, including colorectal cancer (see Chapter 1, sections 1.1.3.2 and 1.1.1).

The loss in the proximal 1p area between D1S57 and the centromere, centred on the D1S73 locus, correlates with that established by Bièche *et al.* (1993) extending between D1S14 and D1S11 and centred on the loci D1S9 and D1S73. The order of these loci with respect to those used in this project is

pter- D1S57 - D1S14 - D1S9 - D1S73 - D1S11 - cen.

Therefore, the two regions may suggest targeting of the same sequences for deletion on chromosome 1 in breast tumours. This may be also the same area of the short arm identified by Devilee *et al.* (1991b) in which 50% of tumours (n=8) showed allele loss at D1S33 situated at 1p31-p21 and mapping between D1S57 and D1S73 (Collins *et al.*, 1992). However, D1S57 and D1S73 map ~110Mb apart (Drapacoli *et al.*, 1991), so that these three sets of deletions from the three groups of patients may not actually coincide; filling the intervening area with additional probes should resolve this question. If they do coincide, then proximal 1p becomes more convincing as being targeted for deletion in breast tumours.

There are no firm data for the existence of a targeted deletion between the centromere and 1q32 from the data generated in this study as small deletions do not cluster in this region of the chromosome and so the 26cM SRO identified by Chen *et al.* (1989) can be neither confirmed nor reduced in size. Nonetheless, the level of allelic loss at loci here is relatively high in this study (table 5.1 and figure 5.9) as a result of gross deletions of 1q. Since this region is widely regarded to be of significance in breast cancer (Merlo *et al.*, 1989; Gendler *et al.*, 1990; Borg *et al.*, 1992b), the lack of localized proximal 1q deletions identified could be interpreted in a number of ways. The first interpretation is that although the incidence of allelic loss in the 1q21-q23 area of chromosome 1 is also high in other studies (Merlo *et al.*, 1989; Gendler *et al.*, 1990; Borg *et al.*, 1992b), these deletions may have been the proximal ends of large-scale deletions of 1q, since the examination of more distal loci on 1q was carried out in only a few patients. Consequently proximal 1q is not the target of specific deletion in breast tumours. Alternatively, it may be that although these sequences are specifically targeted, the deletions most often result in loss of the entire arm as a hitchhiker effect, hence the rare tumour with a regional deletion in the proximal 1q area, namely FY.

The second interpretation is more questionable in light of the higher incidence of small proximal 1p deletions observed in tumours from the panel where distal sequences are retained. It is clear from the patterns of imbalance observed for chromosome 1 loci, in accordance with the extensive karyotype data available on breast tumours from the literature, that the two arms are subject to different kinds of rearrangements and different forces may be acting on their sequences. The short arm appears to be involved in primarily small-scale losses, possibly as a result of simple (interstitial) deletions or as unbalanced translocations. The long arm almost as frequently undergoes amplification as it does deletion and is far more often the target of large-scale rearrangements than 1p. Thus chromosome 1 appears to be very unstable in the breast tumours studied and no firm conclusions can be drawn from the data available here.

The alternative interpretations of data from tumours belonging to different studies highlight several problems. Due to the complex rearrangements occurring within the nuclei of breast tumours and the problems of low levels of heterozygosity with some probes, there is a need to assemble large panels of patients for study to identify those few tumours with small deletions that are required for deletion mapping. The probes used need to be well-mapped genetically (preferably physically too) and ideally highly polymorphic in nature, covering the entire length of the chromosome at regular intervals. Full disclosure of the results would enable cross-referencing for deletion mapping

analysis between different research groups, especially when the loci studied are duplicated between studies.

The frequency of large-scale deletions of chromosome 1 sequences becomes a problem in deletion mapping by masking peaks of allelic loss (table 5.1 and figure 5.9), so that small local deletions are most useful. The larger deletions tend to straddle one or more SROs, perhaps not completely covering either, thereby misleading analysis by focusing away from a possible target sequence. Similarly, tumours such as PB 80, PB 96 and RW 874 with one or two loci lost that are flanked only by uninformative loci, generating apparently large-scale changes, also frustrate attempts at deletion mapping. The frequency of occurrence of such cases is much reduced by concentrating on VNTRs rather than RFLPs to reduce the cumulative effect of homozygosity. Areas within some of these apparent large-scale deletions may have been retained, but are not detected by the lack of polymorphic markers within that region. A higher density of loci along the chromosome could uncover such areas. The possibility of chromosome fragmentation should not necessarily preclude a tumour from deletion mapping analysis as the regions lost may be situated on different homologues rather than the same copy; the tumours RW 647, PB 40 and PB 56 (figures 5.11 and 5.20) are such examples. To establish which homologue is targeted by each event, family studies would have to be carried out for the loci in question. The parental origin of each of the alleles lost can be determined by studying the parents' constitutional DNA.

5.3.2 Concomitant Gain and Loss of Alleles on Chromosome 1

The simplest way to reveal a recessive mutant allele at a tumour-suppressor locus may be by loss of the corresponding homologous wild-type allele through interstitial deletion, unbalanced translocation or mitotic nondisjunction (and loss of the whole homologue). These result in hemizyosity at the locus and flanking loci associated with the event, the most frequent alteration to chromosome 1 loci detected in the panel. Although this mechanism is commonly referred to as loss of heterozygosity, strictly it should be termed reduction to hemizyosity, as loss of heterozygosity can occur by other pathways to uncover mutant tumour-suppressor alleles, as proposed by Cavenee *et al.* (1983) for the retinoblastoma susceptibility gene. The concomitant gain and loss of alleles observed in four patients at chromosome 1 loci appear to exhibit the characteristics of two of these proposed mechanisms, namely mitotic recombination, followed by reduplication of the remaining homologue, or somatic recombination. In two tumours, PB 5

and PB 106, the whole of the chromosome appears to be involved in the event suggesting that the former mechanism is indeed operating in some tumours. Unfortunately, since the whole chromosome is involved, it is impossible to use these patients for deletion mapping, but they do suggest that there is a target(s) for inactivation on the chromosome. RW 686 and RW 895 display an interstitial GAL event, where although multiple loci are involved, they are situated in a common area. Therefore, it appears that somatic recombination has occurred to produce these specific patterns of alterations whereby part of the wild-type homologue has been lost during somatic recombination with the mutant homologue. The localization of the rearrangement to the proximal half of the long arm contrasts the lack of small-scale deletions found here. It may be that the localized GAL event does confirm that a sequence here is targeted for inactivation, but as the data, both GAL and LOH, are scanty, mapping of a possible site is not possible.

5.3.3 Gain in Copy Number of Regions of Chromosome 1

There were considerable levels of GCN on chromosome 1 in the panel of tumours. Although three discrete regions were identified, none were focused and the numbers of patients defining each regional gain were low. Whether these regions would be confirmed by extending the panel of patients through further study is debatable. The overall frequency of GCN on the chromosome does suggest that there may be specific targets for these events, despite the large-scale GCN events observed.

PB 134 appears to have an isochromosome of 1q; the formation of such a structure is implied by the loss of heterozygosity for sequences on the short arm and the gain in copy number for sequences on the long arm of the chromosome. This becomes important to malignancy if one or more tumour-suppressor genes are present on 1p since mutant alleles will be uncovered by loss of the wild-type alleles through LOH. In addition, proto-oncogenes mapping to 1q may be increased in dosage (amplified) through the GCN event, thereby conferring a growth advantage on cells containing the i(1q). Isochromosomes have been previously identified in breast tumours, through karyotyping experiments (Kovacs, 1981; Hill *et al.*, 1987; Dutrillaux *et al.*, 1991), so that it was likely that such a structure would be suggested by the data generated in allelic imbalance studies.

5.3.4 Future Work

It is possible that if more tumour-lymphocyte pairs were examined, the incidence of the small-scale independent regions of deletion identified on chromosome 1 would be increased. Clearly, the frequency of unsuccessfully-tested loci must be reduced and more probes included in the study, especially to the 110Mb interval between D1S57 and the centromere. The use of RFLPs to generate data in regions between minisatellite loci had limited success as the numbers of patients uninformative at these sites is far higher than at minisatellites; the successful adaptation of microsatellite analysis by PCR for AI studies of tumour panels (Bianchi *et al.*, 1991; Oka *et al.*, 1991; Ganly *et al.*, 1992; Louis *et al.*, 1992; Peinado *et al.*, 1992; Peter *et al.*, 1992; Cawkwell *et al.*, 1993; Gruis *et al.*, 1993; Linnenbach *et al.*, 1993) is a more attractive alternative to the use of RFLPs. Thus it might be more profitable to analyze additional markers by this method once an internal control has been worked into a multiplex amplification protocol.

CHAPTER 6

Fluorescence *In Situ* Hybridization to Tissue Sections

6.1 Introduction

Karyotyping of solid tumours is normally fraught with many technical problems so that high quality metaphase figures are rarely obtained from which complex chromosome rearrangements can be identified. Consequently, it has been difficult to establish the existence of repeated chromosomal aberrations or to gain insight into the nature of cytogenetic inter- or intratumour heterogeneity.

Fluorescence *in situ* hybridization with chromosome-specific satellite probes permits the assessment of the copy number of a specific chromosome not only on poor quality metaphase figures (Kokalj-Vokac *et al.*, 1993), but also importantly in interphase nuclei, providing a strong, easily-detectable hybridization signal (Pinkel *et al.*, 1986), in the form of distinct spots or clusters of fluorochrome. Hence, 'interphase cytogenetics' is proving to be a powerful tool in the study of tumours.

Therefore, FISH with satellite sequences as probes to interphase nuclei was developed using nine tumours from the panel of patients, supplied as formalin-fixed paraffin-embedded tissue sections by Dr. Rosemary Walker. This technique enabled the scoring of copy number in a large number of cells within archival tissue sections whilst preserving the tissue architecture of the samples. Ploidy levels within each tumour could be inferred, and their cytogenetic heterogeneity clearly observed by the incidence of differing copy number within separate areas of the sections. The nine tumours had previously been found to undergo a variety of rearrangements to chromosome 1 as determined by Southern hybridization studies, and so were subjected to analysis by FISH in order to gain further insight into these changes.

6.2 Results

6.2.1 Developing the Technique of FISH to Tissue Sections

Initial work to produce a FISH signal in tumour sections was carried out on surplus formalin-fixed paraffin-embedded breast tumour sections, either fibroadenomas or carcinomas, as well as freshly-prepared sections of formalin-fixed paraffin-embedded normal breast tissue. A range of four concentrations of proteinase K was decided upon to generate a reliable signal due to optimal penetration by the probe(s) used: 100, 200, 350 and 500 µg/ml. This range was applied to adjacent sections from each of the specimens to be scored. When under-digested with proteinase K, very little penetration of the probe(s) into the nuclei was obtained, however over-digestion produced a high noise-to-signal ratio. Optimal proteinase K concentrations varied between the specimens used in the study and produced very little or no background unless the probe concentration and/or the antibodies for detection were vastly increased (ten and five times, respectively, the amounts stated in chapter 2, section 2.3.7).

Two-colour FISH was to be used in order to count the number of signals for the two satellites, D1Z1 and D2Z1, situated on chromosomes 1 and 2 respectively. Initial trials of tissue sections and lymphocyte splash preparations (figure 6.1) with this two-colour system showed that the signal from the α -satellite of D2Z1 was far smaller in size than the satellite III of the D1Z1 locus. Attempts were made to reduce the signal of D1Z1 with respect to that of D2Z1, but met with failure. Reduction of the amount of probe and layers of detection of D1Z1 reduced the brightness of the signal but not its diameter; increasing the amount of probe and antibodies for detection of D2Z1 increased the brightness of the signal but not the diameter. Therefore the physical size of the target sequence had a great bearing on the characteristics of the signal produced and it was not possible to match the signal intensities for the two probes. The result was that D2Z1 was often undetectable, most probably masked by the strength of the D1Z1 signal within the nuclei. Reciprocal dual-probe hybridization experiments using the two hapten and detection systems for each probe showed that the digoxigenin, rhodamine system was optimal for D2Z1 since it produced a stronger signal for either probe than the biotin-avidin, FITC system.

Eventually, it was decided to attempt the dual-probe system on the nine tumours obtained from the panel to see if a reliable signal for D2Z1 could be generated in these specimens. On nearly every slide, the D2Z1 signals were undetectable, however the D1Z1 probe generated a reliable signal with at least one proteinase K

Legend to figure 6.1: D1Z1 (FITC, green) and D2Z1 (TRITC, red) co-hybridized to normal human lymphocyte splash preparations and counterstained with DAPI.

a) Two interphase nuclei, one without any detectable signal for either locus. The second nucleus contains both a strong and a weak signal for each locus (arrowheads). With lymphocyte splash preparations, the variation in signal detected in interphase nuclei was variable for each locus; in this case the signals for the two probes were unusually well-matched. The specimen was photographed with a double filter for FITC/TRITC only so that the DAPI counterstain is not detectable. Magnification 2×10^4 .

b) A metaphase spread (not complete) with two copies of chromosome 1 identified by the D1Z1 locus and a single copy of chromosome 2 identified by D2Z1 (arrowheads). The second homologue of chromosome 2 is not present in the group of chromosomes, being outside the field of view. The DAPI counterstain is visible with the use of a triple filter for FITC/TRITC/DAPI. Magnification 2×10^4 .

Figure 6.1: Normal lymphocyte splash preparations

a.



b.



concentration in every specimen. Over-digestion of a section, as determined by D1Z1, did slightly enhance the visibility of D2Z1, but background for both signals was too high to permit counting. Therefore, only signals of D1Z1 were scored in the optimal proteinase K concentrations for each of the nine tumours.

A single probe hybridization and detection was then repeated in duplicate with D1Z1 alone (biotin-avidin, FITC system) at the optimal proteinase K concentrations for each tumour. The duplicated sections were divided into two groups for counterstaining with either DAPI or propidium iodide (PI) to see with which the green FITC signal was optimal for counting. It was clear that while the PI-counterstained nuclei were easier to identify within sections, the FITC signals were often easier to distinguish within the DAPI-counterstained nuclei as the red PI was more intense than the blue DAPI and tended to swamp the FITC signal. This is in contrast to the lymphocyte preparations (figure 6.2), probably because these nuclei were pre-swollen in a hypotonic solution while those in the sections could not be treated in this way, remaining compact and embedded in stromal elements.

The criteria for scoring the signals in a section were established before the results of the hybridizations were examined. Although, scoring of the sections was made without reference to the Southern hybridization data to reduce the likelihood of bias, a truly blind analysis of the FISH results could not be made since only I did the counting from the sections. However, the numbers generated were only compared to the Southern hybridization data once the FISH analysis was completed.

Multiple areas of the sections from each of three slides were included in the counting for each tumour. Unfortunately, very few nuclei could be found for tumour RW 867, hence less than 100 nuclei were successfully scored in this case. The incidence of minor binding sites mentioned in some publications (Hopman *et al.*, 1988; Kim *et al.*, 1993), resulting from cross-hybridization to other regions of the genome containing similar satellite sequences, was eliminated by the stringency of the final set of post-hybridization washes used (0.5x SSC at 45°C) which also removed any remnants of a non-specific background signal. Criteria implemented for scoring the sections to reduce bias were derived from a number of sources (Cremers *et al.*, 1988; Devilee *et al.*, 1988; Hopman *et al.*, 1988, 1991; Nederlof *et al.*, 1989; Kim *et al.*, 1993). Nuclei were dealt with individually, such that the number of spots per nucleus was noted. Each nucleus was scored according to the following criteria:

- 1) the nucleus was uniformly and strongly counterstained

Legend to figure 6.2: Single hybridization of D1Z1 (FITC) to normal human lymphocyte splash preparations, carried out at the same time as the single hybridization of D1Z1 to the tissue sections.

a) Specimen counterstained with DAPI. A single interphase nucleus with two green (FITC) signals for D1Z1, counterstained with DAPI. Magnification 2×10^4 .

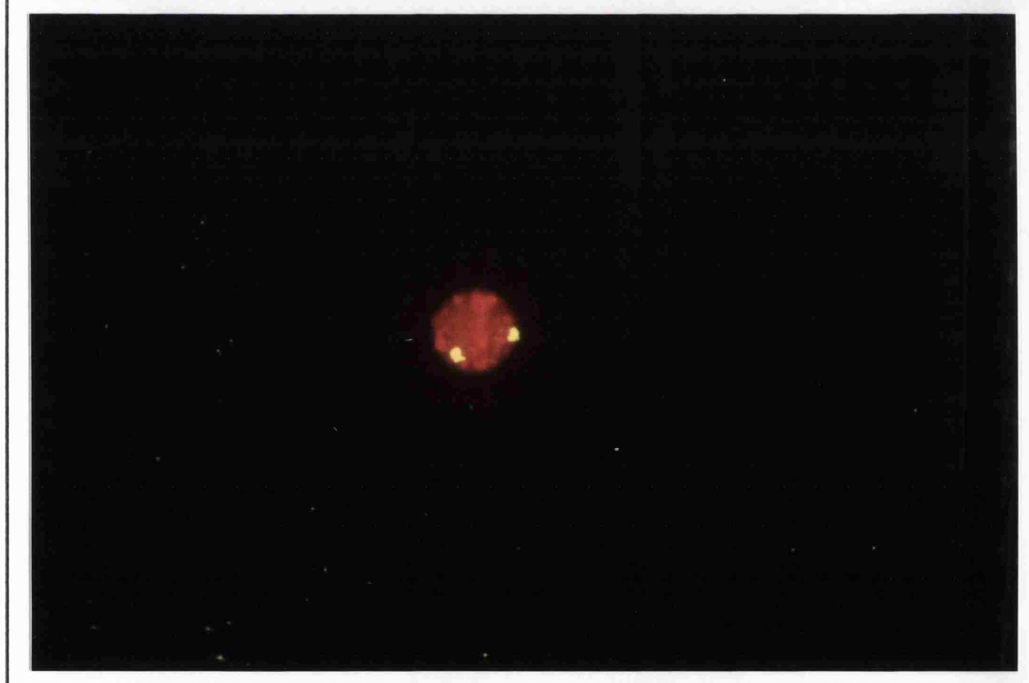
b) Specimen counterstained with PI. A single interphase nucleus with two yellow (FITC) signals for D1Z1, counterstained with PI. Magnification 8×10^3 .

Figure 6.2: Normal lymphocyte splash preparations

a.



b.



- 2) the nucleus appeared intact, well-shaped, remaining adhered to the surface of the slide
- 3) the nucleus was free of cytoplasmic material and overlapping folds of stromal material
- 4) the nucleus was not overlapping with any other; clusters of cells were ignored
- 5) within the nucleus, the signals were approximately homogeneous in intensity
- 6) within a nucleus, paired or split spots situated very close to one another were counted as one
- 7) a nucleus containing patches of signal, rather than specific spots, was scored only when the signals were completely separated from each other.

A small number of nuclei containing no signals are noted in table 6.1. These all occurred within patches of non-overlapping cells that displayed strong hybridization signals and so are unlikely to be due to poor penetration of the probe or incomplete denaturation of the probe or target sequence. Instead, loss of part of the nucleus had probably occurred during sectioning of the tumour when part of the nucleus was physically cut away as the majority of the nucleus was not precisely within the 5µm-thick plane of the section. The good counterstaining of the remains would occur due to the total access of the counterstain to the DNA adhered to the slide. The incidence of these 0-spot nuclei was below 1.25% in each of the five tumours containing them and are not considered to be significant.

D1Z1 maps to the heterochromatic region of chromosome 1 at 1q12 and should be regarded as an indication of the number of copies of the intact centromere of chromosome 1. D2Z1 mapped to the centromere of chromosome 2 itself and would have acted as an internal control to indicate ploidy of the nucleus.

To aid consideration of chromosome dosage in the following sections, let the maternally-inherited homologue of chromosome 1 be designated 1_M and the paternally-derived homologue 1_P . The maternal homologue is routinely assigned the imbalance(s) although the true parental origin of the homologue involved in the events is unknown. Tables 6.1 and 6.2 summarize the results for all tissue sections scored, table 6.2 showing the principal range of copy number and their ratio of incidence in each specimen. Figure 6.3 shows the imbalances to chromosome 1 for each tumour and these are summarized in table 6.2 for comparison to the predominant copy number counts for each.

Table 6.2: A summary of the primary ranges of copy number counts for D1Z1 from FISH and the data obtained by Southern analysis. The normal section was

Legend to figure 6.1: D1Z1 (FITC, green) and D2Z1 (TRITC, red) co-hybridized to normal human lymphocyte splash preparations and counterstained with DAPI.

a) Two interphase nuclei, one without any detectable signal for either locus. The second nucleus contains both a strong and a weak signal for each locus (arrowheads). With lymphocyte splash preparations, the variation in signal detected in interphase nuclei was variable for each locus; in this case the signals for the two probes were unusually well-matched. The specimen was photographed with a double filter for FITC/TRITC only so that the DAPI counterstain is not detectable. Magnification 2×10^4 .

b) A metaphase spread (not complete) with two copies of chromosome 1 identified by the D1Z1 locus and a single copy of chromosome 2 identified by D2Z1 (arrowheads). The second homologue of chromosome 2 is not present in the group of chromosomes, being outside the field of view. The DAPI counterstain is visible with the use of a triple filter for FITC/TRITC/DAPI. Magnification 2×10^4 .

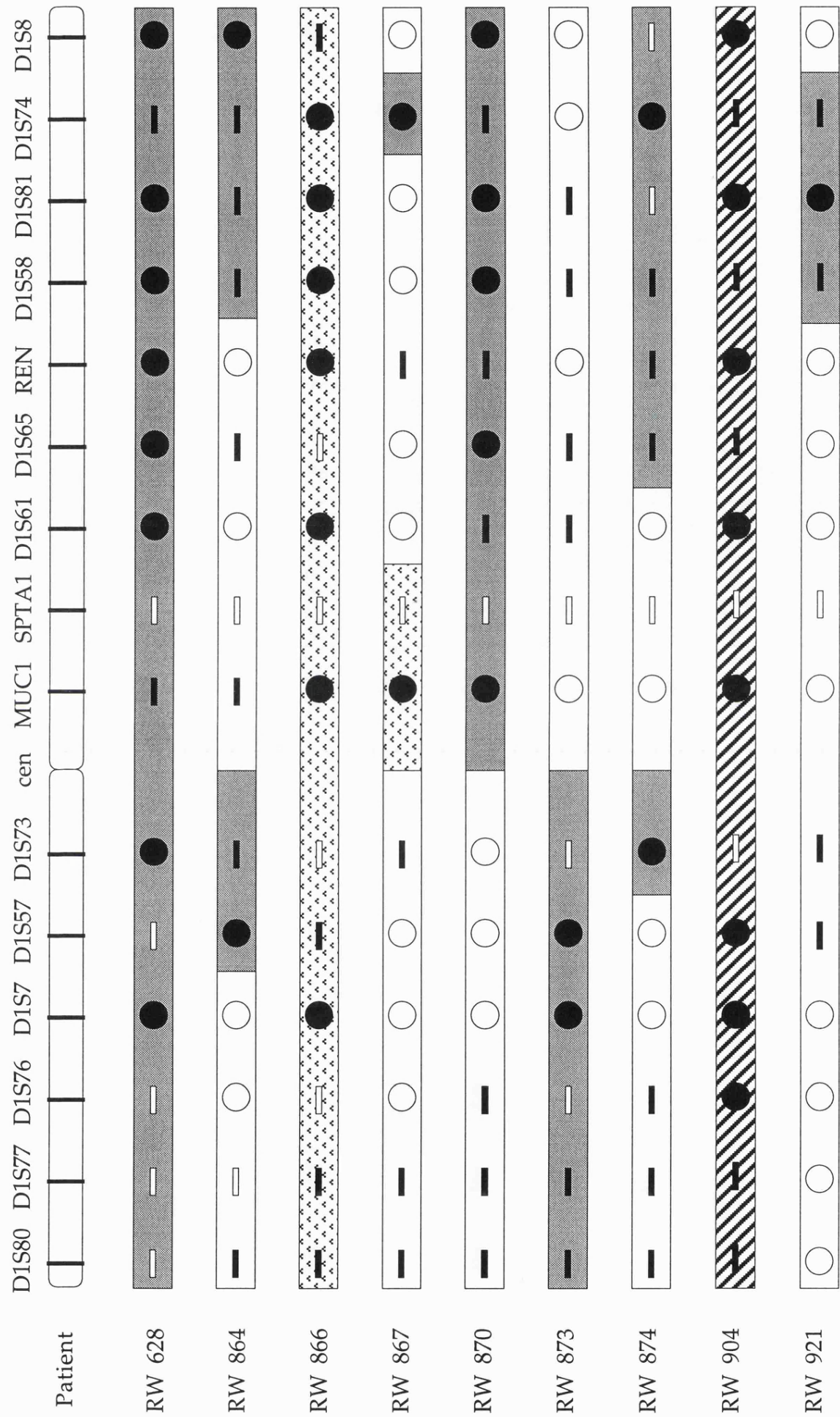
	1,2,3	1,4,1	LOH	whole short arm
RW 871	1,2	1,4	LOH	proximal lp, distal lq
RW 901	1,2,3,4	1,2,1	AI	whole chromosome
RW 921	1,2	1,4	LOH	distal lq

Table 6.2: A summary of the primary ranges of copy number counts for D1Z1 from FISH and the data obtained by Southern analysis. The normal section was taken from a non-tumourous breast and fixed, sectioned and treated as for the tumour specimens.

^a GCN occurred in proximal 1q region, LOH in distal 1q region
n/a, not applicable

Tumour	Signals of D1Z1 per nucleus		AI on chromosome 1	
	Range	Ratio	Imbalance	Extent
Normal	1,2	~1: 4	n/a	n/a
RW 628	1, 2	~1: 1	LOH	whole chromosome
RW 864	1, 2	~1: 3	LOH	proximal 1p, distal 1q
RW 866	1, 2, 3	~1: 3: 2	GCN	whole chromosome
RW 867	1, 2	~1: 3	GCN, LOH ^a	proximal 1q, distal 1q ^a
RW 870	1, 2	~1:1	LOH	whole long arm
RW 873	1, 2, 3	~1: 4: 1	LOH	whole short arm
RW 874	1, 2	~1: 4	LOH	proximal 1p, distal 1q
RW 904	1, 2, 3, 4	~3: 6: 2: 1	AI	whole chromosome
RW 921	1, 2	~1: 4	LOH	distal 1q

Figure 6.3: Summary of Southern hybridization data for chromosome 1 for the tumours studied by FISH also.



6.2.2 Normal Breast Tissue

See also figure 6.4

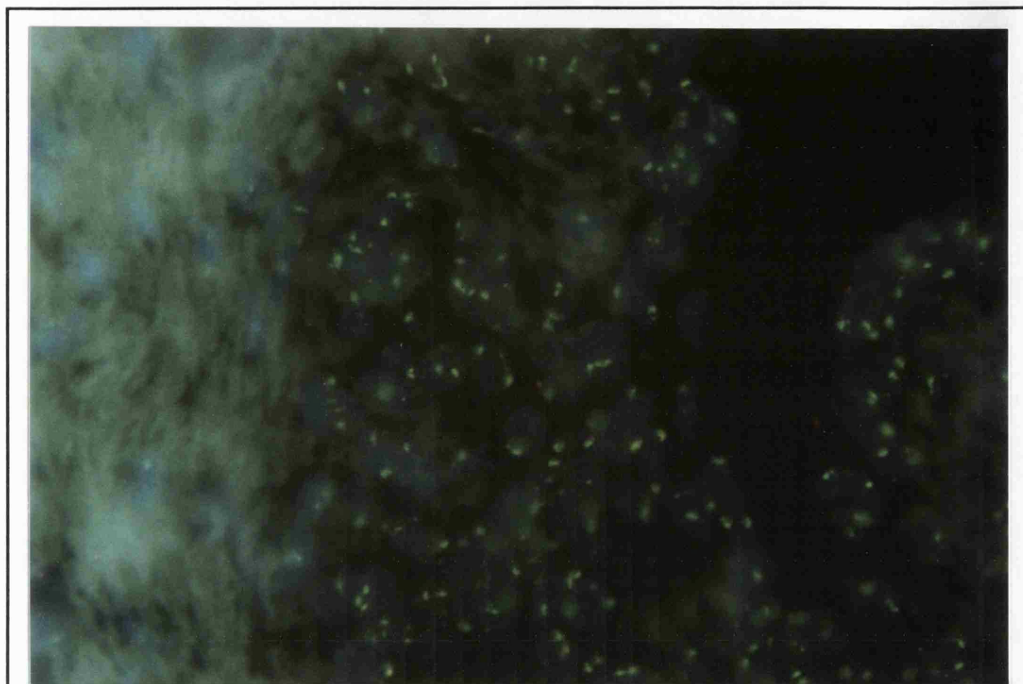
Formalin-fixed paraffin-embedded sections of normal breast tissue from a non-tumourous female were used to provide a positive control for the study. Therefore, the cells from this specimen were expected to contain two signals per nucleus with very little scatter of aneuploidies. The majority of nuclei were found to be disomic for chromosome 1, but 20% of nuclei contained only one signal for D1Z1. This incidence of monosomies is unlikely to be due to actual aneuploidy in the breast cells, but rather to experimental procedures. The former cannot be completely ruled out without the use of D2Z1 as a control.

The whole *in situ* technique relies on the complete denaturation of both target and probe sequences in conjunction with complete penetration into the nuclei by the probe. If either condition is not fully met and only one target is close to the upper surface of the section and easily reached by the probe, then only one spot would be clearly identifiable within a diploid nucleus. The orientation of a nucleus is also important as the two signals may be positioned on top of each other so that only one signal is detected within the nucleus (Devilee *et al.*, 1988). In producing the section, multiple nuclei will have been sliced through since they are not completely within the margins of the 5µm-thick section. Although these partial nuclei are frequently identifiable and excluded from the count, a proportion may in fact appear to be intact and erroneously included in the count thereby skewing the data. The essentially one-sided penetration of the probe into the section is another important factor that has an effect on the fidelity of the results of FISH. Emmerich *et al.* (1989) describe the effects of variation in the actual hybridization conditions of normal diploid lymphocytes. The lymphocytes hybridized on slides as normal showed, in addition to the predominant fraction of nuclei with two signals (~68%), a minor fraction with only one signal (~20%) or even no signal at all (~9%) (Cremer *et al.*, 1988). However, when nuclei were isolated from normal lymphocytes and hybridized in suspension before they were dropped onto slides, 98% of nuclei showed two spots; nuclei with three spots were not observed and only 2% yielded one spot (Emmerich *et al.*, 1989). Repeating this second protocol with tumour cells also generated a higher percentage of diploid cells. If the hybridization of isolated nuclei in suspension is more efficient than on interphase cells already adhered to slides, then hybridization to nuclei contained in tissue sections must be even less effective. Therefore, the incidence of apparent monosomies in normal diploid nuclei is

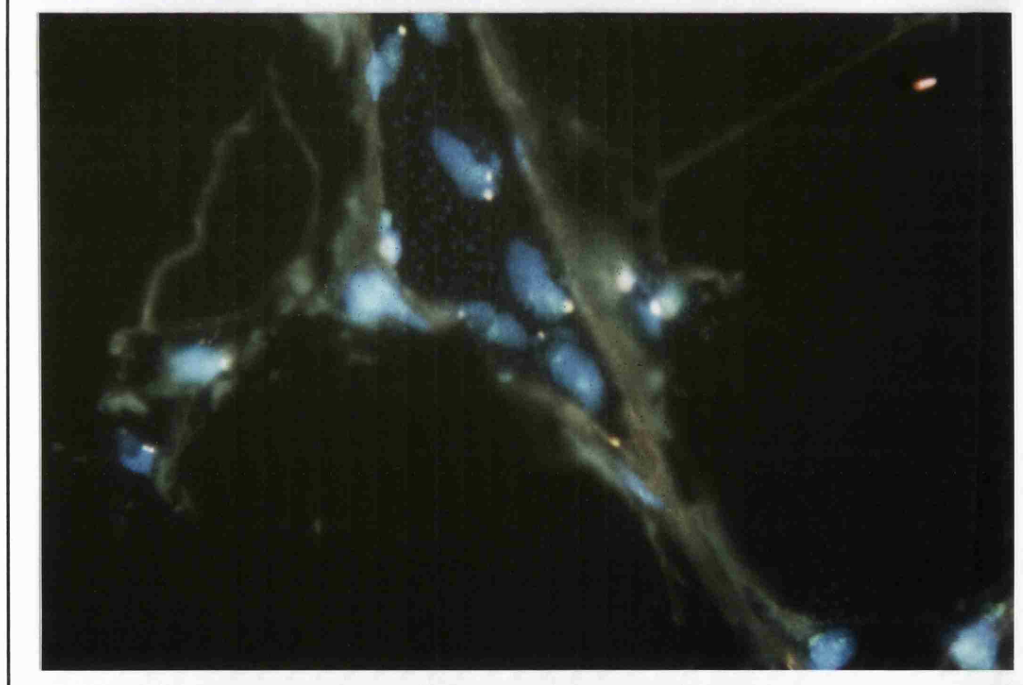
Legend to figure 6.4: D1Z1 (FITC, green) and D2Z1 (TRITC, red) co-hybridized to normal breast tissue sections (digested with 100µg/ml proteinase K) and counterstained with DAPI (blue). Two signals for D1Z1 are evident in most nuclei, although clustering excludes many from the scoring. Signals for D2Z1 were also visible in the nuclei, but are much weaker than those for D1Z1. Hence only D1Z1 signals could be reliably counted in these sections. (a) and (b) represent different areas of the same section. Magnification 8×10^3 .

Figure 6.4: Normal breast tissue

a.



b.



likely to be high when the one-sided penetration and the thickness of the tissue encountered when working on sections is taken into consideration.

The high incidence of nuclei with copy number one less than expected in normal tissues has an important bearing on the interpretation of the data for the tumour sections.

6.2.3 Tumour RW 628

See also figure 6.5

This tumour proved to be heterogeneous with respect to the number of signals of D1Z1 per nucleus. Approximately half the cells contained a single copy of the target while the remainder retained two copies. These results are in close agreement with those obtained by Southern analysis in which LOH extending along the length of chromosome 1 was thought to have occurred. The allele involved at each informative locus studied by Southern hybridization was not completely lost, but clearly reduced in intensity. The residual band seen on the autoradiographs therefore strongly correlates with the FISH scores and is commonly attributed to the presence of normal non-tumourous cells and/or the incidence of tumour cell heterogeneity showing that not all the cells in the tumour mass have the same genetic constitution.

The arrangement of the nuclei with different copy numbers within the sections tended to be patchy rather than interspersed, so that different areas of the section contained cells of differing copy number for chromosome 1. These patches may represent either areas of different tumour sub-clones or areas of tumourous (monosomy-1) and non-tumourous cells (disomy-1). Despite the fact that as many as 20% of nuclei may be falsely monosomic, there remains a significant sub-population of tumour cells monosomic for chromosome 1.

Most of the trisomy-1 nuclei in RW 628 were concentrated within a particular sector of the tissue, but were not exclusive here as nuclei of differing copy number were also found mixed among the trisomic nuclei. This region may be an independent sub-clone of tumour cells that has undergone a different rearrangement to the bulk of the tumour. Near to this region a number of tetrasomic nuclei were identified, which may have been the source of the trisomic cells. These tetrasomic nuclei may either have undergone full tetraploidization (as would have been further indicated or discounted by a

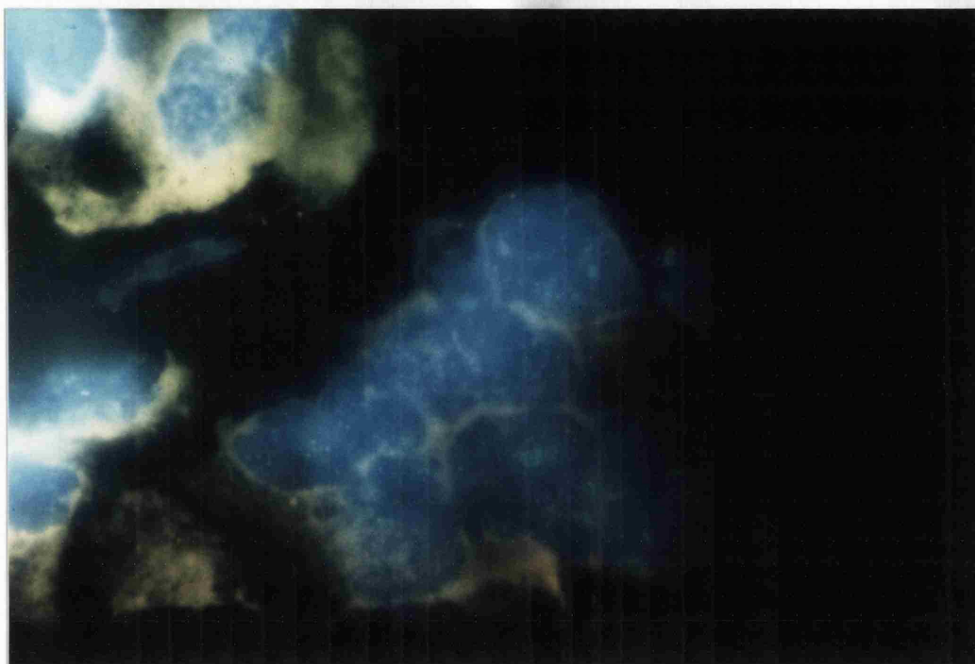
Legend to figure 6.5: D1Z1 (FITC, green) and D2Z1 (TRITC, red) co-hybridized to tumour RW 628 (digested with 100µg/ml proteinase K) and counterstained with DAPI (blue). (a) and (b) are the same area of the section photographed through different filter blocks.

a) DAPI counterstain visible with the triple filter for FITC/TRITC/DAPI. The DAPI shows how the cells are largely overlapping so that most must be excluded from the analysis. Magnification 2×10^4 .

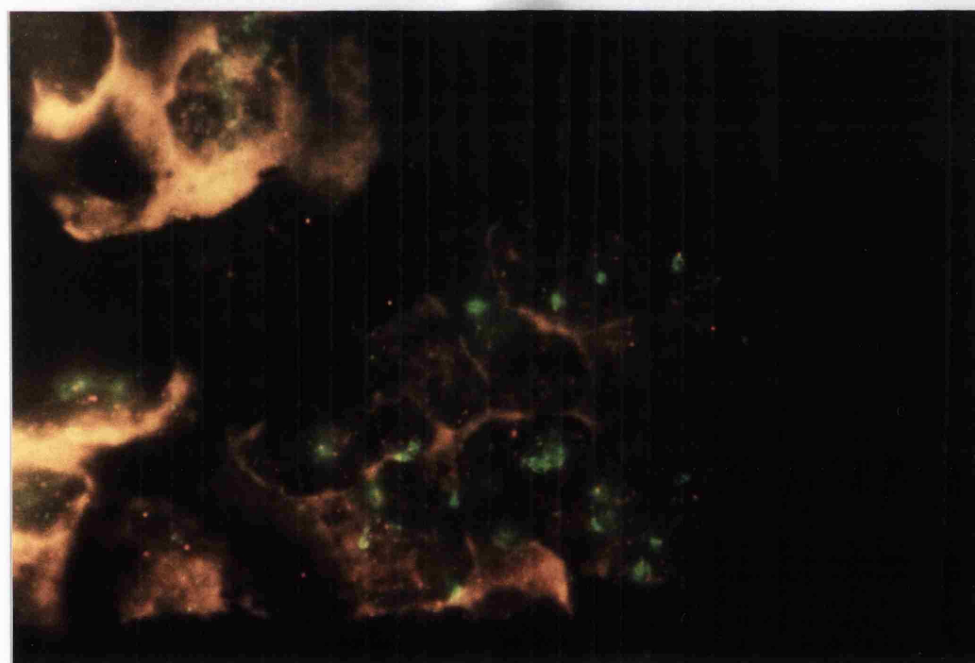
b) Double filter for FITC/TRITC only, prevents DAPI fluorescence. The FITC signals for D1Z1 in this section was obscured by the DAPI with the triple filter block. The non-specific graininess of the failed D2Z1 hybridization is also uncovered by the use of the double filter block. Magnification 2×10^4 .

Figure 6.5: RW 628

a.



b.



successful D2Z1 co-hybridization) or a region of the tumour that is fast-growing so that these cells are currently in G2 of the cell cycle between the S phase of DNA replication and mitosis. If these are genuinely tetraploid cells, then the trisomy-1 nuclei could have developed from them as a result of the loss of one homologue of chromosome 1 through non-disjunction. If so, then these cells would be tetrasomic for chromosome 2 (unknown) and would show up as LOH for chromosome 1 loci compared to D2S44 in Southern analysis. The clear LOH of the entire length of chromosome 1 in RW 628 as indicated by Southern analysis could favour this series of events rather than a gain of either the whole of chromosome 1 or just 1q (D1Z1 maps to 1q12) in nuclei that are otherwise diploid.

If those nuclei containing two D1Z1 signals are assumed to have undergone no changes to chromosome 1, such that one signal is for 1_M, the other for 1_P, then dosage in these cells is

$$1 \times 1_M + 1 \times 1_P.$$

The chromosomal dosage of the monosomy-1 nuclei is

$$0 \times 1_M + 1 \times 1_P.$$

If the two genotypes occur in approximately equal numbers, then the combined ratio of homologues is

$$1:2 (1_M:1_P),$$

but if 20% of cells are incorrectly counted as monosomies due to experimental factors, then the ratio of genotypes is closer to 1:3 (monosomy: disomy) than 1:1 and the combined ratio of homologues becomes

$$3:4 (1_M:1_P).$$

Therefore, both models indicate a clear LOH event at informative loci on chromosome 1.

6.2.4 Tumour RW 864

See also figure 6.6

The predominant copy number for D1Z1 detected in this tumour was two, suggesting these cells are diploid, whether they are tumourous or constitutional, and have two intact copies of the centromeric region of chromosome 1. However, approximately one third of the nuclei contained only one signal suggesting that there is an additional sub-clone present. The majority of these nuclei may be falsely monosomic and so the ratio of monosomics to disomics becomes essentially 0:1. The chromosomal dosage of the cells in the tumour would be

Legend to figure 6.6: Single hybridization to detect D1Z1 (FITC) in tumour RW 864 (digested with 100µg/ml proteinase K).

a) Section counterstained with DAPI. A number of single cells conforming to the criteria for scoring situated in the centre of the field of view. Arrowheads indicate the positions of the D1Z1 signals, although some are not completely in the plane of focus. Magnification 2×10^4 .

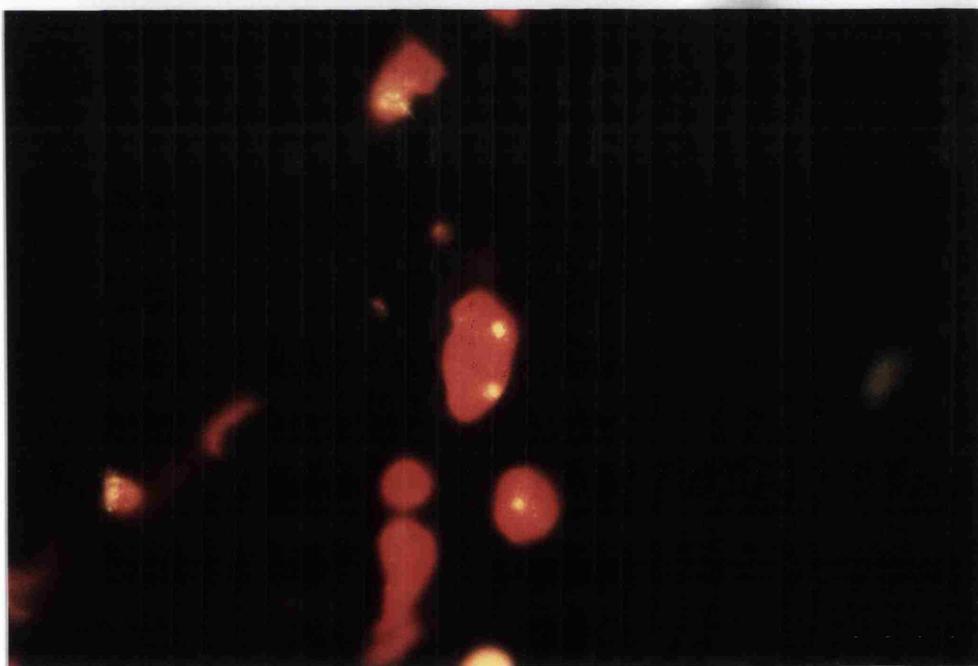
b) Section counterstained with PI. Two cells conforming to criteria, one with two yellow (FITC) signals, the other with a single spot. Magnification 2×10^4 .

Figure 6.6: RW 864

a.



b.



$$1 \times 1_M + 1 \times 1_P,$$

generating an overall ratio of homologues of

$$1:1 (1_M:1_P),$$

from which no imbalance would be detectable by Southern analysis in the region of the centromere. Thus the small imbalances detected on chromosome 1 leave the majority of the chromosome intact preventing distinction between disomic tumour nuclei and normal disomic nuclei by FISH in the sections.

6.2.5 Tumour RW 866

See also figure 6.7

Disomy-1 nuclei are prevalent in this tumour, but a high incidence of trisomy-1 and monosomy-1 nuclei were also scored. Without the presence of a chromosome 2 probe to act as an internal control, the trisomic nuclei may be either diploid or tetraploid. If diploid, then there is an extra copy of chromosome 1 present in these nuclei as indicated by the fully-controlled Southern data. However, if they have in fact undergone tetraploidization, then a subsequent nondisjunction event would have generated the three signals observed by FISH and indicated LOH by Southern hybridization. The Southern data suggest that these are probably diploid cells with an additional copy of chromosome 1 to generate the GCN events. Approximately 4% of the nuclei counted in this tumour contained four signals for D1Z1. These may either be in G2 of the cell cycle before cell division, be genuinely tetraploid, be tetrasomic with a duplication of both chromosome 1 homologues, or tetrasomic with a two-fold amplification of one of homologue of chromosome 1; the latter lending weight to the Southern data for amplification of one particular homologue.

Monosomy-1 nuclei may be falsely scored in this section also, being actually disomics. As they form less than 20% of the total number of nuclei scored in this tumour, it is possible that a proportion of the disomic nuclei may also have been falsely scored and are in fact trisomics. Therefore two populations of cells would exist in this tumour, namely tumourous trisomy-1 cells and disomics that may be normal cells and/or tumour cells without the additional copy of chromosome 1.

The chromosomal dosage of the disomic cells would be

$$1 \times 1_M + 1 \times 1_P.$$

The trisomy-1 nuclei have undergone a nondisjunction event in which an extra homologue of chromosome 1 was gained, such that the dosage in these cells is

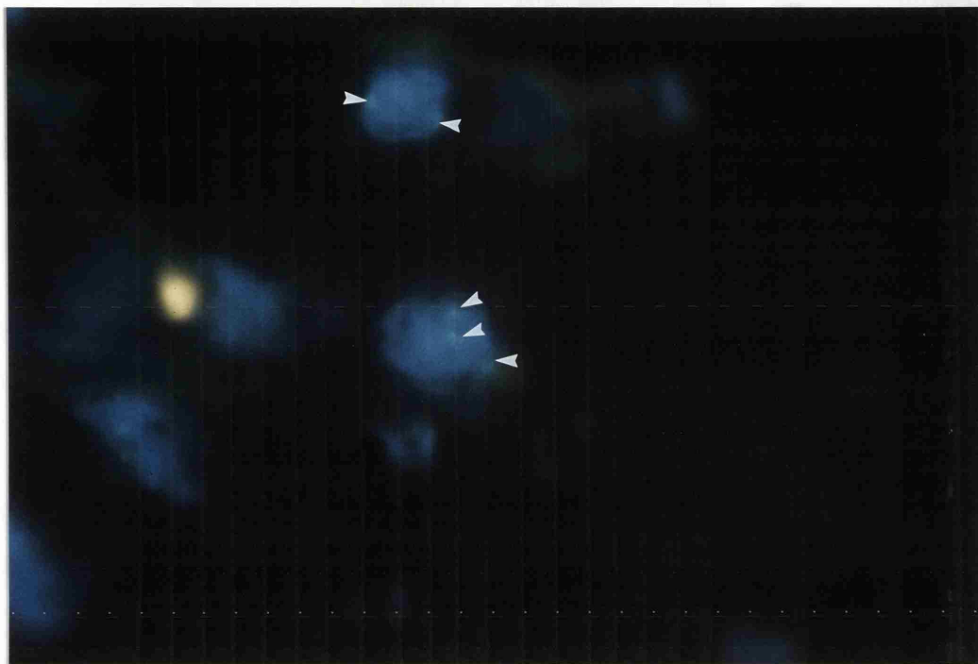
Legend to figure 6.7: Sections of tumour RW 866 digested with 350µg/ml proteinase K. Arrowheads indicate the positions of the D1Z1 signals, some of which are not entirely in the plane of focus.

a) Section co-hybridized with D1Z1 (FITC, green) and D2Z1 (TRITC, red) and counterstained with DAPI. D2Z1 failed to produce a consistent signal but D1Z1 signals were clearly detectable. The nucleus in the centre of the field of view contains three D1Z1 signals, while the nucleus at the top of the field has two signals. Magnification 2×10^4 .

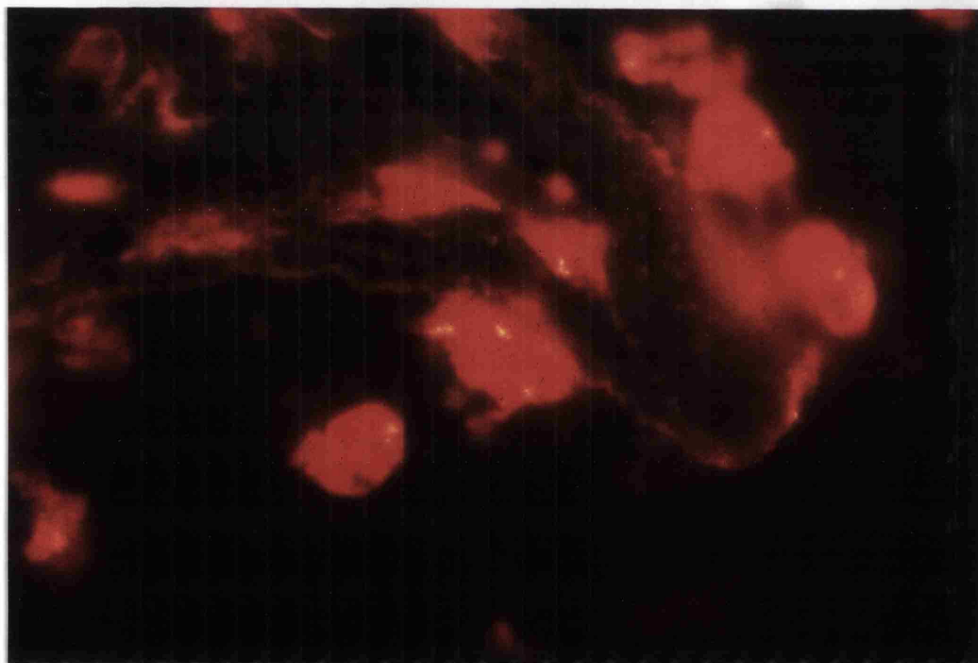
b) Single hybridization of D1Z1 (FITC) to the section, counterstained with PI. Two nuclei conforming to set criteria, one with a single spot for D1Z1, another containing three signals. The background stromal elements also fluoresce red, thereby partially obscuring the precise limits of the trisomic nucleus. Magnification 2×10^4 .

Figure 6.7: RW 866

a.



b.



$$2 \times 1_M + 1 \times 1_P.$$

If the true ratio of monosomies: disomies: trisomies is 0: 3: 2, then the ratio of homologues is

$$7: 5 (1_M: 1_P).$$

Southern analysis of informative probes on chromosome 1 would indicate a GCN of one homologue when fully controlled against an informative locus on chromosome 2.

6.2.6 Tumour RW 867

See also figure 6.8

Very little material was found on the sections from this tumour, but when cells were found, they were invariably solitary in their distribution and predominantly contained two signals for D1Z1. Although a significant proportion of cells were found to have only one signal, these could be false-monosomics so that the nuclei within this tumour are disomic for chromosome 1 and confirms that the small-scale rearrangements detected by Southern analysis have left the remainder of the chromosome intact. In particular, the lack of trisomic nuclei shows that the GCN event at MUC1 is very localized and must be confined to 1q21. The disomic nuclei therefore consist of tumourous and non-tumourous nuclei indistinguishable by the application of D1Z1. Whether there are multiple tumour cell sub-populations is unknown and would only be revealed by FISH with appropriate probe(s).

The chromosomal dosage of the disomies is

$$1 \times 1_M + 1 \times 1_P,$$

and as this is the major apparent genotype, the ratio of homologues in the section is

$$1: 1 (1_M: 1_P).$$

Thus the copy number of chromosome 1 is unaffected by the small-scale rearrangements that have taken place on the long arm.

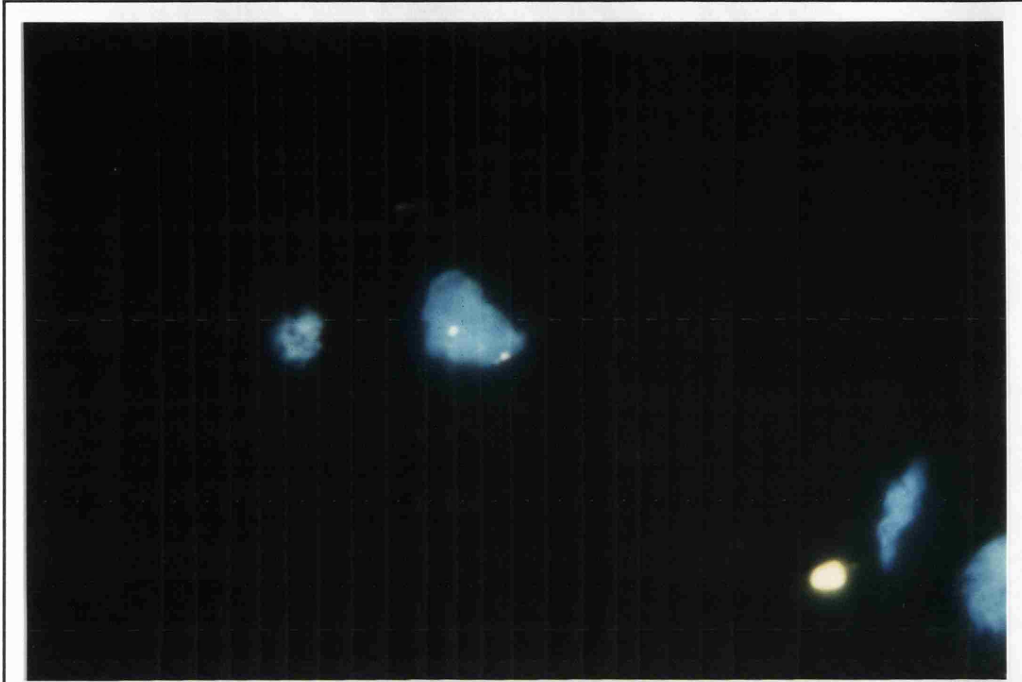
Legend to figure 6.8: Co-hybridization of D1Z1 (FITC, green) and D2Z1 (TRITC, red) to tumour RW 867 (digested with 200µg/ml proteinase K) and counterstained with DAPI.

a) A single intact nucleus containing two red signals for D2Z1. Magnification 2×10^4 .

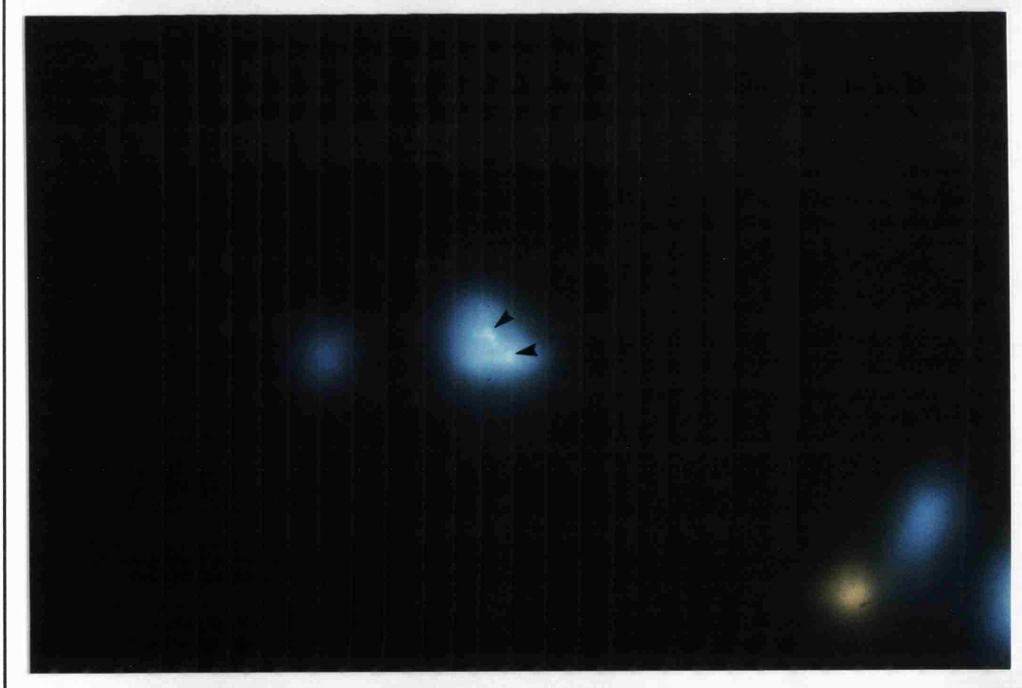
b) The same nucleus as in (a), in a different plane of focus to make the two green signals for D1Z1 visible (arrowheads). Magnification 2×10^4 .

Figure 6.8: RW 867

a.



b.



6.2.7 Tumour RW 870

See also figure 6.9

Similar to RW 628, there were approximately equivalent numbers of monosomic and disomic nuclei scored in RW 870. Therefore it appears that one copy of chromosome 1 has been lost from a significant proportion of cells from this tumour. Southern analysis showed that all informative loci mapping to the long arm of chromosome 1 had undergone LOH while loci on the short arm were retained and unaffected. Together, the data suggest that the whole of the long arm has been lost from half the tumour cells. Whether the actual centromere of the homologue is also involved is unknown as D1Z1 maps to 1q12, but the short arm could easily have been broken away from the centromeric sequences and retained in the cells by translocation onto another chromosome, the remainder of the homologue becoming unstable and lost from the genome.

The disomic nuclei may be either a separate clone of tumour cells or be normal constitutional cells excised along with the tumour. These would have a normal chromosome dosage of

$$1 \times 1_M + 1 \times 1_P.$$

The apparently monosomic nuclei would have the chromosome dosage

$$0 \times 1_M + 1 \times 1_P,$$

while loci on the short arm of the chromosome are retained elsewhere in the genome by another centromere. The combined dosage of these two approximately equal populations of cells is therefore

$$1:2 (1_M: 1_P).$$

If there is an overall error rate of 20% due to experimental factors then the ratio of disomics to monosomics shifts from 1: 1 to 2: 1, and the ratio of homologues becomes

$$2:3 (1_M: 1_P).$$

Both ratios are variant enough to be clearly established by Southern analysis.

6.2.8 Tumour RW 873

See also figure 6.10

Nuclei in this section were predominantly disomic for chromosome 1, but there was a considerable scatter of aneuploid cells counted, with an equivalent number of monosomies and trisomies of chromosome 1 present. 5% of nuclei were

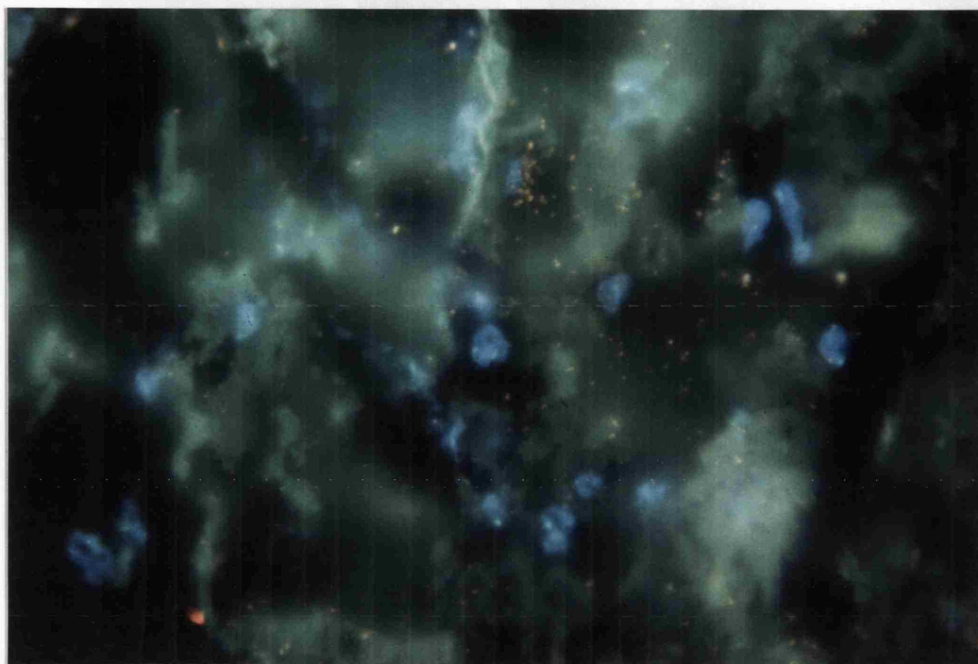
Legend to figure 6.9: Co-hybridization of D1Z1 (FITC, green) and D2Z1 (TRITC, red) to tumour RW 870 (digested with 500µg/ml proteinase K) and counterstained with DAPI.

a) Single nuclei embedded in stromal elements, many with two green signals for D2Z1; D2Z1 failed to produce signals specific to the nuclei on the section. Magnification 8×10^3 .

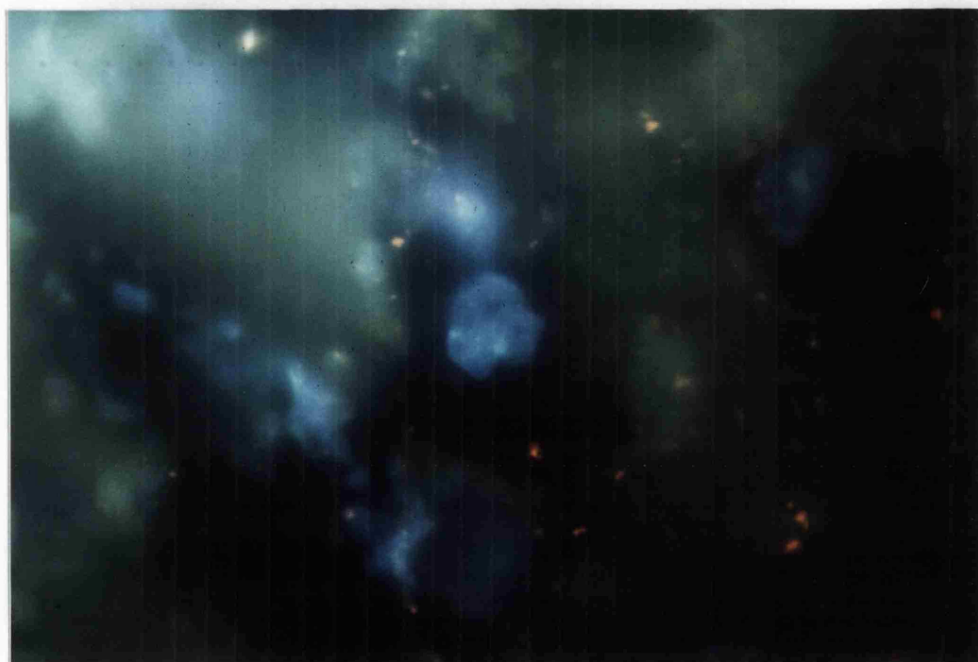
b) The same field of view under higher magnification to show the specificity of D1Z1 signals. D2Z1 signals do not coincide with the counterstain and can be regarded as non-specific in nature. Magnification 2×10^4 .

Figure 6.9: RW 870

a.



b.



Legend to figure 6.10: Sections of tumour RW 873, digested with 100µg/ml proteinase K.

a) Co-hybridization of D1Z1 (FITC, green) and D2Z1 (TRITC, red) to the section with DAPI counterstaining. A single nucleus with two green signals for D1Z1 and two red signals for D2Z1. Magnification 2×10^4 .

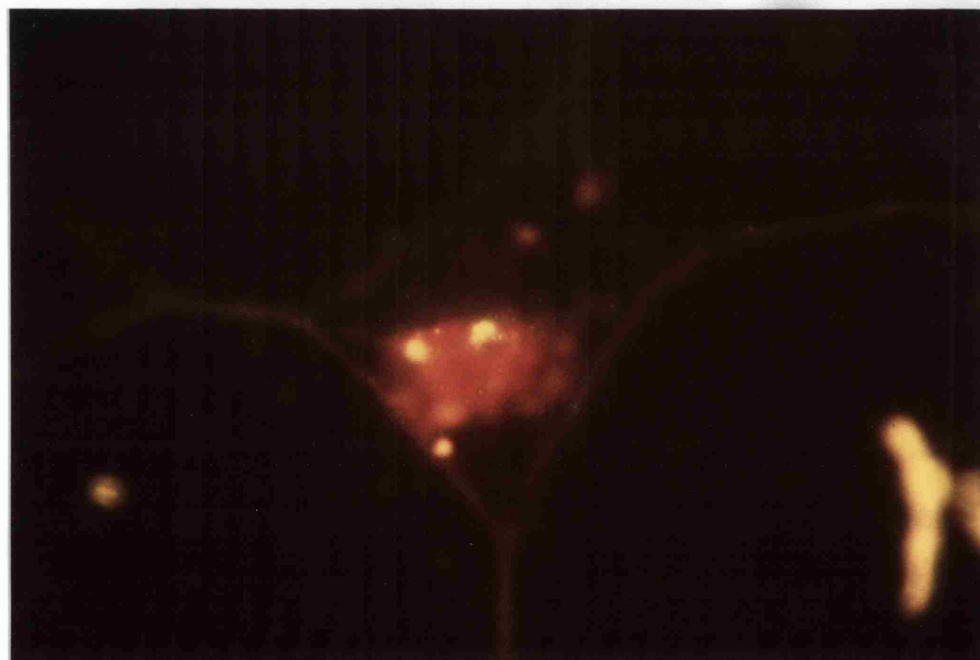
b) A single probe hybridization to detect D1Z1 (FITC), counterstained with PI. A single cell containing two D1Z1 signals is shown. Magnification 2×10^4 .

Figure 6.10: RW 873

a.



b.



tetrasomic for chromosome 1 and may well be either genuinely tetraploid or be fast-growing cells currently in G2 phase of the cell cycle.

Southern data indicated that the short arm of chromosome 1 had undergone LOH with loci on the long arm unaffected by any imbalance. Whether the LOH event includes the actual centromeric sequences is unknown as D1Z1 maps to 1q12 and breakpoints could have occurred immediately either side of the centromere. Karyotypes of breast tumours frequently site breakpoints in 1q12 (Gebhart *et al.*, 1986; Gerbault-Seureau *et al.*, 1987; Dutrillaux *et al.*, 1990), although these are usually associated with changes to the long arm, and breakpoints for deletions of most of the short arm cluster at 1p11 and 1p13 (Gerbault-Seureau *et al.*, 1987; Mitchell and Santibanez-Koref, 1990; Hainsworth *et al.*, 1991, 1992). Therefore, it is most likely that the deletion of the short arm in RW 873 is a result of an unbalanced translocation where the breakpoint is sited between D1S73 and the centromere. The signals for D1Z1 in the disomic nuclei were invariably strong and equivalent in size suggesting that the breakpoint has certainly not occurred at 1q12.

The similar numbers of monosomies and trisomies for chromosome 1 suggest that they are the reciprocal products of a nondisjunction event. One homologue did not segregate correctly at mitosis and both copies moved into one daughter cell along with one copy of the second homologue while a single copy of the second homologue moved into the other daughter cell. Therefore overall no imbalance would be detected as chromosome dosage for the monosomies is

$$0 \times 1_M + 1 \times 1_P,$$

while for the trisomies it is

$$2 \times 1_M + 1 \times 1_P.$$

The chromosomal dosage for the apparently disomic nuclei is

$$1 \times 1_M + 1 \times 1_P,$$

and the overall ratio of different nuclei is 4: 1: 1 (2-spot: 1-spot: 3-spot). Therefore, the ratio of homologues is

$$6: 6 (1_M: 1_P)$$

and no AI detectable by Southern hybridization except at informative loci on the short arm.

However, the 20% error due to false-monosomics would cause all the monosomies to disappear, leaving the ratio of disomics and trisomics at 13: 2 and the ratio of homologues becomes

$$17: 14 (1_M: 1_P).$$

It is doubtful that such a variation would be enough to be detected as this would mean a 15-20% increase in one allele over the other three alleles under scrutiny. As only clear imbalances were scored in the Southern analysis, borderline cases such as these may be suspected but were not sufficient to be scored as AI. The trisomy-1 cells form a small proportion of the total number of cells within the tumour and may be forming a new sub-clone where after the short arm is lost, additional changes are taking place where the long arm is being duplicated, but either did not gain a growth advantage over the general tumour cell population or surgery was performed before it could begin to grow. If additional sections were scored, the percentage of trisomics could possibly fall further to correlate better with the Southern data to show a dominant population of apparently disomic nuclei.

6.2.9 Tumour RW 874

See also figure 6.11

The majority of nuclei displayed two signals of D1Z1, although there was a small scatter of aneuploidies also found. 5% of cells were counted as tetrasomic for chromosome 1 and may be either in G2 of the cell cycle or fully tetraploid in nature; successful implementation of the dual-hybridization system with D2Z1 would have helped to resolve this question. It can be assumed that the remaining cells are probably diploid in nature with changes in copy number occurring to chromosome 1. The predominance of the disomy-1 nuclei suggests that the centromeric region of the chromosome is intact despite the proximal short arm LOH and distal long arm LOH events detected by Southern hybridization in this tumour. The incidence of monosomies is attributable to the 20% error rate in scoring of false-monosomics, leaving a single peak of disomic nuclei and showing that the small-scale losses on either arm have not induced changes in gross chromosomal structure affecting copy number. Therefore the chromosomal dosage of disomic nuclei is

$$1 \times 1_M + 1 \times 1_P,$$

generating an overall ratio of homologues of

$$1:1 (1_M: 1_P)$$

as expected from the Southern hybridization data.

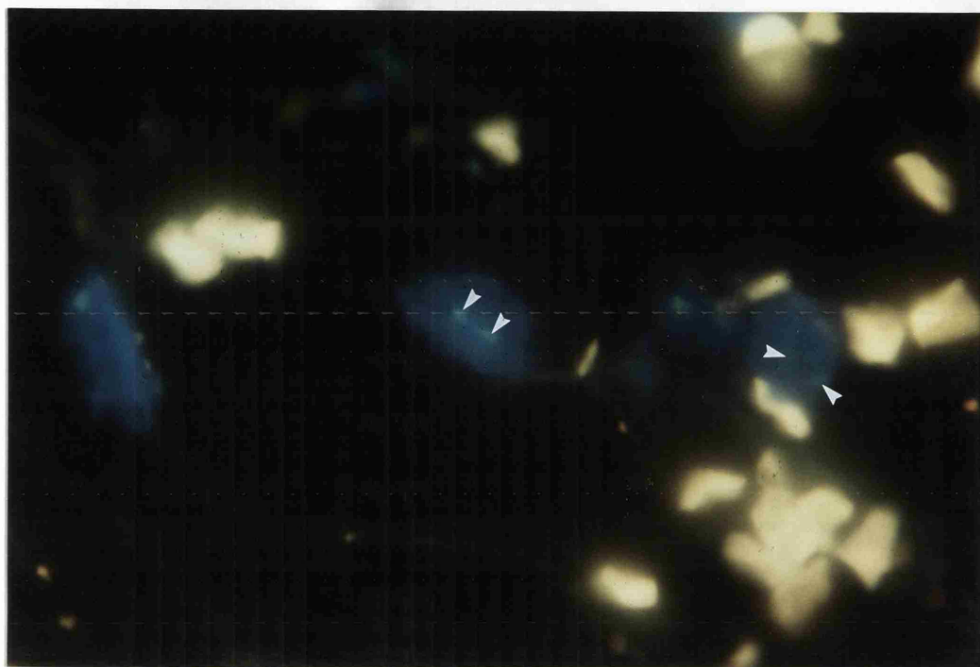
Legend to figure 6.11: Single hybridization to detect D1Z1 (FITC) in sections of tumour RW 874, digested with 100µg/ml proteinase K.

a) Section counterstained with DAPI. Intact nuclei contain two green signals for D1Z1 each (arrowheads), although some signals are out of the plane of focus. Magnification 2×10^4 .

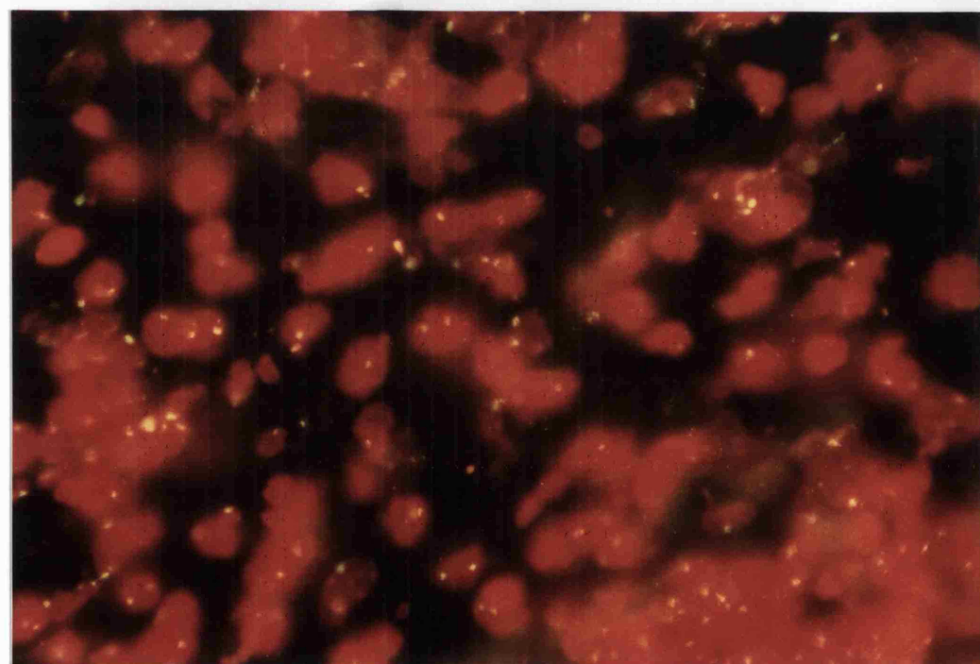
b) Section counterstained with PI. A field of cells mostly containing two signals per nucleus, although there is some clustering of the nuclei which excludes those involved from the scoring. Magnification 8×10^3 .

Figure 6.11: RW 874

a.



b.



6.2.10 Tumour RW 904

See also figure 6.12

There is a significant scatter in the copy number of D1Z1 within the cells of RW 904, although the peak copy number of chromosome 1 was two. The high incidence of monosomies, trisomies, tetrasomies (perhaps diploids in G2 of the cell cycle), as well as nuclei with five and six copies of the chromosome suggests that there are a number of diverse tumour cell sub-populations. Those nuclei with three to six copies of D1Z1 were loosely grouped in the same region of the tumour, although not exclusive to it with monosomic and disomic nuclei interspersed here. The three 0-spot nuclei were all closely situated in this region of differing aneuploid cell types. Therefore, it would appear that this sector of the tumour was experiencing gross genomic instability, possibly as a result of rapid tumour evolution. Monosomies were found primarily elsewhere in the sections and tended to be grouped, as were the predominant disomies. Even if 20% of the nuclei were incorrectly scored due to experimental factors, then there would remain a large scatter in copy number for RW 904. How these nuclei would be distributed in the counting is not clear, but the overall picture of copy number of chromosome 1 may shift upward, so reducing the incidence of monosomies and increasing the number of both disomies and trisomies. The imbalance remains clear and as Southern data implicate the whole chromosome in the event, this may be an increase in copy number as highlighted by the trisomic nuclei and possibly those containing four, five and six signals.

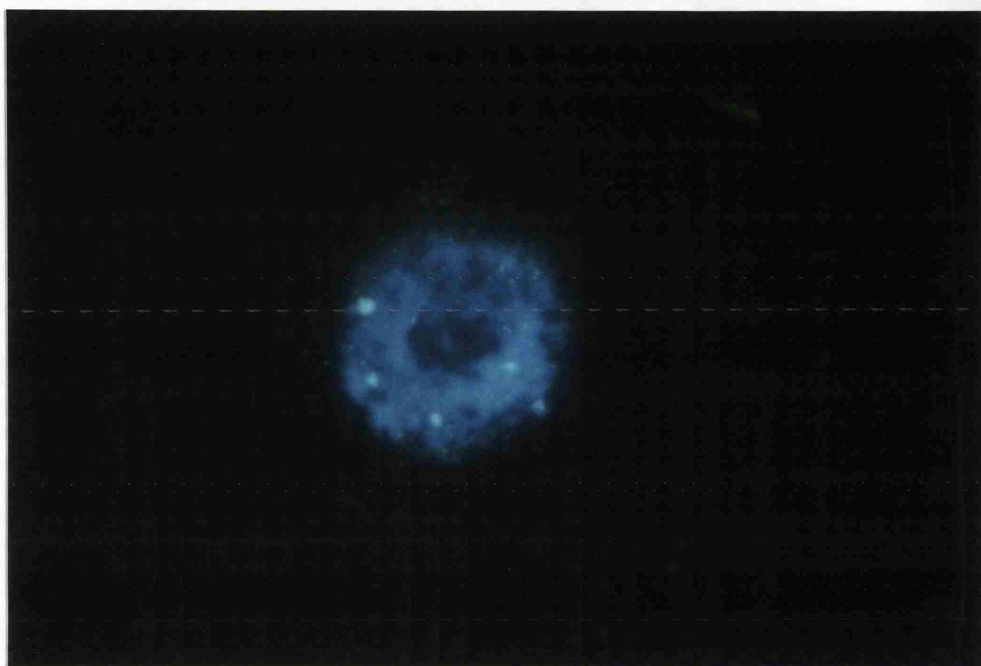
The ploidy status of the tumour may be predominantly diploid, but that of the various sub-populations is unknown. The tumour had undergone AI on chromosome 1 and at D2S44, so that the nature of the imbalance involving the whole of chromosome 1 could not be established from the autoradiographs. If the D2Z1 probe had been reliably working in the co-hybridization protocol, then it would have been possible to see how the copy number of both chromosomes 1 and 2 varied throughout the section. Interestingly, RW 904 shows AI on every chromosome successfully studied in this project, i.e., chromosomes 1, 2, 7, 17, 20 and X (the RB1 locus on chromosome 13 was untested in this patient), so that an additional control locus to another chromosome must be uncovered that is not affected by AI in RW 904. Until then, the overall picture of this tumour remains unclear except that changes have affected a surprising cross-section of chromosomes in the human genome including the often rearranged regions and others that serve as good controls in most breast tumours.

Legend to figure 6.12: Single hybridization to detect D1Z1 (FITC) in sections of tumour RW 904, digested with 200µg/ml proteinase K.

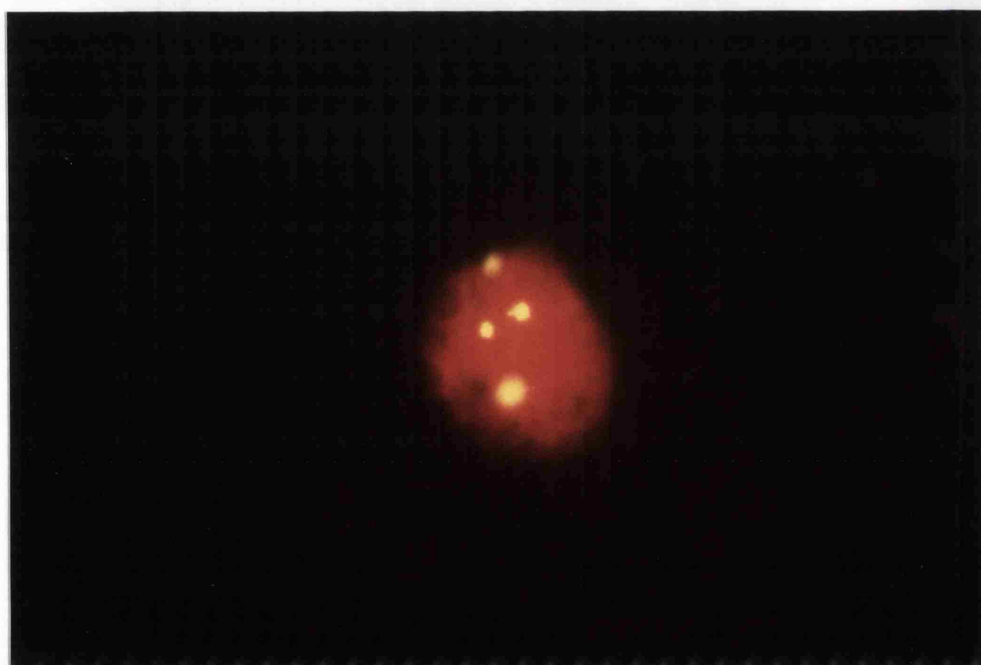
- a) Section counterstained with DAPI. A single intact nucleus is shown containing five distinct green signals for D1Z1. Magnification 2×10^4 .
- b) Section counterstained with PI. A solitary cell with four discrete signals in the nucleus representing D1Z1. Magnification 2×10^4 .

Figure 6.12: RW 904

a.



b.



6.2.11 Tumour RW 921

See also figure 6.13

The majority of nuclei were found to contain two signals of D1Z1 in this tumour. The level of monosomies is in accordance with the 20% error in normal tissue, so that the small LOH event on the long arm of chromosome 1 has occurred in diploid cells and has left the affected homologue intact as expected. Therefore chromosomal dosage of the disomics would be

$$1 \times 1_M + 1 \times 1_P$$

to generate the expected ratio of homologues

$$1:1 (1_M:1_P).$$

6.3 Discussion

6.3.1 Copy Number of D1Z1 in Tissue Sections

The analysis of these tumours by FISH has helped to clarify and confirm the Southern data on chromosome 1, but it has also raised some questions that are unanswerable without the successful use of the D2Z1 locus. Many of the scenarios proposed could have been quickly confirmed by defining the copy number of chromosome 2 in the nuclei scored, although the situation for RW 904 is complex and would probably require additional probes to other chromosomes. If the dual-hybridization system is not optimal for D1Z1 and D2Z1, then single-probe hybridizations to a number of sections should generate results of interest, although this would not serve as an internal control within each nucleus scored for D1Z1 copy number. Perhaps substitution of the probe recognizing D1Z1 with one detecting the α -satellite on chromosome 1 would generate more reliable results by the dual-probe protocol since the size of the target sequences would be matched.

The scatter of aneuploidies, especially monosomies, for RW 864, RW 866, RW 867, RW 873, RW 874 and RW 921 was unexpected in light of the Southern data. However, the similar level of monosomies in the normal breast tissue sections provided an adequate explanation. Therefore, corroboration between the datasets becomes more certain if up to 20% of nuclei have also been 'mis-counted' in these tumours due to the problems with tissue sections. The degree of correlation of data for RW 628 and RW 870 is not greatly affected when a 20%

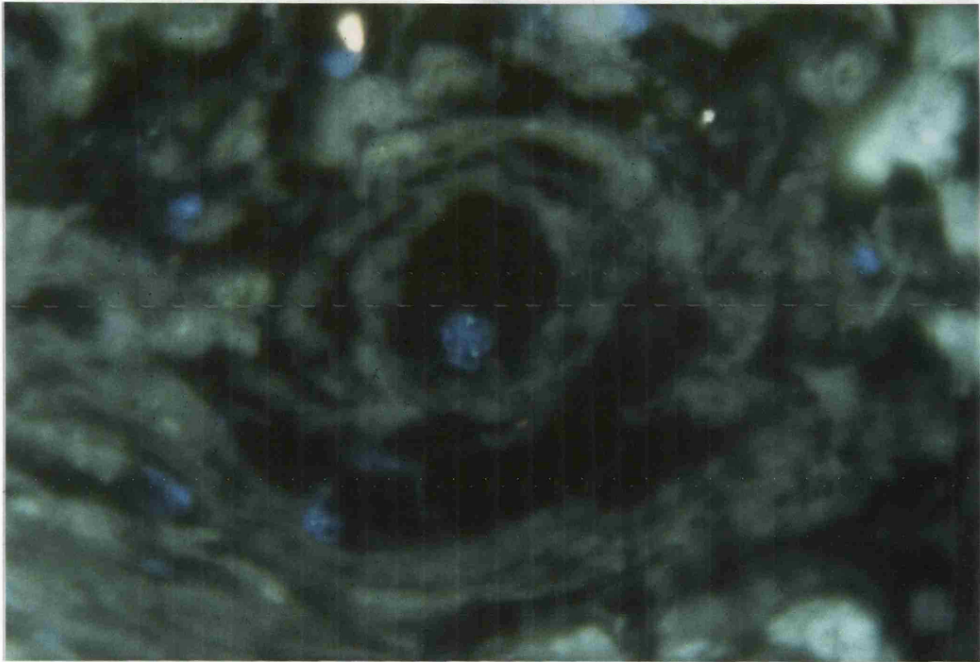
Legend to figure 6.13: Single hybridization to detect D1Z1 (FITC) in sections of tumour RW 921, digested with 200µg/ml proteinase K; DAPI is used as the counterstain.

a) In the centre of the field, a solitary nucleus conforming to the set criteria containing two green signals for D1Z1. Other nuclei in the field of view do not appear to be intact. Magnification 8×10^3 .

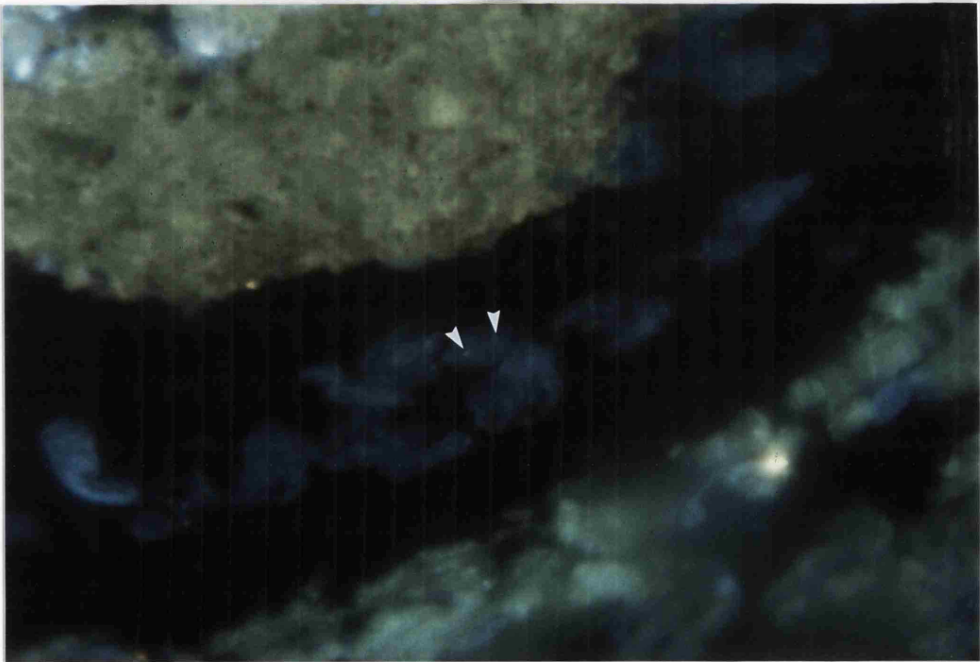
b) A group of cells, many overlapping and therefore not satisfying the criteria. However, at the centre of the field, there is one discrete nucleus containing two signals of FITC (arrowheads). Magnification 8×10^3 .

Figure 6.13: RW 921

a.



b.



inaccuracy is incorporated into the FISH scores as the required imbalances are retained. Since the data for RW 904 are complex, little can be made of the results until unaffected loci can be discovered that can serve to control for the imbalances observed in the tumour.

That the small-scale deletions observed in RW 864, RW 867, RW 874 and RW 921 are localized and have no consequences for the rest of the chromosome, shows that the chromosome is not always subject to changes in copy number during tumourigenesis. The copy number changes thought to have occurred in RW 628, RW 866 and RW 904 are confirmed by FISH, although more work must be carried out on RW 904 to elucidate the ploidy levels and incidence of sub-populations within the tumour.

The major peak of D1Z1 copy number in all specimens scored is two, suggesting that not only are there normal diploid cells, but the tumour cells, except in RW 904, are also principally diploid in nature and are composed of different sub-clones as a result of cells undergoing different genetic rearrangements. Tumour heterogeneity is also evident from the residual bands observed on the autoradiographs obtained by Southern hybridization. Normal cells present must have been enveloped by the invasive spread of the tumours, in some cases still forming a high percentage of the total number of cells. If there are tumour cell sub-populations which have not undergone the imbalance event on chromosome 1 determined by Southern analysis, then other changes involving different chromosomes must have occurred to enable them to proceed to their tumourous state.

Similar to the findings of Devilee *et al.* (1988), the frequency distributions of signals in this study are typical in two ways. Firstly, the major peak appears to indicate the modal number of chromosomes present in that cell population, and secondly the percentage of nuclei with one spot less than the peak is always higher than the percentage of nuclei with one spot more than the peak value. Devilee *et al.* (1988) also reported that in the second point, the percentage of nuclei with one less signal was slightly more variable than those with one signal more between different observers, presumably due to marginally different interpretations of the criteria. Therefore it is likely that the high score of monosomics in each of the ten essentially disomic specimens is due to experimental factors and the incorporation of the 20% error rate from the normal specimen into the models for the tumours is valid. Ideally though, additional normal breast tissue sections from unrelated non-tumourous females should be scored to confirm that this error is standard.

An alternative method of scoring signals per nucleus was employed by Dhingra *et al.* (1992), in which the total number of spots in a field of view was divided by the number of nuclei to obtain a chromosome index (CI). In order to control for hybridization efficiency between sections, a normalized chromosome index (NCI) was also calculated. The NCI was obtained by dividing the CI of the epithelial cells by the CI for the signal from normal diploid lymphocytes present in the section. Although it is probably quicker to count the number of signals within a field of view and then the number of nuclei, this method may be more prone to error due to not matching signals with nuclei conforming to set criteria. In addition, signals may be uncounted as they are situated in different planes of focus within the nuclei. It is probable that the use of CI and NCI calculations will ultimately mask regional differences between sub-populations, unless the CI and NCI for each field of view was calculated separately. Therefore, the identification of each nucleus according to set criteria and their individual scoring, as carried out in this project, should provide the most reliable information on the cell population in the tumour mass.

It is clear from this limited study that FISH analysis of tumour samples, or interphase cytogenetics as it is increasingly referred to, can enhance the amount of information available on each tumour in the panel and clarify the Southern blot data from their DNA. Reliable implementation of the dual probe technique to tissue sections would probably reduce the small discrepancies observed in some tumours between the two techniques and help resolve the data for tumours with AI on multiple chromosomes such as RW 904.

Unfortunately, the parental origin of each of the target sequences detected cannot be established by FISH such that four-spot nuclei may be either diploid with two additional copies of the chromosome derived from either or both homologues, diploid but currently in G2 phase of the cell cycle, or truly tetraploid in nature. Southern hybridization of DNA from patients and their relatives would identify the parental origin of the alleles involved and thereby also the origin of the homologue(s) involved, but the copy number for each homologue is difficult to determine even by laser scanning densitometry. Therefore, FISH and Southern analysis do work hand-in-hand to generate models to explain the results of each for a particular tumour. RW 866 is a prime example of how to generate a model for the tumour based on fitting two different kinds of data together. This could be a questionable practice without a full range of controls on both datasets, but the potential of merging the two techniques is obvious. If flow cytometry data were also available, an additional dimension would be added to the developing picture of each tumour since there would be copy number (somy) data on each

chromosome of interest, ploidy levels would also be known, as well as heterogeneity within the tumour mass. It would be possible to establish whether adjacent tumour sub-populations do contain some related chromosome rearrangements and possibly detect a gradation of genetic changes through a tumour that could lead to further tumour evolution and potentially to metastasis. Where the reciprocal products of non-disjunction events are concentrated, their variation in frequency could indicate which clone has a selective advantage possibly brought on by the change in copy number.

6.3.2 Future work

The success of FISH to the tissue sections warrants further work in this area. First, the incidence of apparent monosomies in normal tissue sections needs to be confirmed by the study of additional unrelated individuals with no evidence of breast cancer; ten individuals should provide an adequate answer. If the level of monosomies remains constant, then it can be attributed to the use of sections in the hybridization protocol through reduced hybridization efficiency, dissected nuclei etc., as detailed above. One extra consideration should be made: it is possible that such monosomy is characteristic of breast tissue in general. FISH analysis of tissues sections derived from other sites in the body would help resolve this, as would implementation of some of the modifications discussed below.

Whilst single probe hybridization generated useful data, dual-hybridization techniques would be optimal by providing an internal control for the technique to aid analysis. Perhaps using a satellite locus other than D1Z1 for chromosome 1 would balance the signals generated with D2Z1 in a dual-hybridization protocol. Another change made could be the procedures by which the cells are prepared for hybridization. Several reports mention the use of sodium isothiocyanate during the preparation of formalin-fixed, paraffin-embedded sections to dissociate the protein-DNA complex (Hopman *et al.*, 1991; de Wit *et al.*, 1992; Steiner *et al.*, 1993). Alternatively, instead of hybridizing the probes direct to thin sections of tissue, it may be better in future to take touch preparations of fresh tumours at several sites in the mass by sectioning the tumour immediately after collection and applying microscope slides to each surface produced. Thus, cells would adhere to the glass slide while the stromal elements of the tissue are largely left behind. However, this is not now possible for the present panel of tumour specimens as these have all been frozen and/or formalin-fixed, paraffin-embedded for storage. Thus, these changes to the protocols would help determine whether the

monosomies were artifactual, providing data on apparent monosomies of chromosome 2 in normal and cancerous tissues and altering the incidence of monosomies for both chromosomes by altering hybridization efficiency and the percentage of intact nuclei present. If the incidence of apparent monosomies is as assumed above, then similar levels would be found for a control locus by the current protocol, but improvements to the technique would result in reduction of the overall incidence of monosomies in any tissue with any probe.

In addition to studying copy number of chromosome 1, the X chromosome could be analyzed in tumours by the use of an α -satellite mapping to the centromere of the chromosome. This should confirm that the X chromosome is not subject to increases in copy number and generally exhibits only small-scale deletions that do not span the centromere. Thus, two dual-hybridization protocols could be developed, firstly D1Z5 (α -satellite on chromosome 1) and D2Z1, and secondly DXZ1 and D2Z1.

CHAPTER 7

Correlation of Southern Data with Clinico-Pathological Variables

7.1 Introduction

It is common to try to correlate the changes seen at specific sites in the genome with various features of the tumour obtained by pathological examination and/or clinical parameters of the patient herself. In this way additional clues to the aetiology of breast cancer can be uncovered as the timing of specific events in tumourigenesis is established.

The Southern data was assembled for comparison and three statistical tests were applied, namely the chi-square (χ^2) test, Yate's correction for the chi-square and the Fisher-Irwin test. The chi-square was the primary test of association, being used in every case while the others were implemented only as deemed appropriate. These tests are designed to try to disprove a null hypothesis, i.e. an hypothesis of no association between the variables.

In the majority of cases, the tests could not disprove independence of the variables, suggesting there was no association. However, there were two highly significant results obtained and a number of borderline cases were also identified, which suggest association.

7.2 Results

7.2.1 Datasets for Statistical Analysis and Contingency Tables

The consensus rearrangements determined from the Southern hybridization data and subsequently subjected to statistical analysis are presented in table 7.1. The full details of the clinical data for all patients in the panel under study are listed in Appendix V. It was decided not to compare the proportion of the seven chromosomes (chromosomes 1, 2, 7, 13, 17, 20 and X) undergoing allelic

Table 7.1: Rearrangements observed in tumours by Southern hybridization and subjected to tests of association to each other and the clinical data available. Some regional rearrangements were not included, those identified on chromosome 1 (except when grouped together), the small deletions on Xp outside the pseudoautosomal region and the proximal Xq region, since these were derived from very few tumours.

Chromosome	Specific alteration
Chromosome 1	Small deletions in any region Whole 1q arm deletion Whole chromosome loss Large deletions Small regional gain events (GCN) Large gain events (GCN)
X chromosome	Distal PAR deletion (DXYS78) DXYS89-DXS278 deletion (assuming single common region of deletion) PAR deletions (including those above) Distal Xq deletion (DXS438-DXS52) Small deletions (including those above) Large deletions
Chromosome 13	Allele loss at RB1 (LOH and GAL)
Chromosome 17	Allele loss at D17S5 (LOH and GAL)

imbalance in each tumour with the clinical information for a number of reasons. Not all patients were found to have informative loci on all chromosomes included in the study, so that the population sample available for statistical testing would not be accurate if all patients were used. Also, the different combinations of chromosomes undergoing AI in the patients defied attempts at grouping for placement within a contingency table. The five independent regions of deletion on chromosome 1 were grouped as a single variable (table 7.1), since the data are not sufficient to support the targeting of specific sequences for deletion on this chromosome at this time. The two independent sites of deletion on Xp that occurred in sufficient numbers to permit statistical analysis, i.e. distal PAR and DXYS89-DXS278 consensus deletions, were not tested for association. In such a case, bias in observation is unavoidable when drawing up the tables because of the incidence of events involving the whole of the PAR or the whole of Xp that may or may not have the same target(s) as the smaller deletions and also because two 'adjacent' localized events are usually identified in different tumours.

Contingency tables can be drawn to display all the data available on the frequency of two variables, so that all combinations that are possible between the variables are stated and observations recorded as appropriate. Such contingency tables were drawn up for each pair of samples under test of association (see Appendix VI for examples). In no case did the total sample reach 71, the size of the panel; different numbers of patients were excluded from each table since they did not have a full set of clinical data or did not have informative loci in the region of the chromosome under test.

7.2.2 The Chi-Square Distribution and Test of Association

For speed, due to the large number of tests to be carried out by hand, the chi-squared formula was applied to every table initially to obtain an approximation of the probability of the null hypothesis being correct, i.e. that there was no association between the variables (see Appendix VI.1 for the formulas and a worked example). The chi-squared distribution can be used for a number of different problems, including giving critical values for the goodness-of-fit tests and tests of association in contingency tables (Neave, 1989). The chi-square test is thought to work well in most conditions usually providing a conservative estimate of the probability of association (Lewis *et al.*, 1984). Unfortunately in this project, most of the derived tables contain low values and chi-square does not perform well under such circumstances by providing an overestimation when

Ex.<5 (Lewis *et al.*, 1984). Therefore, although all tables were analyzed by the chi-square test, any apparent significantly deviant result, as well as borderline cases, required testing by other methods (see below). However, the chi-square test is still regarded as applicable provided that at most one cell out of five or more (or two cells out of ten or more) have $1 \leq \text{Ex.} < 5$ (Lewis *et al.*, 1984). Therefore, although the 2x2 tables do not qualify, 3x2 tables do qualify for this exception (see Appendix VI.1). Regrouping of the observed frequencies can achieve the requirement that $\text{Ex.} \geq 5$, thereby reducing the number of cells in the table through amalgamation by row or by column. 3x2 contingency tables can be reduced by this method but 2x2 contingency tables cannot be re-grouped since no table would exist to enable a meaningful comparison and alternative procedures must be used for these cases (see below).

Although most pairs of data were extremely unlikely to have any association due to the very low χ^2 values obtained, there are a number of borderline cases (table 7.2) that will be worth re-testing when additional patients have been identified through further study. In particular, there is a single table that shows a highly significant result at the <1% level by chi-square test: the frequency of small deletions on the X chromosome compared to the lymph node status of the patients.

7.2.3 Yates' Correction for the Chi-Square Distribution

When $\text{Ex.} < 5$ in a 2x2 contingency table, Yates' correction can be substituted for the standard chi-square calculation (Campbell, 1975). Therefore, this correction was applied as appropriate in order to obtain a closer approximation to the chi-square curve when $\text{Ex.} < 5$ (see Appendix VI.2). In fact, Yates' correction increased the probability of no association in all cases shown in table 7.2 and suggested that none of the datasets produced correlations.

7.2.4 The Fisher-Irwin Test for Exact Probability

Alternatively, the Fisher-Irwin test for exact probability can be applied instead of both the chi-square approximation and Yates' correction to 2x2 contingency tables. By hand, this test (also known as the Fisher test) involves a long calculation and so was not applied to all 2x2 tables, but only to those with a chi-square score ≥ 2.0 , when $\text{df} = 1$. Therefore all cases showing significant deviation from the null hypothesis as well as any cases approaching borderline significance as suggested

Table 7.2: Summary of the pairs of data showing possible association. $P(\chi^2)$ represents the standard chi-square probability of no association, $P(\chi\gamma^2)$ the probability after Yates' correction, and $P(F-I)$ the exact probability as determined by the Fisher-Irwin test. The dataset of small deletions on the X chromosome vs. lymph node status did not require Yates' correction since $Ex.\geq 5$ in each cell. Similarly, comparisons of allele loss at RB1 and D17S5 found in this project with published levels did not require Yate's correction.

Variable I	Variable II	χ^2	P (χ^2)	χ_Y^2	P (χ_Y^2)	P (F-I)
Whole 1q arm deletion	Lymph node status	3.845	2.5-5%	2.323	10-20%	8.0%
Small regional gains on chromosome 1	Lymph node status	2.531	10-20%	1.173	10-20%	16.7%
Whole 1q arm deletion	PR status	4.86	2.5-5%	1.951	10-20%	8.8%
Loss of whole chromosome 1	EGFR status	8.399	<0.5%	1.59	20-30%	100%
Distal PAR deletion	Lymph node status	3.383	5-10%	2.151	10-20%	13.3%
DXYS89-DXS278 deletion	Lymph node status	3.628	5-10%	2.097	10-20%	8.0%
PAR deletions	Lymph node status	3.61	5-10%	2.452	10-20%	9.6%
Small deletions on the X chromosome	Lymph node status	8.48	<0.5%	n/a	n/a	0.4%
DXYS89-DXS278 deletion	ER status	3.384	5-10%	1.938	10-20%	9.0%
Large deletions on the X chromosome	EGFR status	4.725	2.5-5%	0.73	30-50%	18.0%
Allele loss at D17S5	Menopausal status	2.329	10-20%	0.854	30-50%	20.5%
Allele loss at D17S5	ER status	2.666	10-20%	1.091	20-30%	24.9%
Distal PAR deletion	Allele loss at RB1	2.075	10-20%	1.033	30-50%	9.0%
Allele loss at RB1	Allele loss at D17S5	3.6	5-10%	1.6	20-30%	20.6%
Small deletions on chromosome 1	Large deletions on the X chromosome	3.715	5-10%	2.324	10-20%	10.3%
Small deletions on chromosome 1	Grade (I; II and III)	4.59	2.5-5%	3.051	5-10%	4.5%
DXYS89-DXS278 deletion	Grade (I and II; III)	2.089	10-20%	0.873	30-50%	31.5%
Large deletions on the X chromosome	Grade (I; II and III)	2.503	10-20%	1.211	20-30%	17.7%
Allele loss at RB1	Grade (I; II and III)	4.189	2.5-5%	2.01	10-20%	7.8%
Allele loss at RB1	This project and literature	4.178	2.5-5%	n/a	n/a	n/a
Allele loss at D17S5	This project and literature	3.311	5-10%	n/a	n/a	n/a

by the uncorrected chi-square calculation ($\chi^2 = 2$, $df = 1$, 10-20% probability of the null hypothesis being correct) were re-tested using the Fisher-Irwin calculation. It was not necessary to retest those conforming closely to the null hypothesis by chi-square approximation, since this test generates an overestimated probability of deviation from the hypothesis under the sub-optimal conditions dominating the datasets in this project (Lewis *et al.*, 1984).

Table 7.2 gives a summary of those pairs of data subjected to the Fisher-Irwin test. In most cases the probability of no association derived by χ^2 is lower than the exact probability, but the probability from Yates' correction is higher than the exact probability. Therefore, Yates' correction is overly-conservative in its estimation and cannot rule out possible deviation from the null hypothesis.

There are two pairs of data that have >95% probability of association (<5% probability of the null hypothesis being correct). It appears that small-scale deletions occurring on the X chromosome may correlate with lymph node status (exact probability of 0.4%). These deletions are found in patients that have no spread of the cancer to the lymph nodes and so correlate with a favourable prognosis. In addition, small deletions occurring on chromosome 1 may correlate with tumour grade (exact probability of 4.5%). These deletions are found exclusively in patients with higher tumour grade (II and III), i.e. poorly-differentiated tumours, and explains why testing tumours of grades I and II grouped against tumours of grade III did not generate a significant result for small deletions on chromosome 1 [$\chi^2 = 0.417$, $P(\text{null hypothesis correct}) = 50\text{-}70\%$]. This highlights the importance of testing all combinations of re-grouped frequencies to ensure that any possible associations are not masked.

The remaining cases with borderline significance by chi-square test all had exact probabilities >5% of deviation from the null hypothesis. These data with borderline significance pose difficulties, in that firm conclusions cannot be drawn until additional cases are available to increase the sample population. These may well have occurred by chance since a large number of tests were carried out, enhancing the chances of detecting significance; hence the importance of defining the borderline between significant and insignificant deviations from the null hypothesis and ensuring that the tests carried out are appropriate to the data. However, although they require confirmation, the following correlations can be inferred and the probabilities stated come from the Fisher-Irwin test.

There are a number of consistent deletions that occur more frequently than expected in tumours which have not spread to the lymph nodes which has a

good prognosis for the patient. These are the deletions encompassing all or most of the long arm of chromosome 1 (9.0% probability), the consensus region of deletion at DXYS89-DXS278 on Xp (8.0% probability) and all deletions localized to the pseudoautosomal region on Xp (9.6% probability). Notably, DXYS89-DXS278 deletions occur exclusively in lymph node-negative patients. Some of the DXYS89-DXS278 deletions are included in the PAR deletions and may be targeting the same sequence, hence both possibly correlating with lymph node status. However, the distal PAR deletion did not produce a result of significance (13.3%), suggesting that PAR deletions in general do not occur for the same reasons (i.e. the same sequences are targeted), but that there are indeed two independent targets for deletion.

Deletions involving all or most of the long arm of chromosome 1 also occur in progesterone receptor-negative tumours (8.8% probability) although only two tumours with this loss had PR data available. The DXYS89-DXS278 consensus deletion occurs exclusively (9.0% probability) in oestrogen receptor-positive tumours. Oestrogen and progesterone receptors are useful predictors of response to endocrine therapy whereby tumours positive for both receptors (ER+, PR+) display the highest response rates but tumours lacking both receptors (ER-, PR-) are usually endocrine independent, regressing infrequently with endocrine therapy (Osborne *et al.*, 1980).

After the 3x2 contingency tables for examining rearrangements and tumour grade were re-grouped into 2x2 tables, one of these generated a result with borderline significance and bears out the argument for testing all combinations of re-grouped frequencies. Allelic loss at the RB1 locus on chromosome 13 was found exclusively in tumours of grade II and III, i.e. poorly-differentiated tumours.

Allelic loss at the RB1 minisatellite on chromosome 13 also correlated with the consensus deletion localized to the distal region of the pseudoautosomal region of the X chromosome, i.e. the deletion centred on DXYS78 primarily occurred in tumours with allelic loss at RB1 (9.0% probability).

The remainder of those tested by the Fisher-Irwin calculation all had exact probabilities of >10%. While some of these also cannot be firmly excluded from association through additional cases, they are more unlikely to be confirmed as significant. One dataset, whole chromosome 1 loss vs. EGFR status, generated an exact probability of 100% despite appearing to be of borderline significance by chi-square analysis. The reason for this appears to be due to the test itself, where in this case the only values of n possible are 0 and 1, given the upper row marginal

total of 1. Therefore the test appears to be unable to cope with such limited values of n and cannot be applied in such cases.

7.2.5 Deviation of Allelic Loss at RB1 and D17S5 from the Literature

Two additional chi-square tests were performed to determine whether the frequencies of allele loss at RB1 and D17S5 were significantly different from the totals derived from the literature. In both cases, $Ex. \geq 5$ for every cell, so that the total population size was large enough for the chi-square test to be operating under optimal conditions, in which case it provides a conservative estimate of the probability of deviation and neither Yate's correction nor the Fisher-Irwin test was applied in either case. While the frequency of allele loss at RB1 in this project is significantly different from the average from the literature (<5% probability), that for D17S5 is only of borderline significance (5-10% probability). Neither pair of variables were subjected to the as the sample sizes involved were well within the limitations of the chi-square test.

7.3 Discussion

Statistical tests have identified two highly significant results. Small-scale deletions occurring on the X chromosome are found in patients that have no spread of cancer to the lymph nodes and so correlate with a favourable prognosis. There may be a corresponding association between these deletions and stages of the disease (non-distant metastatic disease). However, data on tumour stage was too limited to permit any such comparison. The second result shows that small deletions occurring on chromosome 1 occur exclusively in patients with poorly-differentiated tumours (grades II and III). Since the small deletions on chromosomes 1 and X are obtained by summing all the regionalized deletions on the respective chromosome, it would expected that each of the smaller consensus events should also give the same result as the generalized small deletions on the chromosomes. No such results were obtained and this can probably be attributed to low frequency of the relevant alterations available.

The correlation of small deletions on the X chromosome with negative lymph node status suggests that these alterations confer a growth advantage on the tumour cells rather than an ability to invade and metastasize. If this is the case, then sequences that are targeted for inactivation have control over the

proliferative characteristics of cells. Such sequences may also be involved in the aetiology of benign breast neoplasms and hyperplastic lesions and it would be appropriate to examine loci mapping to the X chromosome in such patients for loss of heterozygosity.

Small deletions on chromosome 1 correlate with tumours of high grade. The cells in these tumours are poorly-differentiated and are more likely to metastasize, having a poor prognosis for the patient. Therefore, sequences targeted for inactivation may have control over cell morphology, signal transduction and/or metastatic potential as the structure of the cells does not closely resemble normal epithelial breast cells and they may have become unresponsive to the controls applied to normal cells. The basement membrane is also more likely to be perturbed and even breached since the tumourous cells are so abnormal, thereby aiding the process of metastasis. Such tumours are harder to treat since prior to surgical excision, metastasis will probably already have taken place, proceeding via the lymph nodes, to give a poorer prognosis. Not all patients with high grade tumours are found to be lymph node positive, suggesting that the primary tumour was discovered early on and excised before metastasis had taken place.

Therefore, these two groups of rearrangements are biological markers that appear to define different sub-groups of tumours. These markers may help to predict the aggressive behaviour of a breast tumour and provide a clearer picture of a patient's prognosis. However, because it is likely that as there is more than one target for LOH on each chromosome, additional patients need to be examined and the statistical tests reapplied to see which of the targets, if any, are truly associated.

There are two main points to address the issue of deviation in the frequency of allele loss at RB1 in particular and also at D17S5. First, there does seem to be a bias in the patients in the panel, but as this bias is unintentional, it does not negate the overall results obtained during the project and merely suggests that the incidence of rearrangements on chromosomes 1 and X would be altered slightly if assessed in other panels which conform to the literature averages for RB1 and D17S5. As neither locus correlated well with any of the clinico-pathological variables tested here, so that no particular class of tumour was present at increased frequencies, the issue of bias is not a major consideration. Secondly, a number of factors question the validity of the LOH recorded in the literature, so that it is not comparable to the data presented here, and the latter is more reliable. Most of the allele loss at RB1 and D17S5 recorded in the literature was carried out when LOH studies were first begun; many of these early studies

did not employ controls for the amounts of the DNA in the tracks of autoradiographs, and more importantly, did not always actually compare the tumour DNA profile obtained for a locus with a constitutive DNA profile (e.g. lymphocyte DNA). Thus, it is not really possible to assess how good the two loci RB1 (VNTR) and D17S5 are as positive controls, except perhaps that they generated allelic imbalance of unsurprising frequency for two anonymous DNA loci corresponding to a known tumour-suppressor gene and the suspected site (due to previously-recorded high LOH) of a second tumour-suppressor gene, respectively.

CHAPTER 8

Conclusion

Originally regarded as an opportunity to define regions on chromosome 1 where tumour-suppressor genes may reside that are involved in malignant breast disease, the project has instead yielded new information on other aspects of chromosomal rearrangements. The relationship between chromosome 1 and breast cancer has been found to be as complex as the cytogenetic data suggested. Although small-scale deletions do occur on chromosome 1 and appear to form five discrete regions of loss, it has not been possible to clearly establish that any of these actually specifically targeting sequences. Chromosome 1 is frequently subject to large-scale events involving whole arms or even the whole chromosome, thus affecting the copy number of vast tracts of sequence through LOH, GCN or GAL. Thus, the technique of Southern hybridization can be sensitive enough to distinguish between the different forms of allelic imbalance that can occur in tumours, when the amounts of DNA in the tracks of the autoradiographs can be established. The complex alterations to chromosome 1 in the panel of tumours studied appear to be upheld by the data obtained from *in situ* hybridization to tissue sections from nine tumours. These nine tumours displayed a variety of rearrangements to chromosome 1 as determined by Southern hybridization analysis and the copy number counts for the D1Z1 locus used in FISH fit these data. In sharp contrast to chromosome 1, the Southern hybridization data for the X chromosome are far more easy to interpret. A high frequency of very small deletions occur, principally in the pseudoautosomal region at the tip of the short arm of the X chromosome: the first is centred on the DXYS78 locus in the distal region of the PAR, and the second is more proximal, possibly spanning the pseudoautosomal boundary.

The general supposition that proximal 1q is targeted for specific LOH can be discarded since, although LOH undoubtedly occurs in this region at high frequency, in only one tumour is the event localized to this area of 1q. Instead LOH at proximal 1q predominantly occurs as part of a large-scale event involving huge tracts of sequences which encompass most of the chromosome arm.

The complex patterns of AI identified on chromosome 1 were found to bear striking resemblance to the data available on the karyotypes of breast tumours. The frequency of those forms of AI indicating inactivation of a tumour-suppressor gene do not occur in similar proportions, LOH being the principal form while GAL rarely takes place. This suggests that the complex mechanisms proposed by Cavenee *et al.*, (1983) for the retinoblastoma susceptibility gene, which lead to GAL, can occur in breast tumours, but that simple deletions or unbalanced translocations predominate. It also appears that interstitial GAL events target the same regions of the genome as LOH, particularly shown by such events on chromosome 1 and possibly also those on the X chromosome. For LOH and GAL, the reduction in intensity of the deleted allele varied between tumours. This is attributed to varying numbers of sub-clones within each tumour, not all of which have undergone the event in question and/or varying levels of 'contaminating' normal tissue such as stromal and capillary cells. As an assessment of the degree of normal tissue within the tumours was not within the scope of the project, it is not possible to confirm a correlation between the reduction of an allele and the amount of normal tissue within the tumour blocks from which DNA was extracted.

It is important to point out that because only single loci were assessed on each of chromosomes 13 and 17, it is not possible to confirm that these loci were the actual targets of the imbalances observed, thus GAL involving the RB1 VNTR and D17S5 may not be targeting the same sequences as the LOH events. Indeed formally, it is entirely possible that none of the imbalances recorded at the loci in question were specific to the RB1 gene or the putative distal 17p tumour-suppressor gene.

The labour-intensive work involved in typing patients by Southern hybridization is now being superseded by quantitative PCR techniques that currently appear superior in all respects, except one. Unless internal controls can be incorporated into the technique through multiplex reactions, the nature of allelic imbalance events cannot be determined. This project, by using Southern hybridization techniques, has shown the critical requirement of such controls, especially for polymorphic markers on chromosome 1, by the frequency of small GCN events that occur in the same regions of chromosome 1 as the small-scale deletions. If only LOH had been assumed to occur, then a distinct proximal 1q region of LOH would have evolved from three patients, FY, RW 660 and RW 867. The incidence of GAL events would not have been established without careful analysis of controls either. However, speed and conservation of DNA samples do demand that quantitative PCR be implemented in deletion mapping studies.

By mapping the limits of regional GCN events in the same way that deletion mapping can be carried out, three independent regions subject to GCN have been uncovered on chromosome 1, although they do not occur in sufficient patients to confirm targeted amplification at this time. The large-scale GCN events also observed on the chromosome may coincide with the regional GCN events and thus have similar consequences for tumourigenesis. Notably, the presence of an isochromosome of 1q in PB 134 underlines the dichotomous nature of such a rearrangement where different events on each arm can have opposite consequences for the sequences involved (inactivation or amplification), while each can still drive tumourigenesis.

The unexpected level of LOH on the X chromosome led to an examination of sequences throughout the chromosome and uncovered a high incidence of LOH at several sites. The undetermined order of DXYS17 and DXYS89 with respect to other loci on the chromosome does generate some uncertainty in the exact number of independent regions of deletion near to the boundary of the pseudoautosomal region, but the most parsimonious analysis of the data available results in a single deletion between DXYS89 and DXS278. However, the unknown order of DXS52 and DXS438 remains less of a problem until further deletion mapping with additional loci in the area is carried out.

That the two principal SROs include sequences in the pseudoautosomal region of the X chromosome suggests that the pseudoautosomal region does contain genes that have similar dosage-requirements to conventional autosomal genes. Thus if a tumour-suppressor gene does map to this region, it should behave as a classical autosomal tumour-suppressor gene, requiring two hits by Knudson's hypothesis (Knudson, 1971). There may also be a third site of targeted loss situated on distal Xq which requires confirmation and may even coincide with the second proposed pseudoautosomal region (Freije *et al.*, 1992). If so, then this target would also be expected to behave as a classical tumour-suppressor gene. In contrast, the SRO mapped by Yang-Feng *et al.*, (1992) in ovarian tumours between DXS84 and DXS7 at Xp21.1-p11.3 is clearly situated in the sex-specific sequences on the X chromosome. If the target of this deletion is a tumour-suppressor gene, it should be quite unique in that it can escape X-inactivation while having an active homologue on the Y chromosome. Whether the rarer small deletions in the sex-specific sequences on Xp identified in breast tumours from this project will correspond to this SRO remains to be seen.

The complete absence of GCN events on the X chromosome in the panel suggests that this chromosome is subject to very specialized deletions. GAL events were

recorded in two tumours and, as for chromosome 1, indicate that the predominant alterations to the X chromosome in these tumours must be simple deletions or unbalanced translocations. The high frequency of small LOH events, that would be submicroscopic if these tumours could be karyotyped, could explain why no role for the X chromosome was implied by karyotype analysis of other breast tumours. Since these events occur in breast tumours, it is possible that they may also occur in other tumours which share similar patterns of rearranged chromosomes to breast tumours.

The lack of karyotypes for tumours in the panel of patients was originally regarded as a handicap to the analysis of the patterns of AI observed for chromosome 1. However, it appears that this can be overcome by the use of fluorescence *in situ* hybridization with satellite probes to tumour sections. The success with the application of a single probe to the sections, certainly warrants further efforts to obtain an internal control with a satellite locus mapping to chromosome 2. Thus, the status of chromosome 1 in tumours such as RW 904 in particular would become apparent if data on chromosome 2 could also be generated.

Although the vast majority of the consensus alterations to chromosomes 1, X, 13 and 17 showed no association with other variables, a few results of interest were obtained. Unfortunately, as the numbers for each dataset are critical in obtaining a result, many borderline associations must be formally discarded until further studies can generate additional examples to enable re-testing. Choice of the statistical tests used is clearly important and knowledge of their applications and corrections essential to produce accurate results.

Appendix I

Complete results for the loci D2S44, D7S21, RB1, D17S5 and D20S26 for all of the patients from the panel. Results are designated

u, uninformative

i, informative and unaffected by any imbalance

LOH, informative and showing loss of heterozygosity

GAL, informative and undergoing a concomitant gain and loss of alleles

GCN, informative and showing a gain in copy number of one allele

AI, informative and undergoing allelic imbalance, the nature of which has not been determined

a blank space indicates that the locus has not been successfully tested to date

D2S44 was hybridized to all sets of filters while D7S21 was hybridized to two half sets of *Mbo*I filters and one set of *Taq*I filters and D20S26 applied to one set of each of *Mbo*I, *Taq*I and *Hinf*I filters. When a patient was informative for a locus on any one of the filter sets, but uninformative on others, she is designated informative in the table.

Patient	Chromosome 2 D2S44 (pYNH24)	Chromosome 7 D7S21 (pMS31)	Chromosome 13 RB1 (VNTR)	Chromosome 17 D17S5 (pYNZ22.1)	Chromosome 20 D20S26 (pMS617)
PB 5	i	i	LOH	i	u
PB 37	u	i			i
PB 40	i	u	GAL	u	i
PB 56	i	i	u	i	i
PB 64	LOH	u	i		i
PB 78	i	LOH	GCN	u	i
PB 80	LOH	u	LOH		i
PB 96	i	i	LOH		LOH
PB 103	i	LOH	LOH		u
PB 106	i	LOH	i	u	i
PB 113	i	u	u	i	
PB 121	i	u	LOH	LOH	GCN
PB 124	i	i	u	i	GCN
PB 126	i		i	LOH	LOH
PB 128	AI			AI	
PB 133	i				
PB 134	i	LOH	LOH	LOH	i
PB 137	GCN	GCN	u	u	i
PB140	AI	AI		AI	AI
PB141	i	i	i		i
FW	i	i	u		u
FY	i	i	LOH	LOH	i

Patient	Chromosome 2		Chromosome 7		Chromosome 13		Chromosome 17		Chromosome 20	
	D2S44 (pYNH24)	D7S21 (pMS31)	RB1 (VNTR)	D17S5 (pYNZ22.1)	D20S26 (pMS617)					
JG	i	i	u				u		i	
MI	i	u	i				u		i	
RW 628	i	GCN	LOH				u		LOH	
RW 630	i	i					u		i	
RW 633	LOH	i							i	
RW 647	i	i	u				GAL		u	
RW 659	i	i	u				u		i	
RW 660	i	i	u				i		i	
RW 661	i	i	u				LOH		i	
RW 663	i	LOH	u				i		LOH	
RW 682	GAL	LOH	u				i		i	
RW 686	i	i	i				i		GCN	
RW 690	i	i	i						i	
RW 860	i						LOH		u	
RW 862	i		u				u		u	
RW 864	i		LOH						u	
RW 866	i	i	u				i		i	
RW 867	i	i	u						i	
RW 869	i		u						i	
RW 870	i	i	i				i		i	
RW 872	i		LOH						i	
RW 873	i	i	i				u		i	


Patient	Chromosome 2 D2S44 (pYNH24)	Chromosome 7 D7S21 (pMS31)	Chromosome 13 RB1 (VNTR)	Chromosome 17 D17S5 (pYNZ22.1)	Chromosome 20 D20S26 (pMS617)
RW 874	i	i	LOH	LOH	LOH
RW 875	i	i	LOH	u	i
RW 878	i		u		i
RW 880	u	i	GCN	i	i
RW 883	i		u		i
RW 885	i	u	u	i	GCN
RW 886	i				
RW 888	i				u
RW 894	i	i	i	i	u
RW 895	i		u		i
RW 896	i	u		u	i
RW 898	i	i	u		LOH
RW 900	i		u		
RW 904	AI	AI		AI	AI
RW 905					
RW 906					
RW 907	u		u		
RW 912			u		
RW 913			u		
RW 920	i		u	u	u
RW 921	i			i	i
RW 926	i		i		i

Patient	Chromosome 2 D2S44 (pYNH24)	Chromosome 7 D7S21 (pMS31)	Chromosome 13 RB1 (VNTR)	Chromosome 17 D17S5 (pYNZ22.1)	Chromosome 20 D20S26 (pMS617)
RW 927	i		u		i
RW 928	i		u	u	LOH
RW 935	LOH		u		i
RW 938	i		u		i
RW 942	i		u		i


Appendix II

Cartoons of the X chromosome showing the results for all patients from the panel. Each of the loci studied are indicated along the length of the chromosome at regular intervals, so that the chromosome is not to scale. The order of the loci along the chromosome is given as: pter - DXYS14 - DXYS78 - DXYS28 - DXYS15 - DXYS17 - DXYS89 - DXS278 - DXS9 - DXS84 - DXS7 - DXS255- cen - DXS159 - DXS101 - DXS51 - DXS438 - DXS52 - qter. However, the orientation of DXYS17 and DXYS89 is currently unknown, but is designated as above because this is most parsimonious to the data, generating the fewest number of independent regions of deletion. Similarly, DXS438 and DXS52 are also unordered, but as these are the two most terminal Xq loci studied, all deletions specific to these loci are contained by DXS51. Those tumours affected by the orientation of either pair of loci are discussed in chapter 4.

The imbalances observed are usually drawn to their widest limits on the chromosome as the boundaries are determined by the nearest informative locus in either direction unaffected by the AI event. There are exceptions to this; firstly, when the most distal informative locus studied on either arm undergoes AI, the telomere defines the most distal limit of the event. Also, when the nearest unaffected informative locus is on the other arm of the chromosome to the AI event, the centromere forms the most proximal limit of the event, since the centromere is assumed for simplicity to be intact and functional to maintain those sequences that are unaffected; it is of course entirely possible that the centromere has been lost in such cases of LOH and the retained portions of the chromosome translocated onto another chromosome where they are maintained within the cell by another centromere. Finally, in each of the two cases, PB 37 and RW 900, where a single informative locus has been successfully studied and found to undergo LOH, there is not enough evidence for the involvement of the entire chromosome arm and so that limits of the events are immediately around the locus in question for each tumour. These two cases are not used in the analysis in chapter 4.

 -, not tested

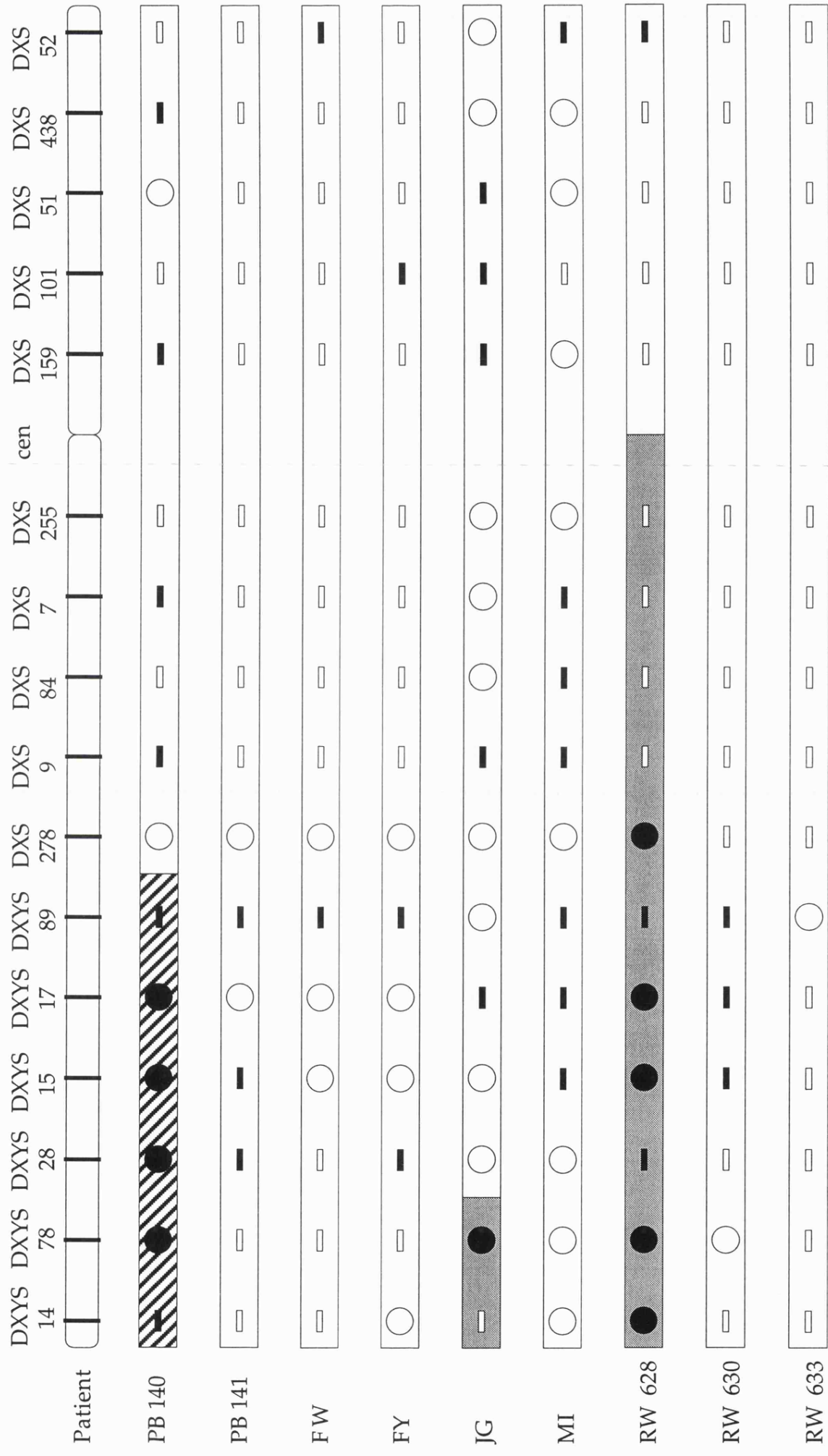
 u, uninformative

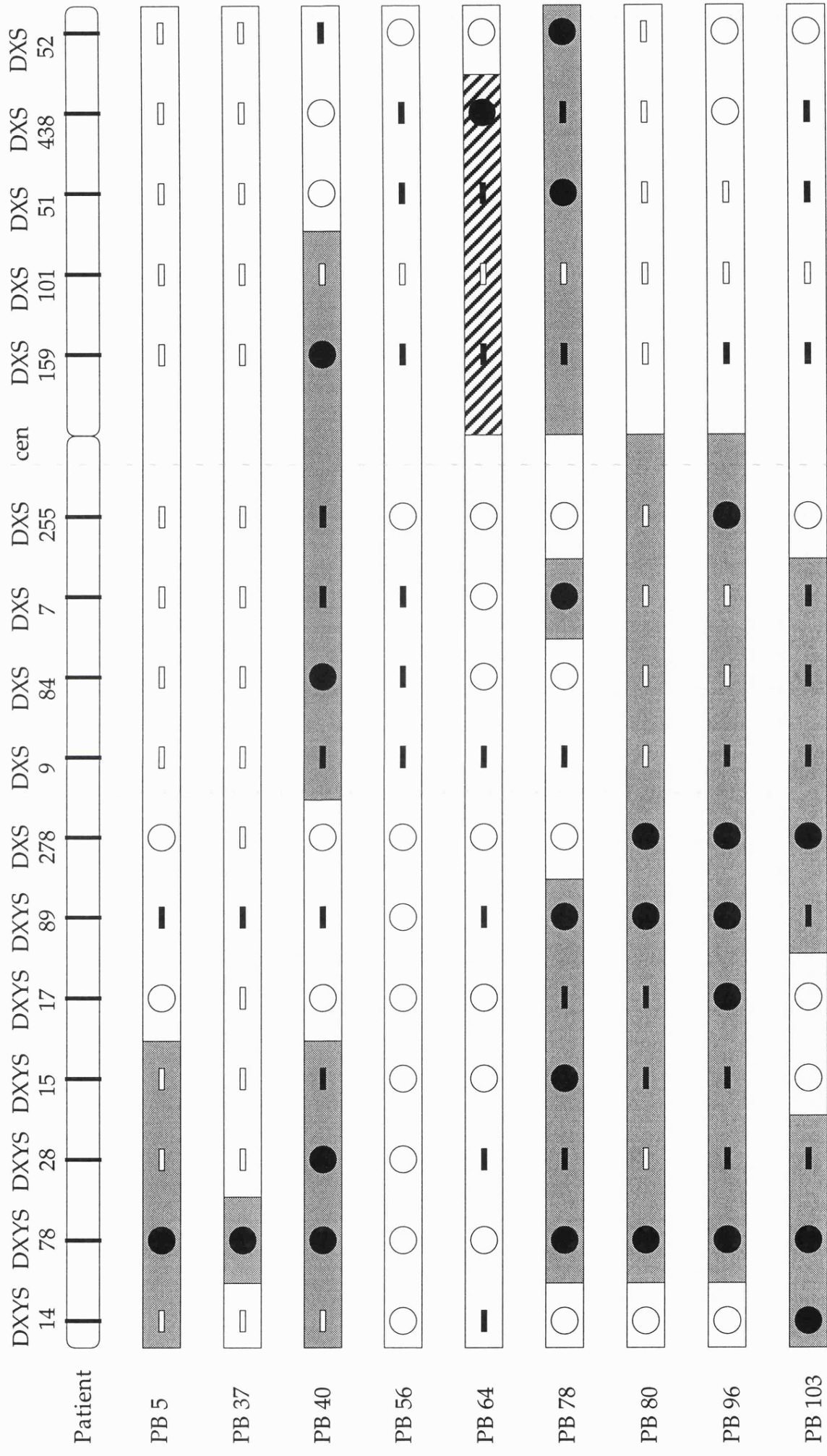
 i, informative and unaffected by imbalance

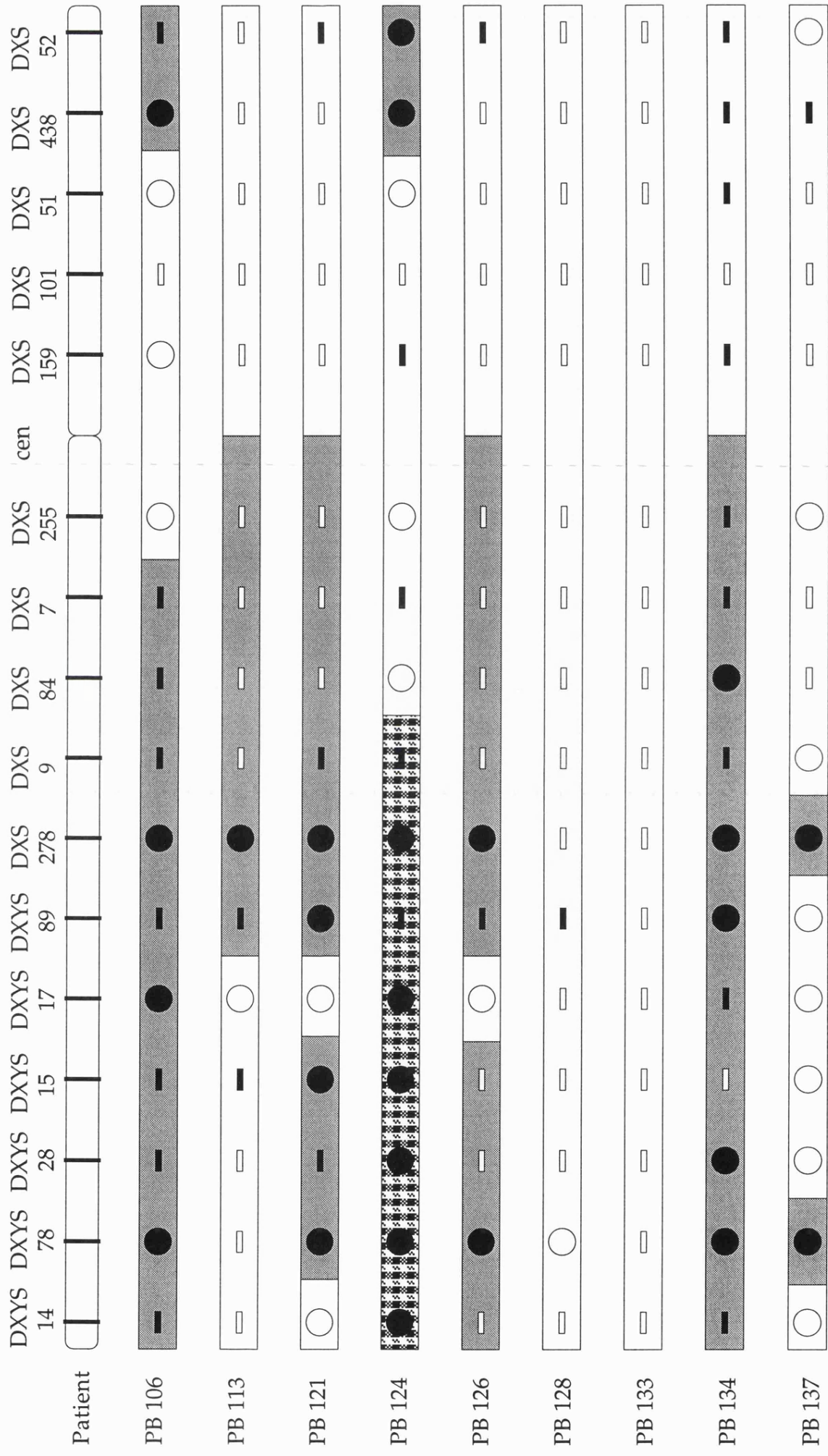
 AI, allelic imbalance, indeterminate as LOH, GAL or GCN

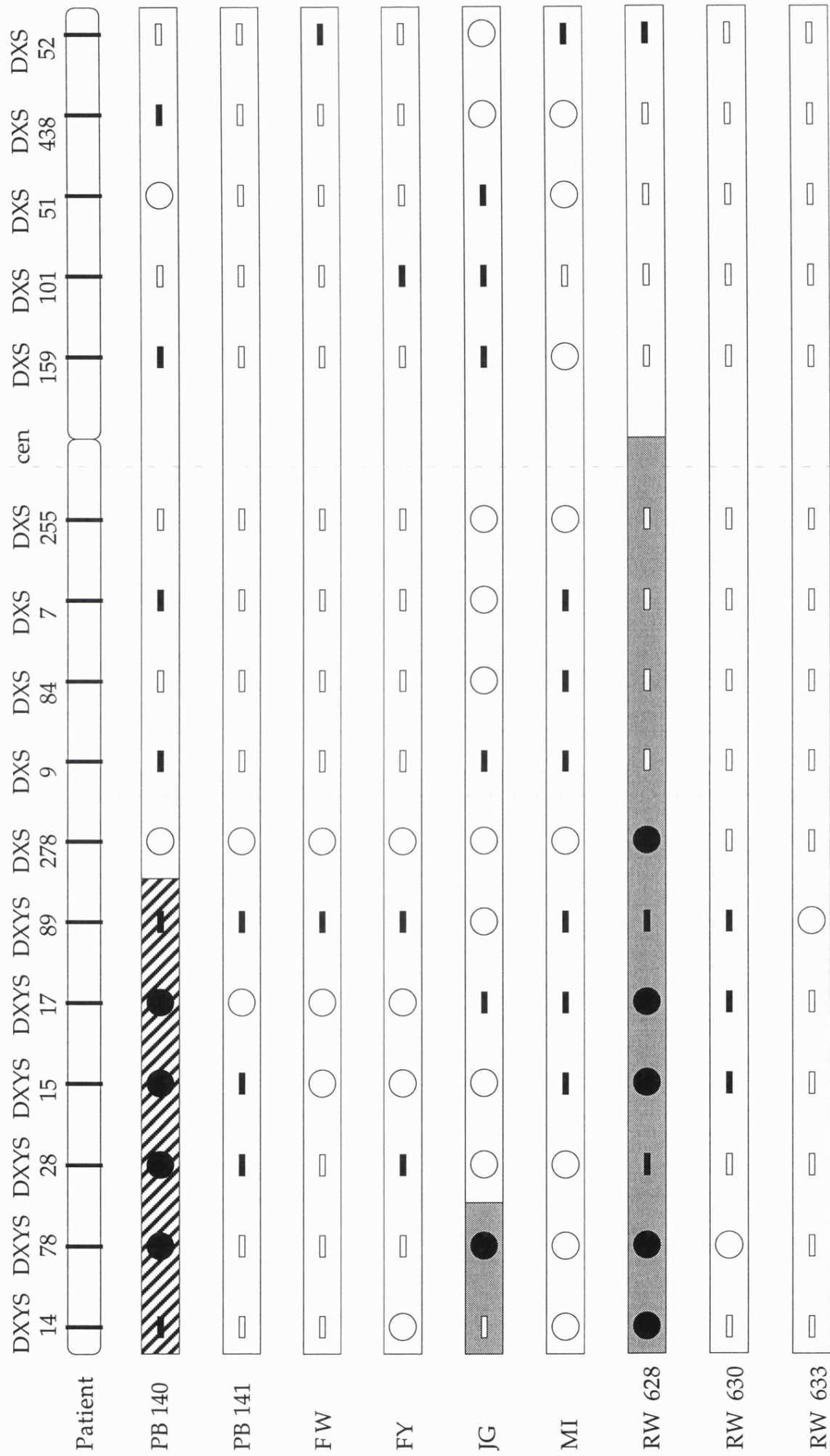
 LOH, loss of heterozygosity

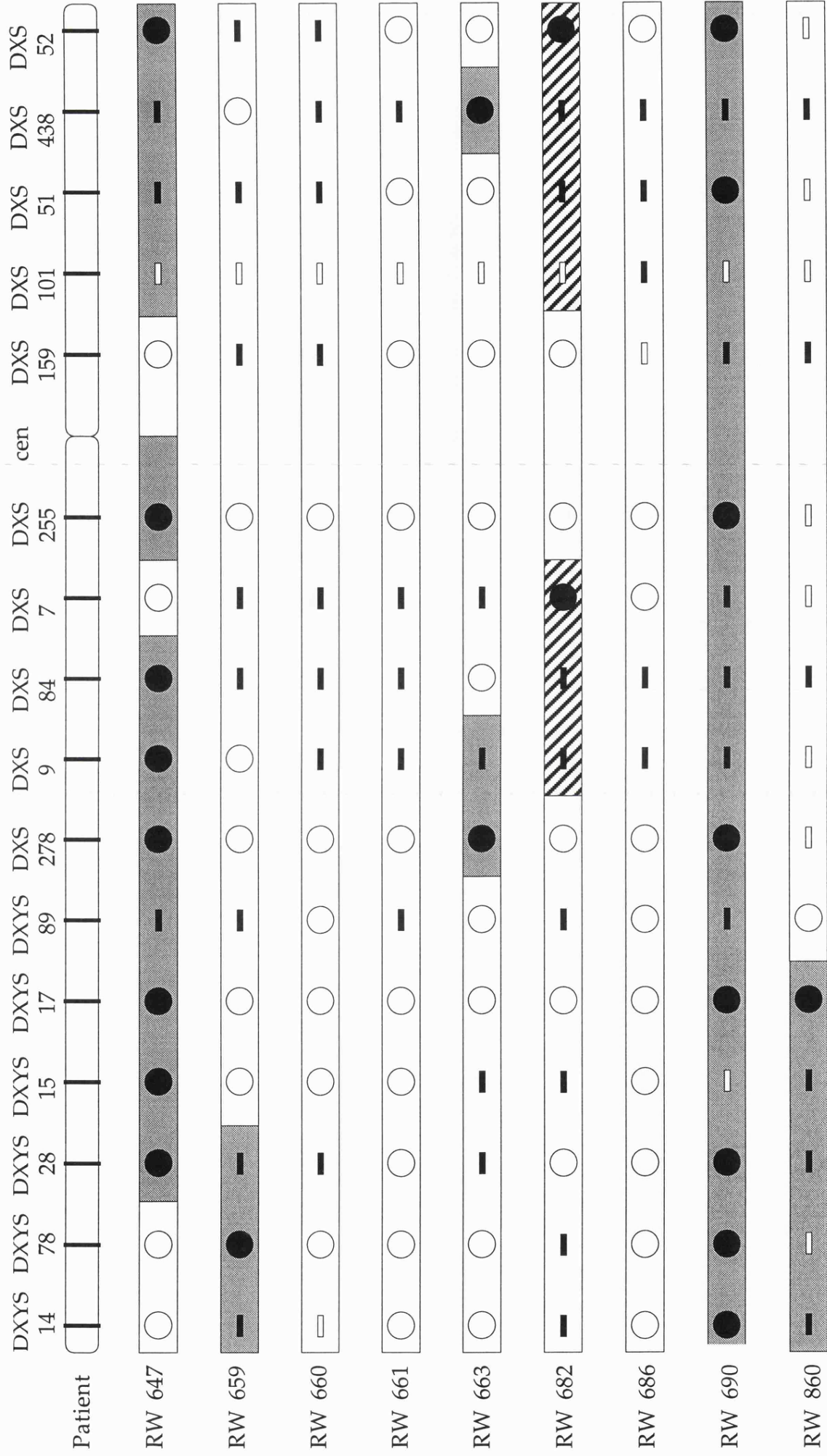
 GAL, concomitant gain and loss of alleles

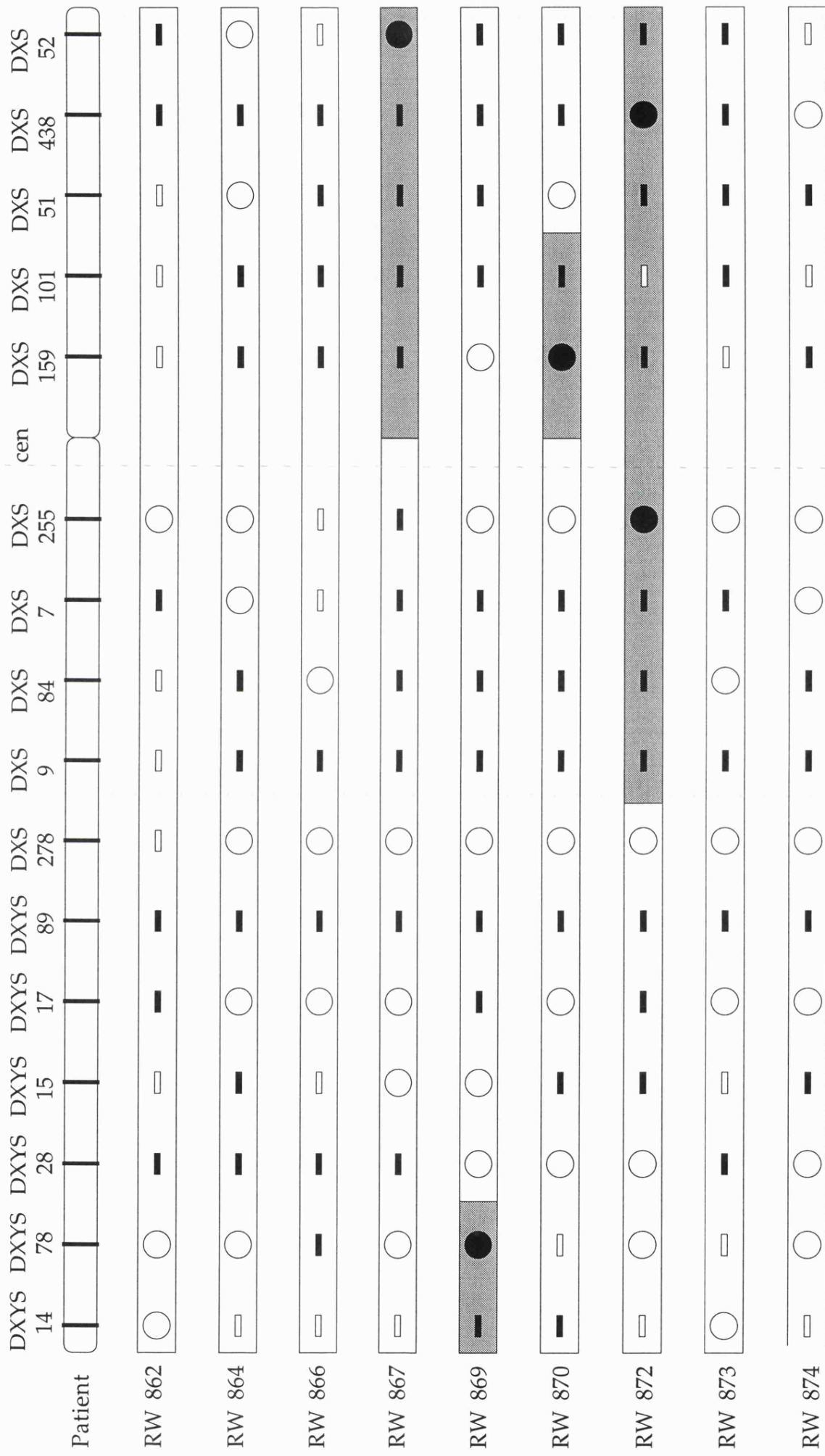


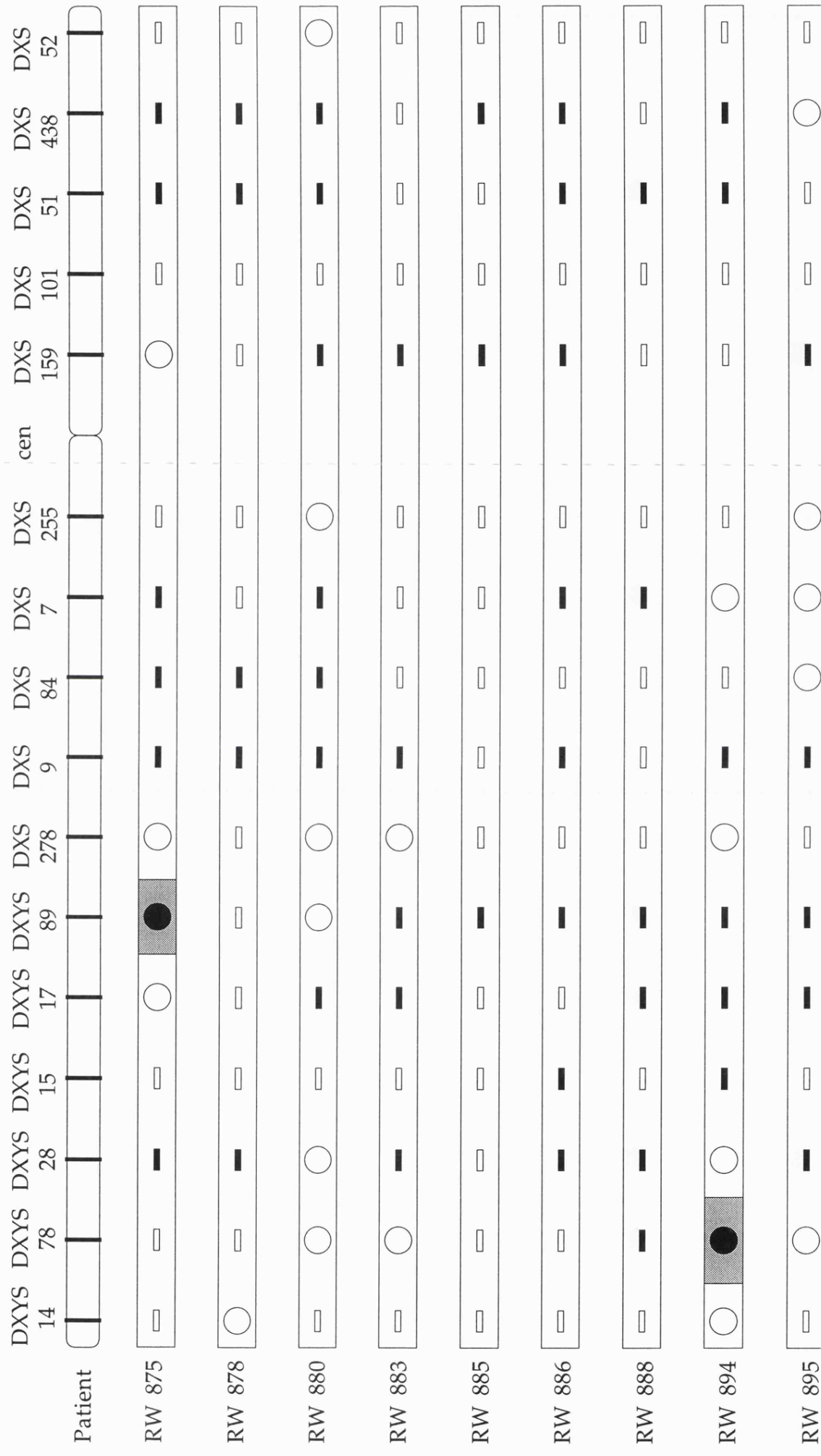


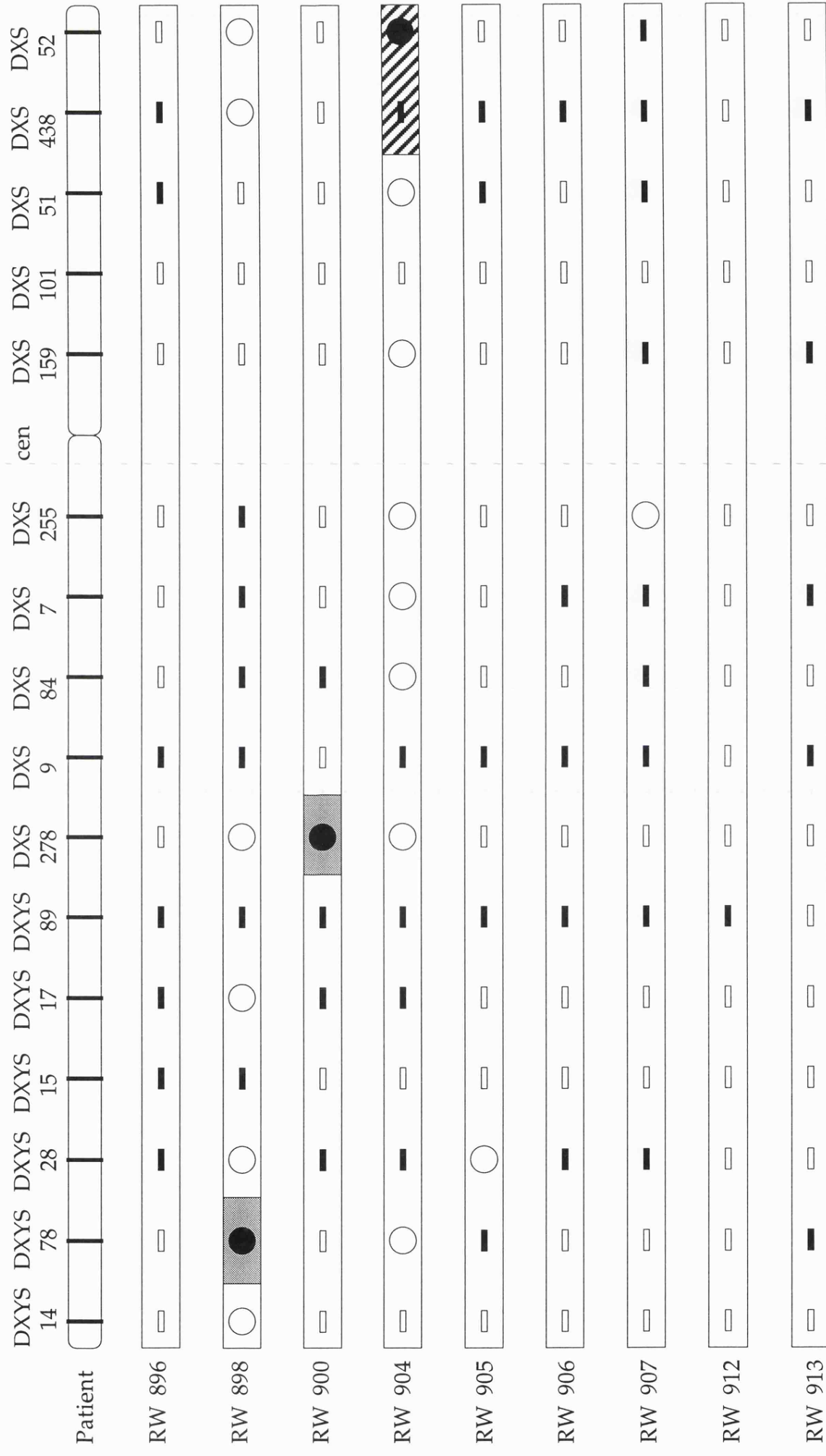


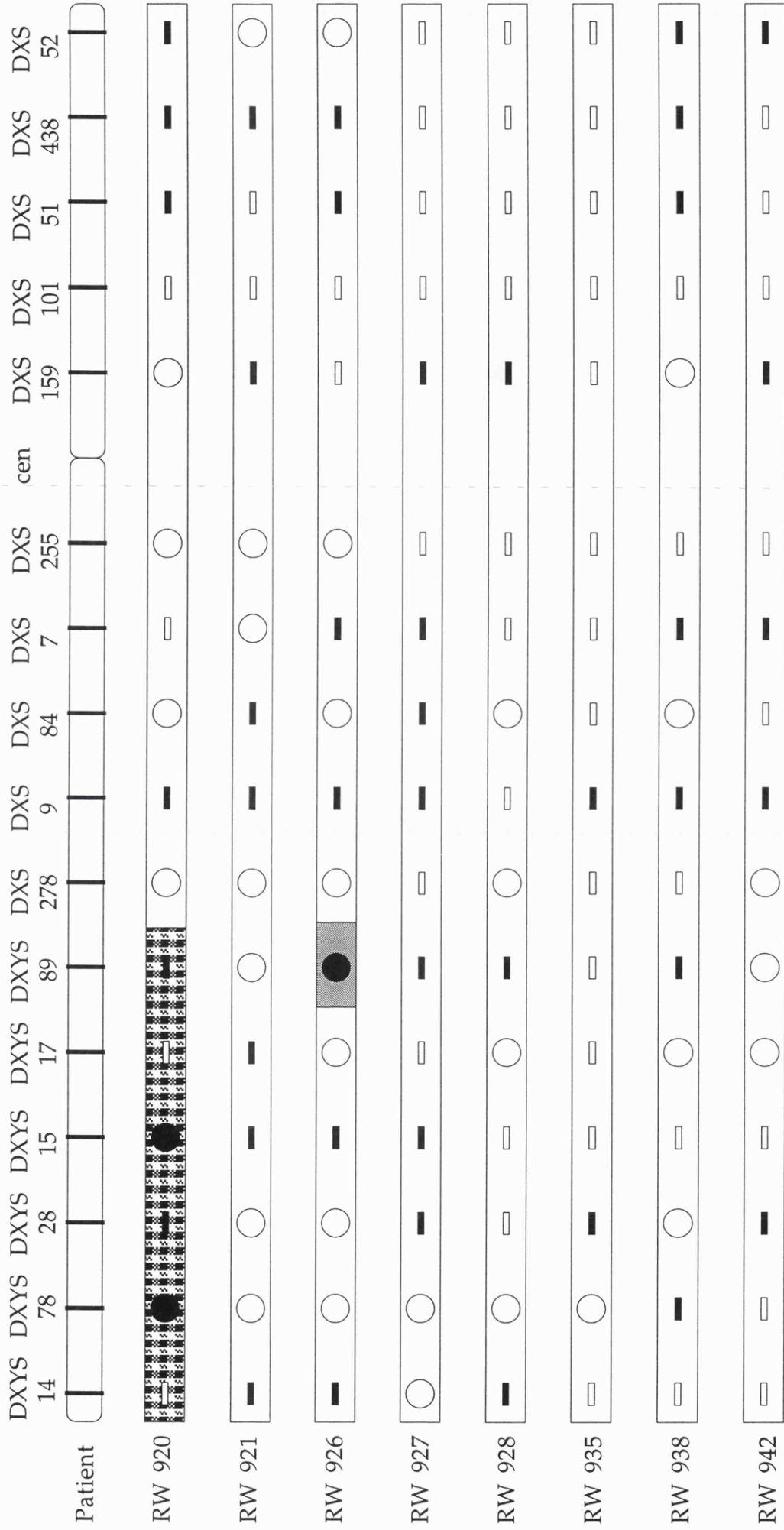













Appendix III

Cartoons of chromosome 1 showing the results for all of the patients from the panel. Each of the loci studied are indicated along the length of the chromosome at regular intervals, so that the chromosome is not to scale. The order of the loci along the chromosome is: pter - D1S80 - D1S77 - D1S76 - D1S7 - D1S57 - D1S73 - cen - MUC1 - SPTA1 - D1S61 - D1S65 - REN - D1S58 - D1S81 - D1S74 - D1S8 - qter, as indicated.

The imbalances observed are usually drawn to their widest limits on the chromosome as the boundaries are determined by the nearest informative locus in either direction unaffected by the AI event. There are exceptions to this; firstly, when the most distal informative locus studied on either arm undergoes AI, the telomere defines the most distal limit of the event. Also, when the nearest unaffected informative locus is on the other arm of the chromosome to the AI event, the centromere forms the most proximal limit of the event, since the centromere is assumed, for simplicity, to be intact and functional to maintain those sequences that are unaffected; it is of course entirely possible that the centromere has been lost in such cases of LOH and the retained portions of the chromosome translocated onto another chromosome where they are maintained within the cell by another centromere.

* patients in which somatic mutations are suspected to have occurred at single loci (see text for details): D1S8 in PB 40, MUC1 in PB 64, D1S8 in RW 898 and D1S7 in RW 921. These mutations take the form of an additional band in the tumour DNA track (see chapter 5)

 -, not tested

 u, uninformative

 i, informative and unaffected by imbalance

 AI, allelic imbalance, indeterminate as LOH, GAL or GCN

 LOH, loss of heterozygosity

 GAL, concomitant gain and loss of alleles

 GCN, gain in copy number of alleles

D1S80 D1S77 D1S76 D1S7 D1S57 D1S73 cen MUC1 SPTA1 D1S61 D1S65 REN D1S58 D1S81 D1S74 D1S8

Patient



PB 5



PB 37



PB 40*



PB 56



PB 64*



PB 78



PB 80



PB 96



PB 103



D1S80 D1S77 D1S76 D1S7 D1S57 D1S73 cen MUC1 SPTA1 D1S61 D1S65 REN D1S58 D1S81 D1S74 D1S8

Patient



PB 106



PB 113



PB 121



PB 124



PB 126



PB 128



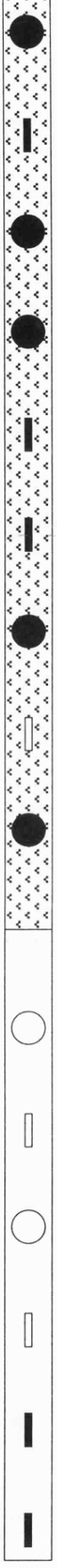
PB 133



PB 134



PB 137



D1S80 D1S77 D1S76 D1S7 D1S57 D1S73 cen MUC1 SPTA1 D1S61 D1S65 REN D1S58 D1S81 D1S74 D1S8

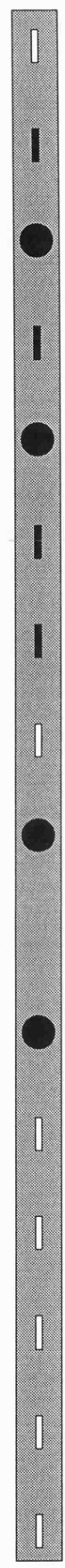
Patient



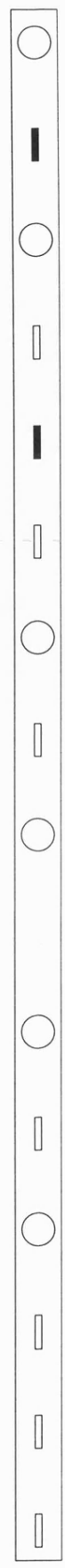
PB 140



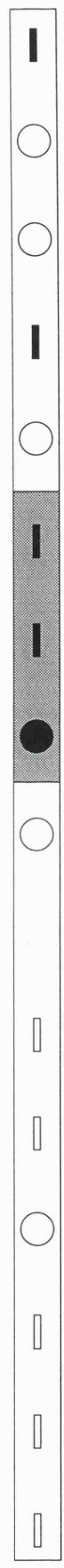
PB 141



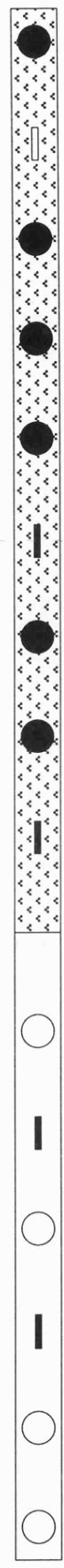
FW



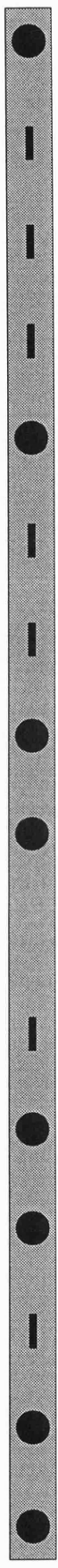
FY



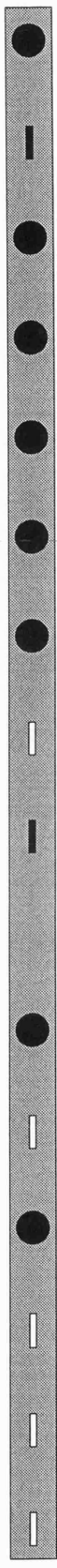
JG



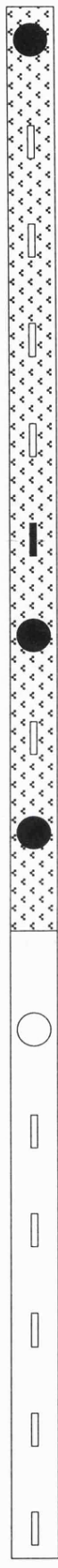
MI



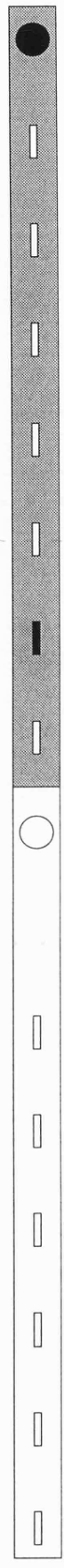
RW 628



RW 630



RW 633



D1S80 D1S77 D1S76 D1S7 D1S57 D1S73 cen MUC1 SPTA1 D1S61 D1S65 REN D1S58 D1S81 D1S74 D1S8



D1S80 D1S77 D1S76 D1S7 D1S57 D1S73 cen MUC1 SPTA1 D1S61 D1S65 REN D1S58 D1S81 D1S74 D1S8

Patient



RW 862



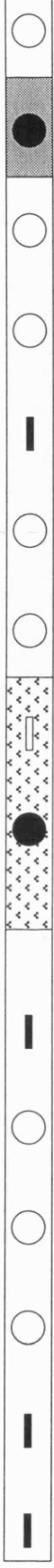
RW 864



RW 866



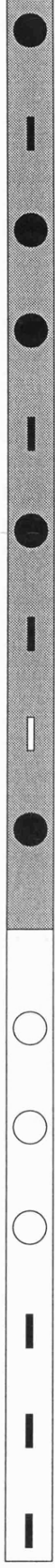
RW 867



RW 869



RW 870



RW 872

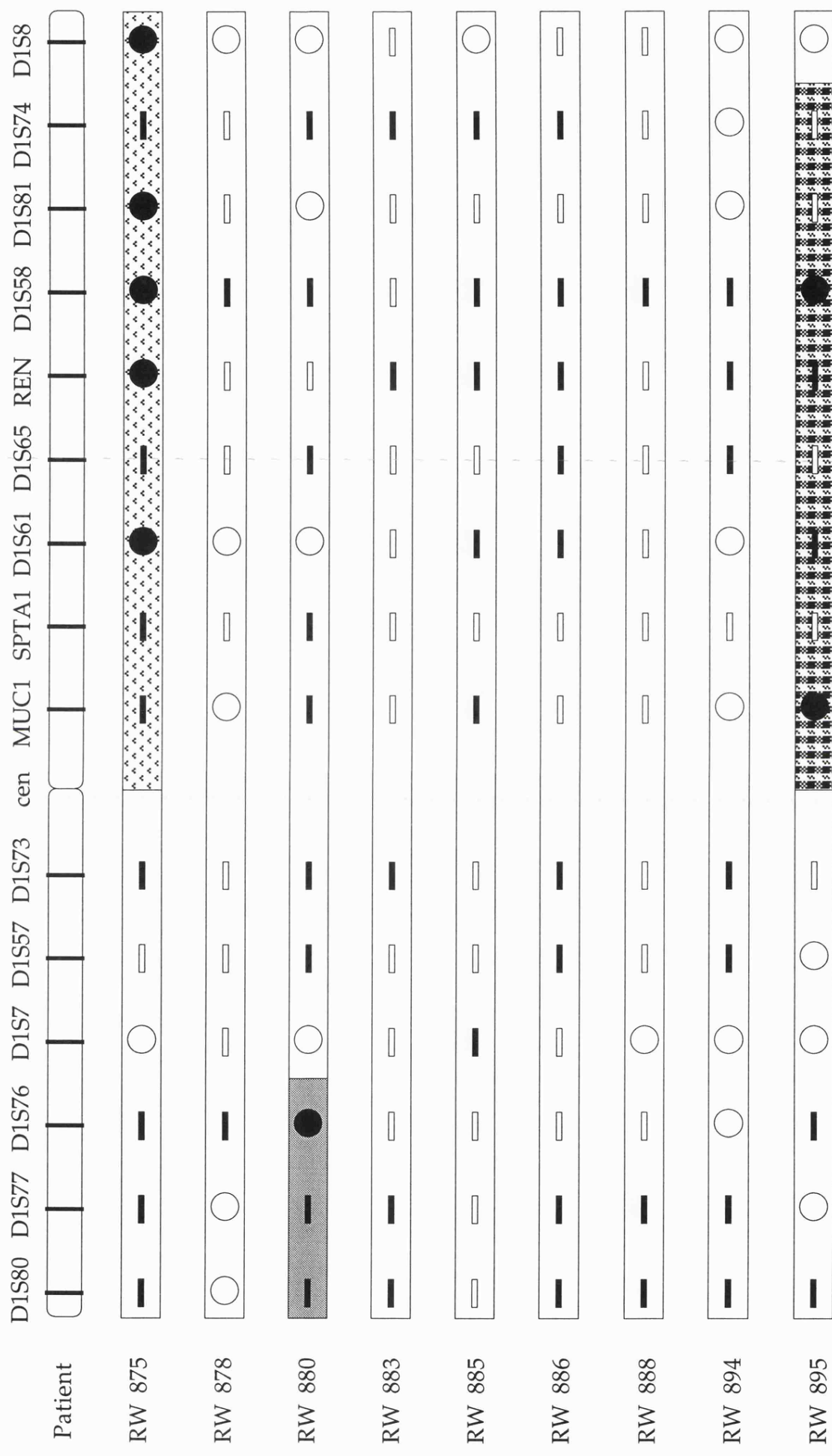


RW 873



RW 874





D1S80 D1S77 D1S76 D1S7 D1S57 D1S73 cen MUC1 SPTA1 D1S61 D1S65 REN D1S58 D1S81 D1S74 D1S8

Patient



RW 896



RW 898*



RW 900



RW 904



RW 905



RW 906



RW 907



RW 912



RW 913



D1S80 D1S77 D1S76 D1S7 D1S57 D1S73 cen MUC1 SPTA1 D1S61 D1S65 REN D1S58 D1S81 D1S74 D1S8

Patient



RW 920



RW 921*



RW 926



RW 927



RW 928



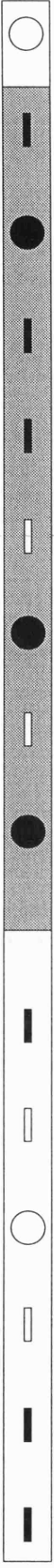
RW 935



RW 938



RW 942



Appendix IV

Examples of the raw data generated by the scanning of loci showing allelic imbalance and appropriate controls. See chapter 3 for a discussion of the results.

Each table cell shows the area under the peak first followed by the percentage of the total intensity of all the peaks in that lane in parentheses. The ratio of alleles in each lane is derived from dividing the value for the lower allele, q, by that of the upper allele, p. The ratio of alleles between lanes is obtained by dividing the value for an allele intensity in the tumour (T) lane by that in the lymphocyte (blood, B) lane. For scans of DXS278, bands are grouped as stated to create two alleles (actually haplotypes), p and q.

When the ratios of alleles for the B and T lanes of the test locus were compared, a difference between them meant that an imbalance had been detected by densitometry. To establish the amounts of DNA in each lane under analysis, the interlane ratio for the alleles of the corresponding control locus were used. When the interlane ratio of an allele of the test locus were close to the control ratio, that allele had not undergone imbalance, since it showed a similar ratio of the amounts of DNA between the lanes as the control. Alleles of the test locus with an interlane ratio lower than for the control had been reduced in copy number in the T lane (LOH); when the ratio was higher than for the control, an increase in copy number had occurred in the T lane (GCN). When one allele ratio for the test locus was lower than the control ratio and the other allele ratio was higher, GAL was taking place. In those cases where the interlane ratios of the alleles of the control locus were different, but no such imbalance was detected by eye, these two ratios were taken to be the rough upper and lower limits for the amounts of DNA between the lanes of the autoradiograph. Thus, interlane ratios for alleles from the test locus within these limits were judged not to be experiencing imbalance while ratios less than the lower limit indicated LOH and ratios higher than the upper limit indicated GCN; similarly, GAL was confirmed by one ratio being higher than the upper limit and the other being less than the lower limit.

e.g. D1S61 in patient PB 5 on the table immediately following:

D1S61 in patient PB 5

D1S61: ratio of alleles q/p in B lane = 0.83; ratio of q/p in T lane = 2.55

=> imbalance

D2S44 control: interlane ratio of p alleles = 1.06; ratio of q alleles = 1.43

=> the limits of the DNA loading between the lanes

D1S61: interlane ratio of p alleles = 1.19; ratio of q alleles = 3.66

=> p allele of T lane within the control limits; q allele of T lane undergoing gain, i.e. GCN at D1S61 in PB 5.

Scoring by eye determined that GAL was occurring in the T lane of PB 5 at D1S61, and indeed at all informative loci on chromosome 1 in this patient. However both of the remaining test loci scanned in this patient did confirm GAL (D1S58 and D1S32).

	Allele	PB 5		Ratio of alleles between lanes
		B	T	
D1S61 (pMLAJ1)	p	6.04 (55%)	7.17 (28%)	1.19
	q	5.00 (45%)	18.30 (72%)	3.66
Ratio of alleles in each lane		0.83	2.55	
D2S44 (pYNH24)	p	16.37 (79%)	17.36 (74%)	1.06
	q	4.36 (21%)	6.24 (26%)	1.43
Ratio of alleles in each lane		0.27	0.36	
D1S58 (pYNZ23)	p	22.28 (48%)	5.90 (24%)	0.26
	q	24.02 (52%)	18.69 (76%)	0.78
Ratio of alleles in each lane		1.08	3.17	
D2S44 (pYNH24)	p	137.9 (55%)	71.37 (54%)	0.52
	q	113.8 (45%)	60.85 (46%)	0.53
Ratio of alleles in each lane		0.83	0.85	
D1S8 (pMS32)	p	47.71 (73%)	95.09 (90%)	1.99
	q	17.48 (27%)	10.98 (10%)	0.63
Ratio of alleles in each lane		0.37	0.12	
D2S44 (pYNH24)	p	16.37 (79%)	17.36 (74%)	1.06
	q	4.36 (21%)	6.24 (26%)	1.43
Ratio of alleles in each lane		0.27	0.36	

	Allele	PB 134		Ratio of alleles between lanes
		B	T	
D1S80 (pMCT118)	p	6.69 (67%)	22.69 (>99%)	3.39
	q	3.29 (33%)	0.13 (<1%)	0.04
Ratio of alleles in each lane		0.49	0.006	
D2S44 (pYNH24)	p	58.61 (44%)	42.40 (36%)	0.72
	q	75.81 (56%)	77.10 (64%)	1.02
Ratio of alleles in each lane		1.29	1.82	
D1S7 (pMS1)	p	116.3 (47%)	41.38 (60%)	0.36
	q	132.1 (53%)	27.53 (40%)	0.21
Ratio of alleles in each lane		1.14	0.67	
D2S44 (pYNH24)	p	98.12 (67%)	33.24 (58%)	0.34
	q	47.42 (33%)	23.88 (42%)	0.50
Ratio of alleles in each lane		0.48	0.72	
D1S57 (pYNZ2)	p	10.93 (60%)	3.26 (26%)	0.30
	q	7.24 (40%)	9.28 (74%)	1.28
Ratio of alleles in each lane		0.66	2.85	
D2S44 (pYNH24)	p	8.38 (28%)	22.88 (32%)	2.73
	q	21.24 (72%)	48.70 (68%)	2.29
Ratio of alleles in each lane		2.53	2.13	
MUC1 (pMUC10)	p	103.8 (92%)	118.1 (63%)	1.14
	q	78.51 (8%)	69.21 (37%)	0.88
Ratio of alleles in each lane		0.76	0.59	
D2S44 (pYNH24)	p	48.32 (62%)	24.94 (60%)	0.52
	q	29.12 (38%)	16.67 (40%)	0.57
Ratio of alleles in each lane		0.60	0.67	
D1S81 (pTHH33)	p	38.36 (53%)	23.15 (36%)	0.60
	q	33.85 (47%)	40.72 (64%)	1.20
Ratio of alleles in each lane		0.88	1.76	
D2S44 (pYNH24)	p	98.12 (67%)	33.24 (58%)	0.34
	q	47.42 (33%)	23.88 (42%)	0.50
Ratio of alleles in each lane		0.48	0.72	
D1S8 (pMS32)	p	39.84 (61%)	21.09 (32%)	0.53
	q	25.70 (39%)	45.05 (68%)	1.75
Ratio of alleles in each lane		0.65	2.14	
D2S44 (pYNH24)	p	48.32 (62%)	24.94 (60%)	0.52
	q	29.12 (38%)	16.67 (40%)	0.57
Ratio of alleles in each lane		0.60	0.67	

	Allele	RW 690		Ratio of alleles between lanes
		B	T	
D1S7 (pMS1)	p	136.6 (61%)	181.0 (69%)	1.33
	q	88.45 (39%)	79.51 (31%)	0.90
Ratio of alleles in each lane		0.65	0.44	
D2S44 (pYNH24)	p	61.25 (36%)	90.68 (37%)	1.48
	q	110.6 (64%)	157.2 (63%)	1.42
Ratio of alleles in each lane		1.81	1.73	

	Allele	RW 864		Ratio of alleles between lanes
		B	T	
D1S57 (pYNZ2)	p	17.87 (48%)	23.59 (73%)	1.32
	q	19.69 (52%)	8.74 (27%)	0.44
Ratio of alleles in each lane		1.10	0.37	
D2S44 (pYNH24)	p	26.73 (67%)	29.27 (68%)	1.09
	q	13.14 (33%)	13.50 (32%)	1.03
Ratio of alleles in each lane		0.49	0.46	
REN (pHRnES1.9)	p	14.47 (68%)	20.49 (66%)	1.42
	q	6.75 (32%)	10.60 (34%)	1.57
Ratio of alleles in each lane		0.47	0.52	

	Allele	RW 867		Ratio of alleles between lanes
		B	T	
MUC1 (pMUC10)	p	17.22 (73%)	34.23 (80%)	1.99
	q	6.23 (37%)	8.43 (20%)	1.35
Ratio of alleles in each lane		0.36	0.25	
D2S44 (pYNH24)	p	32.13 (60%)	35.63 (62%)	1.11
	q	21.06 (40%)	21.64 (38%)	1.03
Ratio of alleles in each lane		0.66	0.61	
D1S74 (cYNA13)	p	2.16 (57%)	50.40 (>99%)	23.34
	q	1.61 (43%)	0.14 (<1%)	0.09
Ratio of alleles in each lane		0.75	0.003	
D2S44 (pYNH24)	p	49.83 (50%)	93.47 (47%)	1.88
	q	50.73 (50%)	103.7 (53%)	2.04
Ratio of alleles in each lane		1.02	1.11	

	Allele	RW 869		Ratio of alleles between lanes
		B	T	
REN (pHRnES1.9)	p	11.53 (56%)	13.81 (44%)	1.20
	q	9.17 (44%)	17.66 (56%)	1.93
Ratio of alleles in each lane		0.80	1.28	
D2S44 (pYNH24)	p	35.22 (56%)	45.75 (54%)	1.30
	q	27.36 (44%)	39.22 (46%)	1.43
Ratio of alleles in each lane		0.78	0.86	
D1S58 (pYNZ23)	p	7.34 (48%)	19.53 (66%)	2.66
	q	7.93 (52%)	10.03 (34%)	1.27
Ratio of alleles in each lane		1.08	0.51	
D2S44 (pYNH24)	p	35.22 (56%)	45.75 (54%)	1.30
	q	27.36 (44%)	39.22 (46%)	1.43
Ratio of alleles in each lane		0.78	0.86	

	Allele	RW 942		Ratio of alleles between lanes
		B	T	
MUC1 (pMUC10)	p	3.31 (58%)	10.77 (52%)	3.26
	q	2.41 (42%)	10.17 (48%)	4.22
Ratio of alleles in each lane		0.73	0.94	
D2S44 (pYNH24)	p	35.40 (61%)	39.65 (41%)	1.12
	q	13.76 (39%)	56.52 (59%)	4.11
Ratio of alleles in each lane		0.39	1.43	
D1S61 (pMLAJ1)	p	7.71 (60%)	12.41 (47%)	1.61
	q	5.08 (40%)	13.97 (53%)	2.75
Ratio of alleles in each lane		0.66	1.13	
D2S44 (pYNH24)	p	35.40 (61%)	39.65 (41%)	1.12
	q	13.76 (39%)	56.52 (59%)	4.11
Ratio of alleles in each lane		0.39	1.43	
D1S81 (pTHH33)	p	1.85 (46%)	8.67 (38%)	4.68
	q	2.16 (54%)	14.39 (62%)	6.66
Ratio of alleles in each lane		1.17	1.66	
D2S44 (pYNH24)	p	9.25 (54%)	62.51 (57%)	6.76
	q	7.95 (46%)	47.26 (43%)	5.95
Ratio of alleles in each lane		0.86	0.76	

	Allele	PB 121		Ratio of alleles between lanes
	(band)	B	T	
DXYS78 (pMS600)	p	100.5 (77%)	36.00 (62%)	0.36
	q	29.85 (23%)	22.11 (38%)	0.74
Ratio of alleles in each lane		0.30	0.61	
D2S44 (pYNH24)	p	14.36 (35%)	13.43 (37%)	0.94
	q	26.50 (65%)	22.57 (63%)	0.85
Ratio of alleles in each lane		1.85	1.68	
DXYS89 (pMS639)	p	13.47 (53%)	7.88 (40%)	0.59
	q	11.71 (47%)	11.58 (60%)	0.99
Ratio of alleles in each lane		0.87	1.47	
D2S44 (pYNH24)	p	14.36 (35%)	13.43 (37%)	0.94
	q	26.50 (65%)	22.57 (63%)	0.85
Ratio of alleles in each lane		1.85	1.68	
DXS278 (CRI-pS232) Group bands 1 and 2 as allele p; 3, 4, 5, 6 and 7 as allele q	1	29.98 (18%)	5.87 (5%)	0.20
	2	25.38 (16%)	9.26 (8%)	0.36
	3	53.96 (33%)	43.63 (37%)	0.81
	4	21.08 (13%)	20.24 (17%)	0.96
	5	7.14 (4%)	8.84 (8%)	1.24
	6	9.92 (6%)	13.61 (12%)	1.37
	7	16.14 (10%)	14.93 (13%)	0.93
Ratio of alleles in each lane q/p		1.96	6.69	
D2S44 (pYNH24)	p	67.33 (51%)	55.62 (48%)	0.83
	q	65.91 (49%)	59.45 (52%)	0.90
Ratio of alleles in each lane		0.98	0.94	

	Allele	PB 124		Ratio of alleles between lanes
		B	T	
DXYS14 (29C1)	p	8.43 (44%)	24.30 (81%)	2.88
	q	10.56 (56%)	5.87 (19%)	0.56
Ratio of alleles in each lane		1.25	0.24	
D2S44 (pYNH24)	p	15.58 (57%)	11.89 (55%)	0.76
	q	11.85 (43%)	9.55 (45%)	0.81
Ratio of alleles in each lane		0.76	0.80	
DXYS28 (pDP411a)	p	29.88 (67%)	116.3 (85%)	3.89
	q	14.58 (33%)	19.79 (15%)	1.36
Ratio of alleles in each lane		0.49	0.17	
D2S44 (pYNH24)	p	97.99 (49%)	116.1 (45%)	1.18
	q	102.5 (51%)	142.9 (55%)	1.39
Ratio of alleles in each lane		1.05	1.23	
DXYS17 (602)	p	15.77 (53%)	40.64 (81%)	2.58
	q	14.07 (47%)	9.41 (19%)	0.67
Ratio of alleles in each lane		0.89	0.23	
D2S44 (pYNH24)	p	96.15 (58%)	94.94 (60%)	0.99
	q	70.91 (42%)	63.57 (40%)	0.90
Ratio of alleles in each lane		0.74	0.67	
DXS84 (754)	p	9.88 (36%)	4.91 (34%)	0.50
	q	17.73 (64%)	9.51 (66%)	0.54
Ratio of alleles in each lane		1.80	1.94	
DXS438 (pMS613)	p	15.56 (41%)	13.79 (31%)	0.89
	q	22.17 (59%)	30.13 (69%)	1.36
Ratio of alleles in each lane		1.43	2.18	
D2S44 (pYNH24)	p	36.66 (50%)	35.31 (49%)	0.96
	q	37.20 (50%)	37.19 (51%)	1.00
Ratio of alleles in each lane		1.01	1.05	

	Allele (band)	RW 647		Ratio of alleles between lanes
		B	T	
DXS278 (CRI-pS232)	1	13.14 (6%)	26.05 (4%)	1.98
	2	17.75 (8%)	42.09 (6%)	2.37
	3	17.22 (7%)	58.87 (9%)	3.42
	4	16.11 (7%)	75.22 (11%)	4.67
Group bands 1, 2, 6, 8, 9 and 10 as allele p; 3, 4, 5, 7 and 11 as allele q	5	16.71 (7%)	73.32 (11%)	4.39
	6	11.36 (5%)	33.78 (5%)	2.97
	7	15.84 (7%)	67.03 (10%)	4.23
	8	37.35 (16%)	74.57 (11%)	2.0
	9	62.55 (26%)	136.6 (20%)	2.18
	10	20.03 (8%)	54.00 (8%)	2.70
	11	8.484 (4%)	41.28 (6%)	4.87
Ratio of alleles in each lane	q/p	0.46	0.86	
D2S44 (pYNH24)	p	42.80 (61%)	94.64 (59%)	2.21
	q	27.76 (39%)	66.14 (41%)	2.38
Ratio of alleles in each lane		0.65	0.70	
DXS84 (754)	p	33.48 (51%)	10.72 (40%)	0.32
	q	31.91 (49%)	15.78 (60%)	0.49
Ratio of alleles in each lane		0.95	1.47	
D2S44 (pYNH24) - 7.12.93	Single	20.02	11.53	0.58
DXS255 (M27β)	p	29.67 (47%)	37.05 (57%)	1.25
	q	33.24 (53%)	28.17 (43%)	0.85
Ratio of alleles in each lane		1.12	0.76	
D2S44 (pYNH24)	Single	52.13	69.09	1.33

	Allele	RW 659		Ratio of alleles between lanes
		B	T	
DXYS78 (pMS600)	p	51.48 (56%)	6.62 (30%)	0.13
	q	40.18 (44%)	15.60 (70%)	0.39
Ratio of alleles in each lane		0.78	2.36	
D2S44 (pYNH24)	Single	70.22	37.18	0.53

	Allele	RW 663		Ratio of alleles between lanes
		B	T	
DXS438 (pMS613)	p	24.80 (56%)	11.66 (37%)	0.47
	q	19.62 (44%)	20.16 (63%)	1.03
Ratio of alleles in each lane		0.79	1.73	
D2S44 (pYNH24)	Single	73.05	61.65	0.84

	Allele	RW 926		Ratio of alleles between lanes
		B	T	
DXYS89 (pMS639)	p	76.87 (54%)	56.81 (62%)	0.74
	q	65.56 (46%)	34.77 (38%)	0.53
Ratio of alleles in each lane		0.85	0.61	
D2S44 (pYNH24)	Single	13.61	12.96	0.95

	Allele	RW 661		Ratio of alleles between lanes
		B	T	
D17S5 (pYNZ22.1)	p	0.50 (48%)	0.82 (56%)	1.63
	q	0.56 (52%)	0.64 (44%)	1.16
Ratio of alleles in each lane		1.10	0.78	
D2S44 (pYNH24)	p	14.35 (28%)	15.77 (26%)	1.10
	q	37.65 (72%)	45.81 (74%)	1.22
Ratio of alleles in each lane		2.62	2.90	

	Allele	RW 663		Ratio of alleles between lanes
		B	T	
D7S21 (pMS31)	p	4.18 (61%)	3.85 (44%)	0.92
	q	2.72 (39%)	4.88 (56%)	1.80
Ratio of alleles in each lane		0.65	1.27	
D20S26 (pMS617)	p	37.27 (50%)	22.64 (34%)	0.61
	q	36.88 (50%)	43.83 (66%)	1.19
Ratio of alleles in each lane		0.99	1.94	
D2S44 (pYNH24)	p	37.80 (56%)	22.47 (50%)	0.59
	q	29.83 (44%)	22.40 (50%)	0.75
Ratio of alleles in each lane		0.79	1.00	

	Allele	RW 682		Ratio of alleles between lanes
		B	T	
D7S21 (pMS31)	p	12.49 (61%)	6.31 (47%)	0.51
	q	8.1 (39%)	7.121 (53%)	0.88
Ratio of alleles in each lane		0.65	1.13	
D20S26 (pMS617)	p	78.80 (51%)	52.82 (48%)	0.67
	q	76.12 (49%)	56.97 (52%)	0.75
Ratio of alleles in each lane		0.97	1.08	
D2S44 (pYNH24)	p	53.31 (51%)	66.43 (68%)	1.25
	q	51.01 (49%)	31.28 (32%)	0.61
Ratio of alleles in each lane		0.96	0.47	

	Allele	RW 686		Ratio of alleles between lanes
		B	T	
D20S26 (pMS617)	p	51.42 (61%)	31.16 (50%)	0.61
	q	32.28 (39%)	30.91 (50%)	0.96
Ratio of alleles in each lane		0.63	0.99	
D2S44 (pYNH24)	p	17.58 (57%)	13.94 (60%)	0.79
	q	13.32 (43%)	9.20 (40%)	0.69
Ratio of alleles in each lane		0.76	0.66	

Appendix V

Detailed clinical data for the 71 patients in the panel

Abbreviations for classification of tumours: ca., carcinoma; DCIS, ductal carcinoma *in situ*; ID ca., infiltrating ductal carcinoma; IL ca., infiltrating lobular carcinoma

EGFR, epidermal growth factor receptor, ER, oestrogen receptor, PR, progesterone receptor

neg., negative for staining, pos. positive for staining

Patient	Menopausal status (age)	Tumour classification	Grade	Node	ER	EGFR	PR
PB 5	post (67)	ID ca.	II	neg.	neg.		pos.
PB 37	post (71)	ID ca.	II	neg.	neg.		neg.
PB 40	post (74)	ID ca. + DCIS	II	neg.	pos.		neg.
PB 56	post (63)	Lobular ca.			neg.		pos.
PB 64	pre (43)	ID ca. + intraductal ca. (Comedo)		neg.	neg.		neg.
PB 78	post (73)	ID ca.	III		neg.		pos.
PB 80	post (60)	ID ca.	III	pos.	pos.		neg.
PB 96	post (63)	ID ca. + intraductal ca.	II	pos.	pos.		pos.
PB 103	post (55)	ID ca.+ lobular ca.	II	neg.	pos.		pos.
PB 106	post (64)	ID ca. + intraductal ca.	II	neg.	neg.		pos.
PB 113	post (72)	Squamous cell ca.	II		pos.		neg.
PB 121	post (65)	IL ca. + DCIS		neg.	pos.		pos.
PB 124	pre (48)	ID ca. + some mucin	II	neg.	pos.		pos.
PB 126	post (75)	ID ca. + some intraductal ca.	I-II	neg.	pos.		neg.
PB 128	post (59)	ID ca. + intraductal ca. (Comedo)	II	pos.	pos.		pos.
PB 133	post (81)	ID ca.		neg.	pos.		neg.
PB 134	post (74)	Anaplastic cells	III	neg.	pos.		pos.
PB 137	post (68)	ID ca.	I	neg.	pos.		pos.
PB 140	post (77)	ID ca.			pos.		pos.
PB 141	pre (41)	ID ca./IL ca.		neg.	pos.		pos.
FW	post?	Tubular ca. + Paget's disease	I				
FY	post	ID ca.	II	pos.			

Patient	Menopausal status (age)	Tumour classification	Grade	Node	ER	EGFR	PR
JG	pre	Intraductal ca. (Cribriform)	I				
MI	(60)	ID ca.	I	pos.			
RW 628	post	ID ca.	II	pos.	neg.		
RW 630	post	ID ca.	II	pos.	pos.		
RW 633	post	IL ca.	II	pos.			
RW 647							
RW 659							
RW 660	post	IL ca.	II	neg.	pos.		
RW 661							
RW 663							
RW 682	pre	ID ca.	III		neg.		
RW 686	pre	ID ca.	II	pos.	pos.		
RW 690							
RW 860	post	ID ca.	I	neg.	pos.	neg.	
RW 862	pre	ID ca.	III	neg.	neg.	neg.	
RW 864	post	ID ca.	III	pos.	pos.	neg.	
RW 866	pre	Tubular ca.	I	neg.	pos.	neg.	
RW 867	post	ID ca.	II	neg.	pos.	neg.	
RW 869	post	ID ca.	I	neg.	pos.	neg.	
RW 870	post	ID ca.	II	neg.	pos.	neg.	
RW 872	pre	ID ca.	III	neg.	neg.	pos.	
RW 873	post	ID ca.	I	pos.	pos.	neg.	

Patient	Menopausal status (age)	Tumour classification	Grade	Node	ER	EGFR	PR
RW 874	post	ID ca.	II	neg.	pos.	neg.	
RW 875	pre	ID ca.	II	neg.	pos.	neg.	
RW 878	post	Mucinous ca.	I				
RW 880	post	ID ca.	III	pos.	neg.	neg.	
RW 883	post	ID ca.	II	pos.	neg.	pos.	
RW 885	post	ID ca.		neg.	pos.	neg.	
RW 886	post	ID ca.	I	neg.	pos.	neg.	
RW 888	post	IL ca.	II	neg.	pos.	neg.	
RW 894	post	IL ca.	II	neg.	pos.	neg.	
RW 895	pre	ID ca.	II	pos.		neg.	
RW 896	post	ID ca.	II	neg.	pos.	neg.	
RW 898	post	ID ca.	III		neg.	pos.	
RW 900	post	IL ca.	II	neg.	pos.	neg.	
RW 904	post	ID ca.	II	neg.	pos.	neg.	
RW 905	post	ID ca.	II	neg.	pos.	neg.	
RW 906		ID ca.	III	neg.	pos.	neg.	
RW 907		ID ca.	III	pos.	pos.	neg.	
RW 912	pre	ID ca.	III	pos.	neg.	pos.	
RW 913	post	ID ca.	II	neg.	pos.	neg.	
RW 920	post	ID ca.	II	neg.	pos.	neg.	
RW 921	pre	DCIS		neg.	neg.	pos.	
RW 926	post	ID ca.	I	neg.	pos.	neg.	

Patient	Menopausal status (age)	Tumour classification	Grade	Node	ER	EGFR	PR
RW 927	post	ID ca.	III	neg.	neg.	pos.	
RW 928	post	Tubular ca.	I	pos.	pos.	neg.	
RW 935		DCIS + ID ca.	III	pos.	pos.	neg.	
RW 938	post	Tubular ca.	I	neg.	pos.	neg.	
RW 942	pre	DCIS		neg.	pos.	neg.	

Appendix VI

Given below are the formulas and a worked example for each test applied to the variables detailed in table 7.1, chapter 7.

VL1 Chi-Square Analysis

A classification scheme is given such that any observation must fall into precisely one class (cell). The data then consist of frequency counts and the statistic used is

$$\chi^2 = \sum \frac{(\text{Ob.} - \text{Ex.})^2}{\text{Ex.}},$$

where the sum is over all the classes (cells), Ob. denoting observed frequencies and Ex. expected frequencies, these being calculated from the appropriate null hypothesis. The null hypothesis is the hypothesis of no association between the classification schemes by rows and by columns. The expected frequency (Ex.) in any cell of the table is

$$\text{Ex.} = \frac{(\text{row marginal}) \times (\text{column marginal})}{\text{total number of observations}},$$

and the number of degrees of freedom (df) in determining the chi-squared value is

$$\text{df} = (\text{number of rows} - 1) \times (\text{number of columns} - 1).$$

In these cases, it is large values of χ^2 which are significant; values corresponding to <5%, or <1%, of the total distribution are regarded as significantly deviant from the null hypothesis of no association on which the tests are based, i.e. 5% or 1% probability of the null hypothesis being correct. The principal values of the chi-square distribution are given in Appendix VII.

Except for the classification of tumour grade, all datasets under test are binary variables, thereby forming 2x2 contingency tables. Binary variables are based on two possible outcomes, such as Yes/No, Positive/Negative, True/False, Present/Absent, On/Off, High/Low, Male/Female. Since tumour grade is based on a categorized scale of increasing abnormality of the tissue, it is possible to rank

this variable, where grade I tumours are well-differentiated, grade II tumours are intermediate and grade III tumours are poorly-differentiated (Harris *et al.*, 1987). Contingency tables based on such data can still be formed, so that if tumour grade is compared to a binary variable, then a 3x2 contingency table would be created, where the frequencies of the binary variable are listed for each of the grades stated.

For the general 2x2 contingency table,

	Classification II		Row marginal
Classification I	a	b	a + b
	c	d	c + d
Column marginal	a + c	b + d	N = a + b + c + d

$$df = (2 - 1)(2 - 1) = 1$$

$$N = a + b + c + d$$

$$Ob. = a, Ex.a = \frac{(a + c)(a + b)}{N}$$

$$\chi^2 = \frac{a - Ex.a}{Ex.a} + \frac{b - Ex.b}{Ex.b} + \frac{c - Ex.c}{Ex.c} + \frac{d - Ex.d}{Ex.d}$$

e.g.

		Lymph node status		Row marginal
		+ve	-ve	
Whole 1q arm deletion	yes	0 (2.23)	7 (4.77)	7
	no	15 (12.77)	25 (27.23)	40
Column marginal		15	32	N = 47

In each table cell, the observed frequency is stated first and is followed by the expected frequency in parentheses.

$$df = 1, \chi^2 = 3.845$$

VI.2 Yates' Correction to the Chi-Square Test

For the general 2x2 contingency table,

$$\chi_Y^2 = \frac{N (|ad - bc| - 0.5 \times N)^2}{(a + c)(b + d)(a + b)(c + d)}$$

e.g. for whole 1q arm deletion vs. lymph node status

$$\begin{aligned}\chi_Y^2 &= \frac{47 (|10 \times 25 - 7 \times 15| - 0.5 \times 47)^2}{15 \times 32 \times 7 \times 40} \\ &= 2.323\end{aligned}$$

VI.3 Fisher-Irwin Exact Test of Probability

For the general 2x2 table,

	Variable II		Row marginal
Variable I	a	b	a + b
	c	d	c + d
Column marginal	a + c	b + d	N

So that for any variable n,

n	(a + b) - n	(a + b)
(a + c) - n	(d - a) + n *	(c + d)
(a + c)	(b + d)	N

$$\begin{aligned} * (d - a) + n &= (b + d) - [(a + b) - n] \\ &= (c + d) - [(a + c) - n] \end{aligned}$$

To find those observations with a deviation at least as great as that actually observed,

$$|n - \text{Ex.}| \geq |\text{Ob.} - \text{Ex.}| \quad (\text{equation 1})$$

where $|n - \text{Ex.}|$ is the absolute difference between the theoretical and expected values and $|\text{Ob.} - \text{Ex.}|$ is the absolute difference between the observed and expected values.

$$\text{i.e. } |n - \frac{(a + c) \times (a + b)}{N}| \geq |a - \frac{(a + c) \times (a + b)}{N}|$$

for $0 \leq n \leq (a + b)$, apply each value of n to this equation and determine which satisfy the conditions.

To determine P (each n satisfying equation 1 | marginal totals are (a + b), (c + d), (a + c), (b + d)) i.e. the probability that for each value of n satisfying equation 1 given that marginal totals are as stated in the contingency table,

$$\text{Probability} = \frac{\binom{a + b}{n} \binom{c + d}{a + c - n}}{\binom{N}{a + c}} \quad \text{for all n satisfying the conditions}$$

$$\text{where } \binom{p}{q} = \frac{p!}{(p-q)! q!}.$$

The total probability derived from summing over all values of n satisfying conditions, gives the exact probability that the null hypothesis (no association between the variables) is correct.

e.g.

		Lymph node status		Row
		+ve	-ve	marginal
Whole 1q arm deletion	yes	0	7	7
	no	15	25	40
Column marginal		15	32	N = 47

n	7 - n	7
15 - n	25 + n	40
15	32	N = 47

$$|n - \frac{15 \times 7}{47}| \geq |0 - \frac{15 \times 7}{47}| \quad \text{i.e.} \quad |n - \frac{105}{47}| \geq \frac{105}{47}$$

$$\text{for } n = 0, \quad \frac{105}{47} \geq \frac{105}{47} \quad \text{yes}$$

$$\text{for } n = 1, \quad \frac{58}{47} \geq \frac{105}{47} \quad \text{no}$$

$$\text{for } n = 2, \quad \frac{11}{47} \geq \frac{105}{47} \quad \text{no}$$

$$\text{for } n = 3, \quad \frac{36}{47} \geq \frac{105}{47} \quad \text{no}$$

$$\text{for } n = 4, \quad \frac{83}{47} \geq \frac{105}{47} \quad \text{no}$$

$$\text{for } n = 5, \quad \frac{130}{47} \geq \frac{105}{47} \quad \text{yes}$$

$$\text{for } n = 6, \quad \frac{177}{47} \geq \frac{105}{47} \quad \text{yes}$$

$$\text{for } n = 7, \quad \frac{224}{47} \geq \frac{105}{47} \quad \text{yes}$$

$$\text{Probability} = \frac{\binom{7}{n} \binom{40}{15-n}}{\binom{47}{15}} \quad \text{for } n = 0, 5, 6, 7$$

$$= \frac{\binom{7}{0} \binom{40}{15}}{\binom{47}{15}} + \frac{\binom{7}{5} \binom{40}{10}}{\binom{47}{15}} + \frac{\binom{7}{6} \binom{40}{9}}{\binom{47}{15}} + \frac{\binom{7}{7} \binom{40}{8}}{\binom{47}{15}}$$

$$\begin{aligned}
\text{Probability} &= \frac{7! \cdot 40! \cdot 32! \cdot 15!}{7! \cdot 0! \cdot 35! \cdot 15! \cdot 47!} + \frac{7! \cdot 40! \cdot 32! \cdot 15!}{2! \cdot 5! \cdot 30! \cdot 10! \cdot 47!} \\
&+ \frac{7! \cdot 40! \cdot 32! \cdot 15!}{1! \cdot 6! \cdot 31! \cdot 9! \cdot 47!} + \frac{7! \cdot 40! \cdot 32! \cdot 15!}{0! \cdot 7! \cdot 32! \cdot 8! \cdot 47!} \\
&= 0.08
\end{aligned}$$

Therefore, 8.0% probability that the null hypothesis of no association is correct.

Appendix VII

Percentage of points of the chi-square distribution.

P (null hypothesis is correct)	Degrees of freedom	
	1	2
99%	0.000157	0.0201
98%	0.000628	0.0404
95%	0.00393	0.103
90%	0.0158	0.211
80%	0.0642	0.446
70%	0.148	0.713
50%	0.455	1.386
30%	1.074	2.408
20%	1.642	3.219
10%	2.706	4.605
5%	3.841	5.991
2.5%	5.024	7.378
2%	5.412	7.824
1%	6.635	9.210
0.5%	7.879	10.597

Bibliography

A

Aaltonen, L.A., Peltomäki, P., Leach, F.S., Sistonen, P., Pylkkänen, L., Mecklin, J.-P., Järvinen, H., Powell, S.M., Jen, J., Hamilton, S.R., Petersen, G.M., Kinzler, K.W., Vogelstein, B. and de la Chapelle, A. (1993) Clues to the pathogenesis of familial colorectal cancer. *Science* **260**, 812-816.

Ali, I.U., Lidereau, R. and Callahan, R. (1989) Presence of two members of c-erbA receptor gene family (c-erbA β and c-erbA2) in smallest region of somatic homozygosity on chromosome 3p21-p25 in human breast carcinoma. *J. Natl. Can. Inst.* **81**, 1815-1820.

Ali, I.U., Lidereau, R., Theillet, C. and Callahan, R. (1987) Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. *Science* **238**, 185-188.

Andersen, T.I., Gaustad, A., Ottestad, L., Farrants, G.W., Nesland, J.M., Tveit, K.M. and Børresen, A.-L. (1992) Genetic alterations of the tumour suppressor gene regions 3p, 11p, 13q, 17p, and 17q in human breast carcinomas. *Genes Chrom. Cancer* **4**, 113-121.

Annab, L.A., Dong, J.-T., Futreal, P.A., Satoh, H., Oshimura, M. and Barrett, J.C. (1992) Growth and transformation suppressor genes for BHK Syrian hamster cells on human chromosomes 1 and 11. *Mol. Carcinogen.* **6**, 280-288.

Armour, J.A.L., Povey, S., Jeremiah, S. and Jeffreys, A.J. (1990) Systematic cloning of human minisatellites from ordered array charomid libraries. *Genomics* **8**, 501-512.

Armour, J.A.L., Vergnaud, G., Crosier, M. and Jeffreys, A.J. (1992) Isolation of human minisatellite loci detected by synthetic tandem repeat probes: direct comparison with cloned DNA fingerprinting probes. *Hum. Mol. Genet.* **1**, 319-323.

Atkin, N.B. (1986) Chromosome 1 aberrations in cancer. *Cancer Genet. Cytogenet.* **21**, 279-285.

B

Bader, S.A., Fasching, C., Brodeur, G.M. and Stanbridge, E.J. (1991) Dissociation of suppression of tumorigenicity and differentiation *in vitro* effected by transfer of single human chromosomes into human neuroblastoma cells. *Cell Growth Differentiat.* **2**, 245-255.

Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., vanTuinen, P., Ledbetter, D.H., Barker, D.F., Nakamura, Y., White, R. and Vogelstein, B. (1989) Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* **244**, 217-221.

Baker, S.J., Preisinger, A.C., Jessup, J.M., Paraskeva, C., Markowitz, S., Willson, J.K.V., Hamilton, S. and Vogelstein, B. (1990) p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Res.* **50**, 7712-7722.

Balazs, M., Mayall, B.H. and Waldman, F.M. (1991) Interphase cytogenetics of a male breast cancer. *Cancer Genet. Cytogenet.* **55**, 243-247.

Barker, P.E. and Hsu, T.C. (1979) Double minutes in human carcinoma cell lines, with special reference to breast tumors. *J. Natl. Can. Inst.* **62**, 257-262.

Barker, P.E., Lau, Y.-F. and Hsu, T.C. (1980) A heterochromatic homogeneously staining region (HSR) in the karyotype of a human breast carcinoma cell line. *Cancer Genet. Cytogenet.* **1**, 311-319.

Barr, M.L. and Bertram, E.G. (1949) A morphological distinction between neurones of the male and female, and the behaviour of the nucleolar satellite during accelerated nucleoprotein synthesis. *Nature* 163, 676-677.

Barrios, L., Caballín, M.R., Miró, R., Fuster, C., Guedea, F., Subias, A. and Egozcue, J. (1991) Chromosomal instability in breast cancer patients. *Hum. Genet.* 88, 39-41.

Bauer, D.dF. and Erickson, R.L. (1955) Male breast cancer. Klinefelter syndrome with prostatic, adrenal and mammary tumors. *Northwest Med.* 54, 472-476.

Bello, M.J. and Rey, J.A. (1989) Cytogenetic analysis of metastatic effusions from breast tumors. *Neoplasma* 36, 71-81.

Benedict, W.F., Banerjee, A., Mark, C. and Murphree, A.L. (1983) Nonrandom chromosomal changes in untreated retinoblastomas. *Cancer Genet. Cytogenet.* 10, 311-333.

Benedict, W.F., Weissman, B.E., Mark, C. and Stanbridge, E.J. (1984) Tumorigenicity of human HT1080 fibrosarcoma x normal fibroblast hybrids: chromosome dosage dependency. *Cancer Res.* 44, 3471-3479.

Bertrand, S., Branger, M.R. and Cheix, F. (1979) Use of G banding technique in the cytogenetic study of a metastatic breast cancer effusion. A case report. *Eur. J. Cancer* 15, 737-743.

Bianchi, A.B., Navone, N.M. and Conti, C.J. (1991) Detection of loss of heterozygosity in formalin-fixed paraffin-embedded tumor specimens by the polymerase chain reaction. *Am. J. Pathol.* 138, 279-284.

Bièche, I., Champègne, M.-H., Matifas, F., Cropp, C.S., Callahan, R. and Lidereau, R. (1993) Two distinct regions involved in 1p deletion in human primary breast cancer. *Cancer Res.* 53, 1990-1994.

Bièche, I., Champègne, M.H., Matifas, F., Hácène, K., Callahan, R. and Lidereau, R. (1992) Loss of heterozygosity on chromosome 7q and aggressive primary breast cancer. *Lancet* 339, 139-143.

Bièche, I., Champègne, M.-H., Merlo, G., Larsen, C.-J., Callahan, R. and Lidereau, R. (1990) Loss of heterozygosity of the L-myc oncogene in human breast tumors. *Hum. Genet.* 85, 101-105.

Biegel, J.A., White, P.S., Marshall, H.N., Fujimori, M., Zackai, E.H., Scher, C.D., Brodeur, G.M. and Emanuel, B.S. (1993) Constitutional 1p36 deletion in a child with neuroblastoma. *Am. J. Hum. Genet.* 52, 176-182.

Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening plasmid DNA. *Nucl. Acids Res.* 6, 1513-1523.

Bishop, J.M. 1991 Molecular themes in oncogenesis. *Cell* 64, 235-248.

Bookstein, R., Shew, J.-Y., Chen, P.-L., Scully, P. and Lee, W.-H. (1990) Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated *RB* gene. *Science* 247, 712-715.

Borg, Å., Zhang, Q.-X., Alm, P., Olsson, H. and Sellberg, G. (1992a) The retinoblastoma gene in breast cancer: allele loss is not correlated with loss of gene expression. *Cancer Res.* 52, 2991-2994.

Borg, Å., Zhang, Q.-X., Olsson, H. and Wenngren, E. (1992b) Chromosome 1 alterations in breast cancer: allelic loss on 1p and 1q is related to lymphogenic metastases and poor prognosis. *Genes Chrom. Cancer* 5, 311-320.

Bouck, N., Stoler, A. and Poverini, P.J. (1986) Co-ordinate control of anchorage independence, actin cytoskeleton, and angiogenesis by human chromosome 1 in hamster-human hybrids. *Cancer Res.* 46, 5101-5105.

Brodeur, G.M. (1990) Neuroblastoma: clinical significance of genetic abnormalities. *Cancer Surv.* 9, 673-688.

Brodeur, G.M., Sekhon, G.S. and Goldstein, M.N. (1977) Chromosomal aberrations in human neuroblastomas. *Cancer* 40, 2256-2263.

Brown, C.J., Ballabio, A., Rupert, J.L., Lafrenière, R.G., Grompe, M., Tonlorenzi, R. and Willard, H.F. (1991) A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature* 349, 38-44.

Brown, C.J., Goss, S.J., Lubahn, D.B., Joseph, D.R., Wilson, E.M., French, F.S. and Willard, H.F. (1989) Androgen receptor locus on the human X chromosome: regional localization to Xq11-12 and description of a DNA polymorphism. *Am. J. Hum. Genet.* 44, 264-269.

Brown, C.J., Hendrich, B.D., Rupert, J.L., Lafrenière, R.G., Xing, Y., Lawrence, J. and Willard, H.F. (1992) The human *XIST* gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell* 71, 527-542.

Brown, C.J. and Willard, H.F. (1990) Localization of a gene that escapes inactivation to the X chromosome proximal short arm: implications for X inactivation. *Am. J. Hum. Genet.* 46, 273-279.

Brown, W.R.A. (1988) A physical map of the human pseudoautosomal region. *EMBO J.* 7, 2377-2385.

Burgoyne, P.S. (1982) Genetic homology and crossing over in the X and Y chromosomes of mammals. *Hum. Genet.* 61, 85-90.

Burns, J., Redfern, D.R.M., Esiri, M.M. and McGee, J.O. (1986) Human and viral gene detection in routine paraffin embedded tissue by in situ hybridisation with biotinylated probes: viral localisation in herpes encephalitis. *J. Clin. Pathol.* 39, 1066-1073.

C

Calabrese, G., Di Virgilio, C., Cianchetti, E., Franchi, P.G., Stuppia, L., Parruti, G., Bianchi, P.G. and Palka, G. (1991) Chromosome abnormalities in breast fibroadenomas. *Genes Chrom. Cancer* 3, 202-204.

Call, K.M., Glaser, T., Ito, C.Y., Buckler, A.J., Pelletier, J., Haber, D.A., Rose, E.A., Kral, A., Yeger, H., Lewis, W.H., Jones, C. and Housman, D.E. (1990) Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* 60, 509-520.

Camargo, M. and Wang, N. (1980) Cytogenetic evidence for the absence of an inactivated X chromosome in a human female (XX) breast carcinoma cell line. *Hum. Genet.* 55, 81-85.

Campbell, R.C. (1975) *Statistics for biologists*. 2nd edition. Cambridge University Press. London.

Caron, H., van Sluis, P., van Hoeve, M., de Kraker, J., Bras, J., Slater, R., Mannens, M., Voûte, P.A., Westerveld, A. and Versteeg, R. (1993) Allelic loss of chromosome 1p36 in neuroblastoma is of preferential maternal origin and correlates with *N-myc* amplification. *Nat. Genet.* 4, 187-190.

Castedo, S.M.M.J., de Jong, B., Oosterhuis, J.W., Seruca, R., Idenburg, V.J.S., Dam, A., te Meerman, G., Schraffordt Koops, H. and Sleijfer, D.T. (1989) Chromosomal changes in human primary testicular nonseminomatous germ cell tumors. *Cancer Res.* 49, 5696-5701.

Cavenee, W.K., Dryja, T.P., Phillips, R.A., Benedict, W.F., Godbout, R., Gallie, B.L., Murphree, A.L., Strong, L.C. and White, R.L. (1983) Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature* 305, 779-784.

- Cawkwell, L., Bell, S.M., Lewis, F.A., Dixon, M.F., Taylor, G.R. and Quirke, P. (1993) Rapid detection of allele loss in colorectal tumours using microsatellites and fluorescent DNA technology. *Br. J. Cancer* 67, 1262-1267.
- Chandley, A.C., Goetz, P., Hargreave, T.B., Joseph, A.M. and Speed, R.M. (1984) On the nature and extent of XY pairing at meiotic prophase in man. *Cytogenet. Cell Genet.* 38, 241-247.
- Chen, L.-C., Dollbaum, C. and Smith, H.S. (1989) Loss of heterozygosity on chromosome 1q in human breast cancer. *Proc. Natl. Acad. Sci. USA* 86, 7204-7207.
- Chen, L.-C., Kurisu, W., Ljung, B.-M., Goldman, E.S., Moore, D. and Smith, H.S. (1992) Heterogeneity for allelic loss in human breast cancer. *J. Natl. Can. Inst.* 84, 506-510.
- Chen, L.-C., Neubauer, A., Kurisu, W., Waldman, F.M., Ljung, B.-M., Goodson, W., Goldman, E.S., Moore, D., Balazs, M., Liu, E., Mayall, B.H. and Smith, H.S. (1991) Loss of heterozygosity on the short arm of chromosome 17 is associated with high proliferative capacity and DNA aneuploidy in primary human breast cancer. *Proc. Natl. Acad. Sci. USA* 88, 3847-3851.
- Chen, Z., Berger, C.S., Morgan, R., Roth, D., Stone, J.F. and Sandberg, A.A. (1992) Cytogenetic and FISH studies of abnormal X chromosomes in a patient with ANLL. *Cancer Genet.-Cytogenet.* 62, 130-133.
- Cheng, J.M., Hiemstra, J.L., Schneider, S.S., Naumova, A., Cheung, N.-K.V., Cohn, S.L., Diller, L., Sapienza, C. and Brodeur, G.M. (1993) Preferential amplification of the paternal allele of the N-myc gene in human neuroblastomas. *Nat. Genet.* 4, 191-194.
- Cheng, K.C. and Diaz, M.O. (1991) Genomic instability and cancer: cause and effect. *Cancer Cell.* 3, 188-192.
- Chirgwin, J.M., Schaefer, I.M., Rotwein, P.S., Piccini, N., Gross, K.W. and Naylor, S.L. (1984) Human renin gene is on chromosome 1. *Somat. Cell Mol. Genet.* 10, 415-421.
- Christiansen, H., Schestag, J., Christiansen, N.M., Grzeschik, K.-H. and Lampert, F. (1992) Clinical impact of chromosome 1 aberrations in neuroblastoma: a metaphase and interphase cytogenetic study. *Genes Chrom. Cancer* 5, 141-149.
- Cliby, W., Ritland, S., Hartmann, L., Dodson, M., Halling, K.C., Keeney, G., Podratz, K.C. and Jenkins, R.B. (1993) Human epithelial ovarian cancer allelotype. *Cancer Res.* 53, 2393-2398.
- Coles, C., Thompson, A.M., Elder, P.A., Cohen, B.B., Mackenzie, I.M., Cranston, G., Chetty, U., Mackay, J., Macdonald, M., Nakamura, Y., Hoyheim, B. and Steel, C.M. (1990) Evidence implicating at least two genes on chromosome 17p in breast carcinogenesis. *Lancet* 336, 761-763.
- Collins, A., Keats, B.J., Dracopoli, N., Shields, D.C. and Morton, N.E. (1992) Integration of gene maps: chromosome 1. *Proc. Natl. Acad. Sci. USA* 89, 4598-4602.
- Cooke, A., Kim, Y.T., Harvey, T.I., Connor, J.M., Nagy, J. and George, W.D. (1993) Loss of heterozygosity on chromosome 7q31 in breast cancer. *Lancet* 341, 1289.
- Cooke, H.J., Brown, W.R.A. and Rappold, G.A. (1985) Hypervariable telomeric sequences from the human sex chromosomes are pseudoautosomal. *Nature* 317, 687-692.
- Cooke, H.J. and Hindley, J. (1979) Cloning of human satellite III DNA: different components are on different chromosomes. *Nucl. Acids Res.* 6, 3177-3197.
- Cornelis, R.S., Devilee, P., van Vliet, M., Kuipers-Dijkshoorn, N., Kersenmaeker, A., Bardoel, A., Meera Khan, P. and Cornelisse, C.J. (1993) Allele loss patterns on chromosome 17q in 109 breast carcinomas indicate at least two distinct target regions. *Oncogene* 8, 781-785.

Cornelisse, C.J., Kuipers-Dijkshoorn, N., van Vliet, M., Hermans, J. and Devilee, P. (1992) Fractional allelic imbalance in human breast cancer increases with tetraploidization and chromosome loss. *Int. J. Cancer* 50, 544-548.

Cowell, J.K. (1991) The genetics of retinoblastoma. *Br. J. Cancer* 63, 333-336.

Cremer, T., Tesin, D., Hopman, A.H.N. and Manuelidis, L. (1988) Rapid interphase and metaphase assessment of specific chromosomal changes in neuroectodermal tumor cells by *in situ* hybridization with chemically modified DNA probes. *Exp. Cell Res.* 176, 199-220.

Cremers, F.P.M., van de Pol, T.J.R., Wieringa, B., Hofker, M.H., Pearson, P.L., Pfeiffer, R.A., Mikkelsen, M., Tabor, A. and Ropers, H.H. (1988) Molecular analysis of male-viable deletions and duplications allows ordering of 52 DNA probes on proximal Xq. *Am. J. Hum. Genet.* 43, 452-461.

Cropp, C.S., Lidereau, R., Campbell, G., Champèrne, M.H. and Callahan, R. (1990) Loss of heterozygosity on chromosomes 17 and 18 in breast carcinoma: two additional regions identified. *Proc. Natl. Acad. Sci. USA* 87, 7737-7741.

D

Darlington, C.D., Haldane, J.B.S. and Koller, P.C. (1934) Possibility of incomplete sex linkage in mammals. *Nature* 133, 417.

Davies, K.E., Pearson, P.L., Harper, P.S., Murray, J.M., O'Brien, T., Sarfarazi, M. and Williamson, R. (1983) Linkage analysis of two cloned DNA sequences flanking the Duchenne muscular dystrophy locus on the short arm of the human X chromosome. *Nucl. Acids Res.* 11, 2303-2312.

Davis, M.P., Dewald, G.W., Pierre, R.V. and Hoagland, H.C. (1984) Hematological manifestations associated with deletions of the long arm of chromosome 20. *Cancer Genet. Cytogenet.* 12, 63-71.

de Bustamante, A.D., Delicado, A., de Miguel, P.G., Darnaude, M.T., de Torres, M.L., Zumel, R. and Pajares, I.L. (1990) Balanced reciprocal translocation (X;20) limited to Wilms' tumor in a Wiedemann-Beckwith syndrome. *Cancer Genet. Cytogenet.* 45, 35-39.

de Jong, B., Molenaar, I.M., Leeuw, J.A., Idenberg, V.J.S. and Oosterhuis, J.W. (1986) Cytogenetics of a renal adenocarcinoma in a 2-year-old child. *Cancer Genet. Cytogenet.* 21, 165-169.

de Leeuw, B., Berger, W., Sinke, R.J., Suijkerbuijk, R.F., Gilgenkrantz, S., Geraghty, M.T., Valle, D., Monaco, A.P., Lehrach, H., Ropers, H.H. and van Kessel, A.G. (1993) Identification of a yeast artificial chromosome (YAC) spanning the synovial sarcoma-specific t(X;18)(p11.2;q11.2) breakpoint. *Genes Chrom. Cancer* 6, 182-189.

de Wit, P.E.J., Hopman, A.H.N., van Muijen, G.N.P., Smeets, D.F.C.M., Beck, J.L.M., Moesker, O. and Ruiter, D.J. (1992) In situ detection of supernumerary aberrations of chromosome-specific repetitive DNA targets in interphase nuclei in human melanoma cell lines and tissue sections. *J. Invest. Dermatol.* 98, 450-458.

Devilee, P., Cornelisse, C.J., Kuipers-Dijkshoorn, N., Jonker, C. and Pearson, P.L. (1990) Loss of heterozygosity on 17p in human breast carcinomas: defining the smallest common region of deletion. *Cytogenet. Cell Genet.* 53, 52-54.

Devilee, P., Thierry, R.F., Kievits, T., Kolluri, R., Hopman, A.H.N., Willard, H.F., Pearson, P.L. and Cornelisse, C.J. (1988) Detection of chromosome aneuploidy in interphase nuclei from human primary breast tumors using chromosome-specific repetitive DNA probes. *Cancer Res.* 48, 5825-5830.

Devilee, P., van den Broek, M., Kuipers-Dijkshoorn, N., Kolluri, R., Meera Khan, P., Pearson, P.L. and Cornelisse, C.J. (1989) At least four different chromosomal regions are involved in loss of heterozygosity in human breast carcinoma. *Genomics* 5, 554-560.

Devilee, P., van den Broek, M., Mannens, M., Slater, R., Cornelisse, C.J., Westerveld, A. and Meera Khan, P. (1991a) Differences in patterns of allelic loss between two common types of adult cancer, breast and colon carcinoma, and Wilms' tumor of childhood. *Int. J. Cancer* 47, 817-821.

Devilee, P., van Vliet, M., Bardoel, A., Kievits, T., Kuipers-Dijkshoorn, N., Pearson, P.L. and Cornelisse, C.J. (1991b) Frequent somatic imbalance of marker alleles for chromosome 1 in human primary breast carcinoma. *Cancer Res.* 51, 1020-1025.

Devilee, P., van Vliet, M., Kuipers-Dijkshoorn, N., Pearson, P.L. and Cornelisse, C.J. (1991c) Somatic genetic changes on chromosome 18 in breast carcinomas: is the DCC gene involved? *Oncogene* 6, 311-315.

Devilee, P., van Vliet, M., van Sloun, P., Kuipers-Dijkshoorn, N., Hermans, J., Pearson, P.L. and Cornelisse, C.J. (1991d) Allelotype of human breast carcinoma: a second major site for loss of heterozygosity is on chromosome 6q. *Oncogene* 6, 1705-1711.

Dhingra, K., Sahin, A., Supak, J., Kim, S.Y., Hortobagyi, G. and Hittelman, W.N. (1992) Chromosome *in situ* hybridization on formalin-fixed mammary tissue using non-isotopic, non-fluorescent probes: technical considerations and biological implications. *Breast Cancer Res. Treat.* 23, 201-210.

Donis-Keller, H., Green, P., Helms, C., Cartinhour, S., Weiffenbach, B., Stephens, K., Keith, T.P., Bowden, D.W., Smith, D.R., Lander, E.S., Botstein, D., Akots, G., Rediker, K.S., Gravius, T., Brown, V.A., Rising, M.B., Parker, C., Powers, J.A., Watt, D.E., Kauffman, E.R., Bricker, A., Phipps, P., Muller-Kahle, H., Fulton, T.R., Ng, S., Schumm, J.W., Braman, J.C., Knowlton, R.G., Barker, D.F., Crooks, S.M., Lincoln, S.E., Daly, M.J. and Abrahamson, J. (1987) A genetic linkage map of the human genome. *Cell* 51, 319-337.

Dracopoli, N.C., Alhadeff, B., Houghton, A.N. and Old, L.J. (1987) Loss of heterozygosity at autosomal and X-linked loci during tumor progression in a patient with melanoma. *Cancer Res.* 47, 3995-4000.

Dracopoli, N.C., Harnett, P., Bale, S.J., Stanger, B.Z., Tucker, M.A., Housman, D.E. and Kefford, R.F. (1989) Loss of alleles from the distal short arm of chromosome 1 occurs late in melanoma tumor progression. *Proc. Natl. Acad. Sci. USA* 86, 4614-4618.

Dracopoli, N.C., Houghton, A.N. and Old, L.J. (1985) Loss of polymorphic restriction fragments in malignant melanoma: implications for tumor heterogeneity. *Proc. Natl. Acad. Sci. USA* 82, 1470-1474.

Dracopoli, N.C., O'Connell, P., Elsner, T.I., Lalouel, J.-M., White, R.L., Buetow, K.H., Nishimura, D.Y., Murray, J.C., Helms, C., Mishra, S.K., Donis-Keller, H., Hall, J.M., Lee, M.K., King, M.-C., Attwood, J., Morton, N.E., Robson, E.B., Mahtani, M., Willard, H.F., Royle, N.J., Patel, I., Jeffreys, A.J., Verga, V., Jenkins, T., Weber, J.L., Mitchell, A.L. and Bale, A.E. (1991) The CEPH consortium linkage map of human chromosome 1. *Genomics* 9, 686-700.

Drayna, D., Davies, K., Hartley, D., Mandel, J.-L., Camerino, G., Williamson, R. and White, R. (1984) Genetic mapping of the human X chromosome by using restriction fragment length polymorphisms. *Proc. Natl. Acad. Sci. USA* 81, 2836-2839.

Dupont, W.D. and Page, D.L. (1985) Risk factors for breast cancer in women with proliferative disease. *New Engl. J. Med.* 312, 146-151.

Dutrillaux, B., Gerbault-Seureau, M., Remvikos, Y., Zafrani, B. and Prieur, M. (1991) Breast cancer genetic evolution: 1. Data from cytogenetics and DNA content. *Breast Cancer Res. Treat.* 19, 245-255.

Dutrillaux, B., Gerbault-Seureau, M. and Zafrani, B. (1990) Characterization of chromosomal anomalies in human breast cancer. A comparison of 30 paradiplod cases with few chromosome changes. *Cancer Genet. Cytogenet.* 49, 203-217.

E

Easton, D.F., Bishop, D.T., Ford, D., Crockford, G.P. and the Breast Cancer Linkage Consortium (1993) Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. *Am. J. Hum. Genet.* **52**, 678-701.

Ellis, N. and Goodfellow, P.N. (1989) The mammalian pseudoautosomal region. *Trend. Genet.* **5**, 406-410.

Ellis, N.A., Goodfellow, P.J., Pym, B., Smith, M., Palmer, M., Frischauf, A.-M. and Goodfellow, P.N. (1989) The pseudoautosomal boundary in man is defined by an *Alu* repeat sequence inserted on the Y chromosome. *Nature* **337**, 81-84.

Ellison, J., Passage, M., Yu, L.-C., Yen, P., Mohandas, T.K. and Shapiro, L. (1992a) Directed isolation of human genes that escape X inactivation. *Somat. Cell Mol. Genet.* **18**, 259-268.

Ellison, J.W., Ramos, C., Yen, P.H. and Shapiro, L.J. (1992b) Structure and expression of the human pseudoautosomal gene XE7. *Hum. Mol. Genet.* **1**, 691-696.

Emmerich, P., Jauch, A., Hofman, M.-C., Cremer, T. and Walt, H. (1989) Interphase cytogenetics in paraffin embedded sections from testicular germ cell tumor xenografts and in corresponding cultured cells. *Lab. Invest.* **61**, 235-242.

Eva, A. and Aaronson, S.A. (1985) Isolation of a new human oncogene from a diffuse B-cell lymphoma. *Nature* **316**, 273-275.

Eva, A., Vecchio, G., Rao, C.D., Tronick, S.R. and Aaronson, S.A. (1988) The predicted *DBL* oncogene product defines a distinct class of transforming proteins. *Proc. Natl. Acad. Sci. USA* **85**, 2061-2065.

Evans, D.B. and Crichlow, R.W. (1987) Carcinoma of the male breast and Klinefelter's syndrome: is there an association? *CA: Cancer J. Clinic.* **37**, 246-251.

F

Farr, C., Fantes, J., Goodfellow, P. and Cooke, H. (1991) Functional reintroduction of human telomeres into mammalian cells. *Proc. Natl. Acad. Sci. USA* **88**, 7006-7010.

Farr, C.J., Stevanovic, M., Thomson, E.J., Goodfellow, P.N. and Cooke, H.J. (1992) Telomere-associated chromosome fragmentation: applications in genome manipulation and analysis. *Nat. Genet.* **2**, 275-282.

Fasano, O., Birnbaum, D., Edlund, L., Fogh, J. and Wigler, M. (1984) New human transforming genes detected by a tumorigenicity assay. *Mol. Cell. Biol.* **4**, 1695-1705.

Fearon, E.R. (1992) Genetic alterations underlying colorectal tumorigenesis. *Cancer Surv.* **12**, 119-136.

Fearon, E.R., Cho, K.R., Nigro, J.M., Kern, S.E., Simons, J.W., Ruppert, J.M., Hamilton, S.R., Preisinger, A.C., Thomas, G., Kinzler, K.W. and Vogelstein, B. (1990) Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science* **247**, 49-56.

Fearon, E.R. and Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. *Cell* **61**, 759-767.

Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analyt. Biochem.* **132**, 6-13.

Ferti-Passantonopoulou, A.D. and Panani, A.D. (1987) Common cytogenetic findings in primary breast cancer. *Cancer Genet. Cytogenet.* **27**, 289-298.

Fey, M.F., Hesketh, C., Wainscoat, J.S., Gendler, S. and Thein, S.L. (1989) Clonal allele loss in gastrointestinal cancers. *Br. J. Cancer* **59**, 750-754.

Fong, C.-T., Dracopoli, N.C., White, P.S., Merrill, P.T., Griffith, R.C., Housman, D.E. and Brodeur, G.M. (1989) Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification. *Proc. Natl. Acad. Sci. USA* **86**, 3753-3757.

Fong, C.-T., White, P.S., Peterson, K., Sapienza, C., Cavenee, W.K., Kern, S.E., Vogelstein, B., Cantor, A.B., Look, A.T. and Brodeur, G.M. (1992) Loss of heterozygosity for chromosome 1 or chromosome 14 defines subsets of advanced neuroblastomas. *Cancer Res.* **52**, 1780-1785.

Foulkes, W.D., Black, D.M., Stamp, G.W.H., Solomon, E. and Trowsdale, J. (1993) Very frequent loss of heterozygosity throughout chromosome 17 in sporadic ovarian carcinoma. *Int. J. Cancer* **54**, 220-225.

Fraser, N.J., Boyd, Y., Brownlee, G.G. and Craig, I.W. (1987) Multi-allelic RFLP for M27 β , an anonymous single copy genomic clone at Xp11.3-Xcen. *Nucl. Acids Res.* **15**, 9616.

Freije, D., Helms, C., Watson, M.S. and Donis-Keller, H. (1992) Identification of a second pseudoautosomal region near the Xq and Yq telomeres. *Science* **258**, 1784-1787.

Fukumoto, M., Suzuki, A., Inazawa, J., Yoshimura, T., Arao, S., Takahashi, T., Nomura, H. and Hiai, H. (1993) Chromosomal location and structure of amplicons in two human cell lines with coamplification of c-myc and Ki-ras oncogenes. *Somat. Cell Mol. Genet.* **19**, 21-28.

Futreal, P.A., Söderkvist, P., Marks, J.R., Iglehart, J.D., Cochran, C., Barrett, J.C. and Wiseman, R.W. (1992) Detection of frequent allelic loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymorphisms. *Cancer Res.* **52**, 2624-2627.

G

Ganly, P.S., Jarad, N., Rudd, R.M. and Rabbitts, P.H. (1992) PCR-based RFLP analysis allows genotyping of the short arm of chromosome 3 in small biopsies from patients with lung cancer. *Genomics* **12**, 221-228.

Gardner, H.A., Gallie, B.L., Knight, L.A. and Phillips, R.A. (1982) Multiple karyotypic changes in retinoblastoma tumor cells: presence of normal chromosome no.13 in most tumors. *Cancer Genet. Cytogenet.* **6**, 201-211.

Gebhart, E., Brüderlein, S., Augustus, M., Siebert, E., Feldner, J. and Schmidt, W. (1986) Cytogenetic studies on human breast carcinomas. *Breast Cancer Res. Treat.* **8**, 125-138.

Gendler, S.J., Burchell, J.M., Duhig, T., Lamport, D., White, R., Parker, M. and Taylor-Papadimitriou, J. (1987) Cloning of partial cDNA encoding differentiation and tumor-associated mucin glycoproteins expressed by human mammary epithelium. *Proc. Natl. Acad. Sci. USA* **84**, 6060-6064.

Gendler, S.J., Cohen, E.P., Craston, A., Duhig, T., Johnstone, G. and Barnes, D. (1990) The locus of the polymorphic epithelial mucin (PEM) tumour antigen on chromosome 1q21 shows a high frequency of alteration in primary human breast tumours. *Int. J. Cancer* **45**, 431-435.

Genuardi, M., Saunders, G.F., Anderson, D.E. and Neri, G. (1989a) Chromosome 1 allelic imbalances in breast cancer. *Acta Med. Rom.* **27**, 148-156.

Genuardi, M., Tsihira, H., Anderson, D.E. and Saunders, G.F. (1989b) Distal deletion of chromosome 1p in ductal carcinoma of the breast. *Am. J. Hum. Genet.* **45**, 73-82.

Gerbault-Seureau, M., Vielh, P., Zafrani, B., Salmon, R. and Dutrillaux, B. (1987) Cytogenetic study of twelve human near-diploid breast cancers with chromosomal changes. *Ann. Génét.* 30, 138-145.

Ghose, T., Lee, C.L.Y., Fernandez, L.A., Lee, S.H.S., Raman, R. and Colp, P. (1990) Role of 1q trisomy in tumorigenicity, growth, and metastasis of human leukemic B-cell clones in nude mice. *Cancer Res.* 50, 3737-3742.

Ghosh, S.N. and Shah, P.N. (1975) Prognosis and incidence of sex chromatin in breast cancer. A preliminary report. *Acta Cytol.* 19, 58-61.

Ghosh, S.N. and Shah, P.N. (1981) Significance of the Barr body in human female tumors. *Cancer Genet. Cytogenet.* 4, 269-274.

Ghosh, S.N., Shah, P.N. and Banerji, A.P. (1979) Loss of X-chromatin and X-chromosome activation in HeLa cells. *Acta Cytol.* 23, 169-172.

Gilbert, F., Feder, M., Balaban, G., Brangman, D., Lurie, D.K., Podolsky, R., Rinaldt, V., Vinikoor, N. and Weisband, J. (1984) Human neuroblastomas and abnormalities of chromosomes 1 and 17. *Cancer Res.* 44, 5444-5449.

Goodfellow, P., Pym, B., Mohandas, T. and Shapiro, L.J. (1984) The cell surface antigen locus, *MIC2X*, escapes X-inactivation. *Am. J. Hum. Genet.* 36, 777-782.

Goodfellow, P.J., Darling, S.M., Thomas, N.S. and Goodfellow, P.N. (1986) A pseudoautosomal gene in man. *Science* 234, 740-743.

Green, A.R. (1988) Recessive mechanisms of malignancy. *Br. J. Cancer* 58, 115-121.

Greig, G.M., England, S.B., Bedford, H.M. and Willard, H.F. (1989) Chromosome-specific alpha satellite DNA from the centromere of human chromosome 16. *Am. J. Hum. Genet.* 45, 862-872.

Gruis, N.A., Abeln, E.C.A., Bardoel, A.F.J., Devilee, P., Frants, R.R. and Cornelisse, C.J. (1993) PCR-based microsatellite polymorphisms in the detection of loss of heterozygosity in fresh and archival tumour tissue. *Br. J. Cancer* 68, 308-313.

H

Haber, A. and Runyon, R.P. (1973) *General statistics*. 2nd edition. Addison-Wesley. London.

Hackenberg, R., Lüttchens, S., Hofmann, J., Kunzmann, R., Hölzel, F. and Schulz, K.-D. (1991) Androgen sensitivity of the new human breast cancer cell line MFM-223. *Cancer Res.* 51, 5722-5727.

Hainsworth, P.J., Raphael, K.L., Stillwell, R.G., Bennett, R.C. and Garson, O.M. (1991) Cytogenetic features of twenty-six primary breast cancers. *Cancer Genet. Cytogenet.* 52, 205-218.

Hainsworth, P.J., Raphael, K.L., Stillwell, R.G., Bennett, R.C. and Garson, O.M. (1992) Rearrangement of chromosome 1p in breast cancer correlates with prognostic features. *Br. J. Cancer* 66, 131-135.

Hall, J.M., Friedman, L., Guenther, C., Lee, M.K., Weber, J.L., Black, D.M. and King, M.-C. (1992) Closing in on a breast cancer gene on chromosome 17q. *Am. J. Hum. Genet.* 50, 1235-1242.

Hall, J.M., Lee, M.K., Newman, B., Morrow, J.E., Anderson, L.A., Huey, B. and King, M.-C. (1990) Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* 250, 1684-1689.

Hansen, M.F., Koufos, A., Gallie, B.L., Phillips, R.A., Fodstad, Ø., Brøgger, A., Gedde-Dahl, T. and Cavenee, W.K. (1985) Osteosarcoma and retinoblastoma: a shared chromosomal mechanism revealing recessive disposition. *Proc. Natl. Acad. Sci. USA* 82, 6216-6220.

Harbour, J.W., Lai, S.-L., Whang-Peng, J., Gazdar, A.F., Minna, J.D. and Kaye, F.J. (1988) Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. *Science* **241**, 353-357.

Harnett, P.R., Kearsley, J.H., Hayward, N.K., Dracopoli, N.C. and Kefferd, R.F. (1991) Loss of allelic heterozygosity on distal chromosome 1p in Merkel cell carcinoma. A marker of neural crest origins? *Cancer Genet. Cytogenet.* **54**, 109-113.

Harris, H. (1990) Tumor suppressor genes: studies with hybrid mouse cells. In: *Tumor suppressor genes*. Ed. Klein, G. 1-13. Marcel Dekker. New York.

Harris, H., Miller, O.J., Klein, G., Worst, P. and Tachibana, T. (1969) Suppression of malignancy by cell fusion. *Nature* **223**, 363-368.

Harris, J.R., Hellman, S., Henderson, I.C. and Kinne, D.W. (1987) *Breast diseases*. J.B. Lippincott. Philadelphia.

Hill, S.M., Rodgers, C.S. and Hultén, M.A. (1987) Cytogenetic analysis in human breast carcinoma. II. Seven cases in the triploid/tetraploid range investigated using direct preparations. *Cancer Genet. Cytogenet.* **24**, 45-62.

Hofker, M.H., Skraastad, M.I., Bergen, A.A.B., Wapenaar, M.C., Bakker, E., Millington-Ward, A., van Ommen, G.J.B. and Pearson, P.L. (1986) The X chromosome shows less genetic variation at restriction sites than the autosomes. *Am. J. Hum. Genet.* **39**, 438-451.

Hopman, A.H.N., Ramaekers, F.C.S., Raap, A.K., Beck, J.L.M., Devilee, P., van der Ploeg, M. and Vooijs, G.P. (1988) In situ hybridization as a tool to study numerical chromosome aberrations in solid bladder tumors. *Histochem.* **89**, 307-316.

Hopman, A.H.N., van Hooren, E., van de Kaa, C., Vooijs, P.G.P. and Ramaekers, F.C.S. (1991) Detection of numerical chromosome aberrations using *in situ* hybridization in paraffin sections of routinely processed bladder cancers. *Mod. Pathol.* **4**, 503-513.

Huebner, K., ar-Rushdi, A., Griffin, C.A., Isobe, M., Kozak, C., Emanuel, B.S., Nagarajan, L., Cleveland, J.L., Bonner, T.I., Goldsborough, M.D., Croce, C.M. and Rapp, U. (1986) Actively transcribed genes in the *raf* oncogene group, located on the X chromosome in mouse and man. *Proc. Natl. Acad. Sci. USA* **83**, 3934-3938.

I

Inglehearn, C.F. and Cooke, H.J. (1990) A VNTR immediately adjacent to the human pseudoautosomal telomere. *Nucl. Acids Res* **18**, 471-476.

Islam, M.Q., Szpirer, J., Szpirer, C., Islam, K., Dasnoy, J.-F. and Levan, G. (1989) A gene for the suppression of anchorage independence is located in rat chromosome 5 bands q22-23, and the rat alpha-interferon locus maps at the same region. *J. Cell Sci.* **92**, 147-162.

J

Jackson, A.W., Muldal, S., Ockey, C.H. and O'Connor, P.J. (1965) Carcinoma of male breast in association with the Klinefelter syndrome. *Br. Med. J.* **1**, 223-225.

Jacobs, I.J., Smith, S.A., Wiseman, R.W., Futreal, P.A., Harrington, T., Osborne, R.J., Leech, V., Molyneux, A., Berchuck, A., Ponder, B.A.J. and Bast, R.C. (1993) A deletion unit on chromosome 17q in epithelial ovarian tumors distal to the familial breast/ovarian cancer locus. *Cancer Res.* **53**, 1218-1221.

Jacobs, P.A., Brunton, M. and Brown, W.M.C. (1964) Cytogenetic studies in leukocytes on the general population: subjects of ages 65 years and more. *Ann. Hum. Genet.* **27**, 353-362.

Jacobs, P.A., Brunton, M., Brown, W.M.C., Doll, R. and Goldstein, H. (1963) Change of human chromosome count distributions with age: evidence for a sex difference. *Nature* **197**, 1080-1081.

Jeffreys, A.J., Wilson, V. and Thein, S.L. (1985) Hypervariable 'minisatellite' regions in human DNA. *Nature* **314**, 67-73.

Jenkyn, D.J. and McCartney, A.J. (1986) Primary cytogenetic abnormality detected in an endometrial adenocarcinoma. *Cancer Genet. Cytogenet.* **20**, 149-157.

Jonasson, J., Povey, S. and Harris, H. (1977) The analysis of malignancy by cell fusion. VII. Cytogenetic analysis of hybrids between malignant and diploid cells and of tumours derived from them. *J. Cell Sci.* **24**, 217-254.

Jones Cruciger, Q.V., Pathak, S. and Cailleau, R. (1976) Human breast carcinomas: marker chromosomes involving 1q in seven cases. *Cytogenet. Cell Genet.* **17**, 231-135.

K

Kajii, T., Tsukahara, M., Fukushima, Y., Hata, A., Matsuo, K. and Kuroki, Y. (1985) Translocation (X;13)(p11.21;q12.3) in a girl with *incontinentia pigmenti* and bilateral retinoblastoma. *Ann. Génét.* **28**, 219-223.

Kallenberger, A., Hagmann, A. and Descoeudres, C. (1968) The interpretation of abnormal sexchromatin incidence in human breast tumors on the basis of DNA measurements. *Eur. J. Cancer* **3**, 439-448.

Kallioniemi, A., Kallioniemi, O.-P., Waldman, F.M., Chen, L.-C., Yu, L.-C., Fung, Y.K.T., Smith, H.S., Pinkel, D. and Gray, J.W. (1992) Detection of retinoblastoma gene copy number in metaphase chromosomes and interphase nuclei by fluorescence *in situ* hybridization. *Cytogenet. Cell Genet.* **60**, 190-193.

Kallioniemi, O.P., Kallioniemi, A., Kurisu, W., Thor, A., Chen, L.-C., Smith, H.S., Waldman, F.M., Pinkel, D. and Gray, J.W. (1992) *ERBB2* amplification in breast cancer analyzed by fluorescence *in situ* hybridization. *Proc. Natl. Acad. Sci. USA* **89**, 5321-5325.

Keith, W.N., Douglas, F., Wishart, G.C., McCallum, H.M., George, W.D., Kaye, S.B. and Brown, R. (1993) Co-amplification of *erbB2*, topoisomerase II α and retinoic acid receptor α genes in breast cancer and allelic loss at topoisomerase I on chromosome 20. *Eur. J. Cancer* **29A**, 1469-1475.

Khosla, S., Patel, V.M., Hay, I.D., Schaid, D.J., Grant, C.S., van Heerden, J.A. and Thibodeau, S.N. (1991) Loss of heterozygosity suggests multiple genetic alterations in pheochromocytomas and medullary thyroid carcinomas. *J. Clin. Invest.* **87**, 1691-1699.

Kim, S.Y., Lee, J.S., Ro, J.Y., Gay, M.L., Hong, W.K. and Hittelman, W.N. (1993) Interphase cytogenetics in paraffin sections of lung tumors by non-isotopic *in situ* hybridization. Mapping genotype/phenotype heterogeneity. *Am. J. Pathol.* **142**, 307-317.

Kinzler, K.W., Zehnauer, B.A., Brodeur, G.M., Seeger, R.C., Trent, J.M., Meltzer, P.S. and Vogelstein, B. (1986) Amplification units containing human *N-myc* and *c-myc* genes. *Proc. Natl. Acad. Sci. USA* **83**, 1031-1035.

Kirucuta, I. and Olinici, C.D. (1975) Significance of the disease of sex chromatin incidence in malignant breast tumors. *Morphol. Embryol.* **21**, 197-201.

Knowles, M.A., Shaw, M.E. and Proctor, A.J. (1993) Deletion mapping of chromosome 8 in cancers of the urinary bladder using restriction fragment length polymorphisms and microsatellite polymorphisms. *Oncogene* **8**, 1357-1364.

Knudson, A.G. (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl. Acad. Sci. USA* 68, 820-823.

Knyazev, P.G., Imyanitov, E.N., Chernitsa, O.I. and Nikiforova, I.F. (1993) Loss of heterozygosity at chromosome 17p is associated with *HER-2* amplification and lack of nodal involvement in breast cancer. *Int. J. Cancer* 53, 11-16.

Kokalj-Vokac, N., Alemeida, A., Gerbault-Seureau, M., Malfoy, B. and Dutrillaux, B. (1993) Two-color FISH characterization of i(1q) and der(1;16) in human breast cancer cells. *Genes Chrom. Cancer* 7, 8-14.

Kovacs, G. (1978) Abnormalities of chromosome no. 1 in human solid malignant tumours. *Int. J. Cancer* 21, 688-694.

Kovacs, G. (1979) Homogeneously staining regions on marker chromosomes in malignancy. *Br. J. Cancer* 23, 299-301.

Kovacs, G. (1981) Preferential involvement of chromosome 1q in a primary breast carcinoma. *Cancer Genet. Cytogenet.* 3, 125-129.

Kubo, K., Yoshimoto, K., Yokogoshi, Y., Tsuyuguchi, M. and Saito, S. (1991) Loss of heterozygosity on chromosome 1p in thyroid adenoma and medullary carcinoma, but not in papillary carcinoma. *Jpn. J. Cancer Res.* 82, 1097-1103.

Kugoh, H.M., Hashiba, H., Shimizu, M. and Oshimura, M. (1990) Suggestive evidence for functionally distinct, tumor-suppressor genes on chromosomes 1 and 11 for a human fibrosarcoma cell line, HT1080. *Oncogene* 5, 1637-1644.

Kumlin-Wolff, E., Nakamura, Y., Hoff, M., O'Connell, P., Leppert, M., Lathrop, G.M., Lalouel, J.-M. and White, R. (1987) Isolation and mapping of a polymorphic DNA sequence pEKH7.4 to chromosome 1 (D1S65). *Nucl. Acids Res.* 15, 9621.

L

La Spada, A.R., Wilson, E.M., Lubahn, D.B., Harding, A.E. and Fischbeck, K.H. (1991) Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature* 352, 77-79.

Lamb, J., Harris, P.C., Wilkie, A.O.M., Wood, W.G., Dauwerse, J.H.G. and Higgs, D.R. (1993) De novo truncation of chromosome 16p and healing with (TTAGGG)_n in the α -thalassemia/mental retardation syndrome (ATR-16). *Am. J. Hum. Genet.* 52, 668-676.

Lambson, B., Affara, N.A., Mitchell, M. and Ferguson-Smith, M.A. (1992) Evolution of DNA sequence homologies between the sex chromosomes in primate species. *Genomics* 14, 1032-1040.

Larsson, C., Byström, C., Skoog, L., Rotstein, S. and Nordenskjöld, M. (1990) Genomic alterations in human breast carcinomas. *Genes Chrom. Cancer* 2, 191-197.

Lasko, D., Cavenee, W. and Nordenskjöld, M. (1991) Loss of constitutional heterozygosity in human cancer. *Ann. Rev. Genet.* 25, 281-314.

Latif, F., Tory, K., Gnarr, J., Yao, M., Duh, F.-M., Orcutt, M.L., Stackhouse, T., Kuzmin, I., Modi, W., Geil, L., Schmidt, L., Zhou, F., Li, H., Wei, M.H., Chen, F., Glenn, G., Choyke, P., Walther, M.M., Weng, Y., Duan, D.-S.R., Dean, M., Glavac, D., Richards, F.M., Crossey, P.A., Ferguson-Smith, M.A., Le Paslier, D., Chumakov, I., Cohen, D., Chinault, A.C., Maher, E.R., Linehan, W.M., Zbar, B. and Lerman, M.I. (1993) Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 260, 1317-1320.

Laureys, G., Speleman, P., Opdenakker, G., Benoit, Y. and Leroy, J. (1990) Constitutional translocation t(1;17)(p32;q12-21) in a patient with neuroblastoma. *Genes Chrom. Cancer* 2, 252-254.

- Le Beau, M.M., Westbrook, C.A., Diaz, M.O. and Rowley, J.D. (1985) c-src is consistently conserved in the chromosomal deletion (20q) observed in myeloid disorders. *Proc. Natl. Acad. Sci. USA* **82**, 6692-6696.
- Lee, W.-H., Bookstein, R., Hong, F., Young, L.-J., Shew, J.-Y. and Lee, E.Y.-H.P. (1987) Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science* **235**, 1394-1399.
- Leister, I., Weith, A., Brüderlein, S., Cziepluch, C., Kangwanpong, D., Schlag, P. and Schwab, M. (1990) Human colorectal cancer: high frequency of deletions at chromosome 1p35. *Cancer Res.* **50**, 7232-7235.
- Levine, A.J. (1993) The tumor suppressor genes. *Ann. Rev. Biochem.* **62**, 623-651.
- Lewis, T., Saunders, I.W. and Westcott, M. (1984) The moments of the Pearson chi-squared statistic and the minimum expected value in two-way tables. *Biometrika* **71**, 515-522.
- Lichter, P. and Ward, D.C. (1990) Is non-isotopic *in situ* hybridization finally coming of age? *Nature* **345**, 93-94.
- Lindblom, A., Skoog, L., Andersen, T.I., Rotstein, S., Nordenskjöld, M. and Larsson, C. (1993) Four separate regions on chromosome 17 show loss of heterozygosity in familial breast carcinomas. *Hum. Genet.* **91**, 6-12.
- Linnenbach, A.J., Pressler, L.B., Seng, B.A., Kimmel, B.S., Tomaszewski, J.E. and Malkowicz, S.B. (1993) Characterization of chromosome 9 deletions in transitional cell carcinoma by microsatellite assay. *Hum. Mol. Genet.* **2**, 1407-1411.
- Linnenbach, A.J., Speicher, D.W., Marchesi, V.T. and Forget, B.G. (1986) Cloning of a portion of the chromosomal gene for human erythrocyte α -spectrin by using a synthetic gene fragment. *Proc. Natl. Acad. Sci. USA* **83**, 2397-2401.
- Litt, M. and Luty, J.A. (1989) A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am. J. Hum. Genet.* **44**, 397-401.
- Lobaccaro, J.-M., Lumbroso, S., Belon, C., Galtier-Dereure, F., Bringer, J., Lesimple, T., Heron, J.-F., Pujol, H. and Sultan, C. (1993a) Male breast cancer and the androgen receptor gene. *Nat. Genet.* **5**, 109-110.
- Lobaccaro, J.-M., Lumbroso, S., Belon, C., Galtier-Dereure, F., Bringer, J., Lesimple, T., Namer, M., Cutuli, B.F., Pujol, H. and Sultan, C. (1993b) Androgen receptor gene mutation in male breast cancer. *Hum. Mol. Genet.* **2**, 1799-1802.
- Looijenga, L.H.J., Gillis, A.J.M., Van Putten, W.L.J. and Oosterhuis, J.W. (1993) *In situ* numeric analysis of centromeric regions of chromosomes 1, 12, and 15 of seminomas, nonseminomatous germ cell tumors, and carcinoma *in situ* of human testis. *Lab. Invest.* **68**, 211-219.
- Louis, D.N., von Deimling, A. and Seizinger, B.R. (1992) A (CA)_n dinucleotide repeat assay for evaluating loss of allelic heterozygosity in small and archival human brain tumor specimens. *Am. J. Pathol.* **141**, 777-782.
- Lundberg, C., Skoog, L., Cavenee, W.K. and Nordenskjöld, M. (1987) Loss of heterozygosity in human ductal breast tumors indicates a recessive mutation on chromosome 13. *Proc. Natl. Acad. Sci. USA* **84**, 2372-2376.
- Lynch, H.T., Kaplan, A.R. and Lynch, J.F. (1974) Klinefelter syndrome and cancer. A family study. *J.A.M.A* **229**, 809-811.
- Lynch, H.T., Kimberling, W., Albano, W.A., Lynch, J.F., Biscone, K., Schuelke, G.S., Sandberg, A.A., Lipkin, M., Deschner, E.E., Mikol, Y.B., Elston, R.C., Bailey-Wilson, J.E. and Danes, B.S.

(1985a) Hereditary nonpolyposis colorectal cancer (Lynch syndromes I and II). I. Clinical description of resource. *Cancer* 56, 934-938.

Lynch, H.T., Schuelke, G.S., Kimberling, W.J., Albano, W.A., Lynch, J.F., Biscione, K.A., Lipkin, M.L., Deschner, E.E., Mikol, Y.B., Sandberg, A.A., Elston, R.C., Bailey-Wilson, J.E. and Danes, B.S. (1985b) Hereditary nonpolyposis colorectal cancer (Lynch syndromes I and II). II. Biomarker studies. *Cancer* 56, 934-938.

Lynch, H.T., Watson, P., Krieglner, M., Lynch, J.F., Lanspa, S.J., Marcus, J., Smyrk, T., Fitzgibbons, R.J. and Cristofaro, G. (1988) Differential diagnosis of hereditary nonpolyposis colorectal cancer (Lynch syndrome I and Lynch syndrome II). *Dis. Colon Rectum* 31, 372-377.

Lyon, M.F. (1961) Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature* 190, 372-373.

Lyon, M.F. (1993) Epigenetic inheritance in mammals. *Trend. Genet.* 9, 123-128.

M

Mackay, J., Elder, P.A., Porteous, D.J., Steel, C.M., Hawkins, R.A., Going, J.J. and Chetty, U. (1988a) Partial deletion of chromosome 11p in breast cancer correlates with size of primary tumour and oestrogen receptor level. *Br. J. Cancer* 58, 710-714.

Mackay, J., Steel, C.M., Elder, P.A., Forrest, A.P.M. and Evans, H.J. (1988b) Allele loss on short arm of chromosome 17 in breast cancers. *Lancet* ii, 1384-1385.

Mandel, M. and Higa, A. (1970) Calcium-dependent bacteriophage DNA infection. *J. Mol. Biol.* 53, 159-162.

Mars, W.M., Genuardi, M., Tsihira, H., Anderson, D.E. and Saunders, G.F. (1991) Genomic changes on the short arm of human chromosome 1 in breast cancer. *Cancer Detect. Preven.* 15, 145-149.

Mars, W.M. and Saunders, G.F. (1990) Chromosomal abnormalities in human breast cancer. *Cancer Metast. Rev.* 9, 35-43.

Marshall, C. 1991 Tumor suppressor genes. *Cell* 64, 313-326.

Mathew, C.G.P., Smith, B.A., Thorpe, K., Wong, Z., Royle, N.J., Jeffreys, A.J. and Ponder, B.A.J. (1987) Deletion of genes on chromosome 1 in endocrine neoplasia. *Nature* 328, 524-526.

Matsumura, K., Kallioniemi, A., Kallioniemi, O., Chen, L., Smith, H.S., Pinkel, D., Gray, J. and Waldman, F.M. (1992) Deletion of chromosome 17p loci in breast cancer cells detected by fluorescence *in situ* hybridization. *Cancer Res.* 52, 3474-3477.

McDonnell, M.W., Simon, M.N. and Studier, F.W. (1977) Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. *J. Mol. Biol.* 110, 119-146.

McGuire, W.L. and Dressler, L.G. (1985) Emerging impact of flow cytometry in predicting recurrence and survival in breast cancer patients. *J. Natl. Can. Inst.* 75, 405-410.

Meloni, A.M., Dobbs, R.M., Pontes, J.E. and Sandberg, A.A. (1993) Translocation (X;1) in papillary renal cell carcinoma. A new cytogenetic subtype. *Cancer Genet. Cytogenet.* 65, 1-6.

Meloni, A.M., Sandberg, A.A., Pontes, J.E. and Dobbs, R.M. (1992) Translocation (X;1)(p11.2;q21). A subtype of renal adenocarcinomas. *Cancer Genet. Cytogenet.* 63, 100-101.

Meltzer, P.S., Guan, X.-Y. and Trent, J.M. (1993) Telomere capture stabilizes chromosome breakage. *Nat. Genet.* 4, 252-255.

Merlo, G.R., Siddiqui, J., Cropp, C.S., Liscia, D.S., Lidereau, R., Callahan, R. and Kufe, D.W. (1989) Frequent alteration of the *DF3* tumor-associated antigen gene in primary human breast carcinomas. *Cancer Res.* 49, 6966-6971.

Merlo, G.R., Venesio, T., Bernardi, A., Canale, L., Gaglia, P., Lauro, D., Cappa, A.P.M., Callahan, R. and Liscia, D.S. (1992) Loss of heterozygosity on chromosome 17p13 in breast carcinomas identifies tumors with high proliferation index. *Am. J. Pathol.* 140, 215-223.

Miller, D.A., Okamoto, E., Erlanger, B.F. and Miller, O.J. (1982) Is DNA methylation responsible for mammalian X chromosome inactivation? *Cytogenet. Cell Genet.* 33, 345-349.

Mitchell, E.L.D. and Santibanez-Koref, M.F. (1990) 1p13 is the most frequently involved band in structural chromosomal rearrangements in human breast cancer. *Genes Chrom. Cancer* 2, 278-289.

Mitelman, F. and Heim, S. (1988) Consistent involvement of only 71 of the 329 chromosomal bands of the human genome in primary neoplasia-associated rearrangements. *Cancer Res.* 48, 7115-7119.

Mitelman, F., Kaneko, Y. and Berger, R. (1993) Report of the committee on chromosome changes in neoplasia. In: *Chromosome Coordinating Meeting 1992*. Ed. Cuticchia, A.J. and Pearson, P.L. 700-726. Karger. Basel.

Mitelman, F., Kaneko, Y. and Trent, J. (1991) Report of the committee on chromosome changes in neoplasia. *Cytogenet. Cell Genet.* 58, 1053-1079.

Miyoshi, J., Kagimoto, M., Soeda, E.-I. and Sakaki, Y. (1984) The human c-Ha-ras2 is a processed pseudogene inactivated by numerous base substitutions. *Nucl. Acids Res.* 12, 1821-1828.

Mohandas, T., Sparkes, R.S. and Shapiro, L.J. (1981) Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. *Science* 211, 393-396.

Moley, J.F., Brother, M.B., Fong, C.-T., White, P.S., Baylin, S.B., Nelkin, B., Wells, S.A. and Brodeur, G.M. (1992) Consistent association of 1p loss of heterozygosity with pheochromocytomas from patients with multiple endocrine neoplasia type 2 syndromes. *Cancer Res.* 52, 770-774.

Morris, C.M., Honeybone, L.M., Hollings, P.E. and Fitzgerald, P.H. (1989) Localization of the *SRC* oncogene to chromosome band 20q11.2 and loss of this gene with deletion (20q) in two leukemic patients. *Blood* 74, 1768-1773.

Moyzis, R.K., Albright, K.L., Bartholdi, M.F., Cram, L.S., Deaven, L.L., Hildebrand, C.E., Joste, N.E., Longmire, J.L., Meyne and Schwarzscher-Robinson, T. (1987) Human chromosome-specific repetitive DNA sequences: novel markers for genetic analysis. *Chromosoma* 95, 375-386.

Mukerjee, D., Bowen, J. and Anderson, D.E. (1970) Simian papovavirus 40 transformation of cells from cancer patient with XY/XXY mosaic Klinefelter's syndrome. *Cancer Res.* 30, 1769-1772.

Mulligan, L.M., Gardner, E., Smith, B.A., Mathew, C.G.P. and Ponder, B.A.J. (1993a) Genetic events in tumour initiation and progression in multiple endocrine neoplasia type 2. *Genes Chrom. Cancer* 6, 166-177.

Mulligan, L.M., Kwok, J.B.J., Healey, C.S., Elsdon, M.J., Eng, C., Gardner, E., Love, D.R., Mole, S.E., Moore, J.K., Papi, L., Ponder, M.A., Telenius, H., Tunnacliffe, A. and Ponder, B.A.J. (1993b) Germ-line mutations of the *RET* proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* 363, 458-460.

Murnane, J.P. and Yu, L.-C. (1993) Acquisition of telomere repeat sequences by transfected DNA integrated at the site of a chromosome break. *Mol. Cell. Biol.* 13, 977-983.

Murray, J.M., Davies, K.E., Harper, P.S., Meredith, L., Mueller, C.R. and Williamson, R. (1982) Linkage relationship of a cloned DNA sequence on the short arm of the X chromosome to Duchenne muscular dystrophy. *Nature* 300, 69-71.

N

- Nakamura, Y. and White, R. (1988) Isolation and mapping of a polymorphic DNA sequence (cYNA13) on chromosome 1p [D1S74]. *Nucl. Acids Res.* **16**, 9369.
- Nakamura, Y., Carlson, M., Krapcho, K., Kanamori, M. and White, R. (1988a) New approach for isolation of VNTR markers. *Am. J. Hum. Genet.* **43**, 854-859.
- Nakamura, Y., Carlson, M., Krapcho, K. and White, R. (1988b) Isolation and mapping of a polymorphic DNA sequence (pMCT118) on chromosome 1p [D1S80]. *Nucl. Acids Res.* **16**, 9364.
- Nakamura, Y., Carlson, M., Krapcho, K. and White, R. (1988c) Isolation and mapping of a polymorphic DNA sequence (pMCT58) on chromosome 1p [D1S77]. *Nucl. Acids Res.* **16**, 9367.
- Nakamura, Y., Culver, M., Gillilan, S., O'Connell, P., Leppert, M., Lathrop, G.M., Lalouel, J.-M. and White, R. (1987a) Isolation and mapping of a polymorphic DNA sequence pYNZ23 on chromosome 1q (D1S58). *Nucl. Acids Res.* **15**, 9620.
- Nakamura, Y., Culver, M., Sergeant, L., Leppert, M., O'Connell, P., Lathrop, G.M., Lalouel, J.-M. and White, R. (1988d) Isolation and mapping of a polymorphic DNA sequence (pYNZ2) on chromosome 1p [D1S57]. *Nucl. Acids Res.* **16**, 4747.
- Nakamura, Y., Gillilan, S., O'Connell, P., Leppert, M., Lathrop, G.M., Lalouel, J.-M. and White, R. (1987b) Isolation and mapping of a polymorphic DNA sequence pYNH24 on chromosome 2 (D2S44). *Nucl. Acids Res.* **15**, 10073.
- Nakamura, Y., Holm, T. and White, R. (1988e) Isolation and mapping of a polymorphic DNA sequence (pTHH33) on chromosome 1q [D1S81]. *Nucl. Acids Res.* **16**, 9363.
- Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fujimoto, E., Hoff, M., Kumlin, E. and White, R. (1987c) Variable number of tandem repeat (VNTR) markers for human gene mapping. *Science* **235**, 1616-1622.
- Nakamura, Y., Martin, C., Myers, R. and White, R. (1988f) Isolation and mapping of a polymorphic DNA sequence (pCMM12) on chromosome 1p [D1S76]. *Nucl. Acids Res.* **16**, 9368.
- Narod, S.A., Feunteun, J., Lynch, H.T., Watson, P., Conway, T., Lynch, J. and Lenoir, G.M. (1991) Familial breast-ovarian cancer locus on chromosome 17q12-q23. *Lancet* **338**, 82-83.
- Neave, H.R. (1989) *Elementary statistics tables*. Unwin Hyman. London.
- Nederlof, P.M., van der Flier, S., Raap, A.K., Tanke, H.J., van der Ploeg, M., Kornips, F. and Geraedts, J.P.M. (1989) Detection of chromosome aberrations in interphase tumor nuclei by nonradioactive in situ hybridization. *Cancer Genet. Cytogenet.* **42**, 87-98.
- Nielsen, K.V. and Briand, P. (1989) Cytogenetic analysis of in vitro karyotype evolution in a cell line established from nonmalignant human mammary epithelium. *Cancer Genet. Cytogenet.* **39**, 103-118.
- Noguchi, T., Galland, F., Batoz, M., Mattei, M.-G. and Birnbaum, D. (1988) Activation of a *mcf.2* oncogene by deletion of amino-terminal coding sequences. *Oncogene* **3**, 709-715.
- Noguchi, T., Mattei, M.-G., Oberlè, I., Planche, J., Imbert, J., Pelassy, C., Birg, F. and Birnbaum, D. (1987) Localization of the *mcf.2* transforming sequence to the X chromosome. *EMBO J.* **6**, 1301-1307.

O

O'Connell, P., Lathrop, G.M., Nakamura, Y., Leppert, M.L., Ardinger, R.H., Murray, J.L., Lalouel, J.-M. and White, R. (1989) Twenty-eight loci form a continuous linkage map of markers for human chromosome 1. *Genomics* 4, 12-20.

Oberlé, I., Drayna, D., Camerino, G., White, R. and Mandel, J.L. (1985) The telomeric region of the X chromosome long arm: presence of a highly polymorphic DNA marker and analysis of recombination frequency. *Proc. Natl. Acad. Sci. USA* 82, 2824-2828.

Ohno, S. and Makino, S. (1961) The single-X nature of sex chromatin in man. *Lancet* i, 78-79.

Oka, K., Ishikawa, J., Bruner, J.M., Takahashi, R. and Saya, H. (1991) Detection of loss of heterozygosity in the *p53* gene in renal cell carcinoma and bladder cancer using the polymerase chain reaction. *Mol. Carcinogen.* 4, 10-13.

Osborne, C.K., Yochmowitz, M.G., Knight, W.A. and McGuire, W.L. (1980) The value of estrogen and progesterone receptors in the treatment of breast cancer. *Cancer* 46, 2884-2888.

Oshimura, M., Kugoh, H.M., Shimizu, M., Yamada, H., Hashiba, H., Horikawa, I. and Sasaki, M. (1990) Multiple chromosomes carrying tumor suppressor activity, *via* microcell-mediated chromosome transfer, for various tumor cell lines. In: *Genetic basis for carcinogenesis: tumor suppressor genes and oncogenes*. Eds. Knudson, A.G., Stanbridge, E.J., Sugimura, T., Terada, M. and Watanabe, S. 249-257. Taylor and Francis Ltd. London.

Owainati, A.A.R., Robins, R.A., Hinton, C., Ellis, I.O., Dowle, C.S., Ferry, B., Elston, C.W., Blamey, R.W. and Baldwin, R.W. (1987) Tumour aneuploidy, prognostic parameters and survival in primary breast cancer. *Br. J. Cancer* 55, 449-454.

P

Page, D.C., Bieker, K., Brown, L.G., Hinton, S., Leppert, M., Lalouel, J.-M., Lathrop, M., Nystrom-Lahti, M., de la Chapelle, A. and White, R. (1987) Linkage, physical mapping, and DNA sequence analysis of pseudoautosomal loci on the human X and Y chromosomes. *Genomics* 1, 243-256.

Pandis, N., Heim, S., Bardi, G., Idvall, I., Mandahl, N. and Mitelman, F. (1992) Whole-arm t(1;16) and i(1q) as sole anomalies identify gain of 1q as a primary chromosomal abnormality in breast cancer. *Genes Chrom. Cancer* 5, 235-238.

Pandis, N., Heim, S., Bardi, G., Idvall, I., Mandahl, N. and Mitelman, F. (1993) Chromosome analysis of 20 breast carcinomas: cytogenetic multiclonality and karyotypic-pathologic correlations. *Genes Chrom. Cancer* 6, 51-57.

Paraskeva, C., Finerty, S. and Powell, S. (1988) immortalization of a human colorectal adenoma cell line by continuous *in vitro* passage: possible involvement of chromosome 1 in tumour progression. *Int. J. Cancer* 41, 908-912.

Paraskeva, C., Hague, A., Rooney, N., Williams, A.C., Harper, S.J., Hanlon, K.A., Atkinson, R.J. and Corfield, A.P. (1992) A single human colonic adenoma cell line can be converted *in vitro* to both a colorectal adenocarcinoma and a mucinous carcinoma. *Int. J. Cancer* 51, 661-664.

Paraskeva, C., Harvey, A., Finerty, S. and Powell, S. (1989) Possible involvement of chromosome 1 in *in vitro* immortalization: evidence from progression of a human adenoma-derived cell line *in vitro*. *Int. J. Cancer* 43, 743-746.

Pathak, S. (1980) Cytogenetic analysis in human breast tumors. *Cancer Genet. Cytogenet.* 1, 281-289.

Pathak, S., Hopwood, V.L., Hortobagyi, G.N., Jackson, G.L., Hughes, J.I. and Melillo, D. (1991) Chromosome anomalies in human breast cancer: evidence for specific involvement of 1q region in lymphocyte cultures. *Anticancer Res.* **11**, 1055-1060.

Pathak, S., Siciliano, M.J., Cailleau, R., Wiseman, C.L. and Hsu, T.C. (1979) A human breast adenocarcinoma with chromosome and isoenzyme markers similar to those of the HeLa line. *J. Natl. Can. Inst.* **62**, 263-271.

Peinado, M.A., Malkhosyan, S., Velazquez, A. and Perusho, M. (1992) Isolation and characterization of allelic losses and gains in colorectal tumors by arbitrarily primed polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **89**, 10065-10069.

Peltomäki, P., Aaltonen, L.A., Sistonen, P., Pylkkänen, L., Mecklin, J.-P., Järvinen, H., Green, J.S., Jass, J.R., Weber, J.L., Leach, F.S., Petersen, G.M., Hamilton, S.R., de la Chapelle, A. and Vogelstein, B. (1993) Genetic mapping of a locus predisposing to human colorectal cancer. *Science* **260**, 810-812.

Peltomäki, P., Lothe, R., Børresen, A.-L., Fosså, S.D., Brøgger, A. and de la Chapelle, A. (1991) Altered dosage of the sex chromosomes in human testicular cancer: a molecular genetic study. *Int. J. Cancer* **47**, 518-522.

Peter, M., Michon, J., Vielh, P., Neuenschwander, S., Nakamura, Y., Sonsino, E., Zucker, J.-M., Vergnaud, G., Thomas, G. and Delattre, O. (1992) PCR assay for chromosome 1p deletion in small neuroblastoma samples. *Int. J. Cancer* **52**, 544-548.

Petit, C., Levilliers, J., Rouyer, F., Simmler, M.C., Nakahori, Y., Nakagome, Y. and Weissenbach, J. (1989) A physical map of the terminal part of the short arm of the X chromosome. *Cytogenet. Cell Genet.* **51**, 1059.

Phillips, N., Ziegler, M., Saha, B. and Xynos, F. (1993) Allelic loss on chromosome 17 in human ovarian cancer. *Int. J. Cancer* **54**, 85-91.

Pinkel, D., Straume, T. and Gray, J.W. (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. *Proc. Natl. Acad. Sci. USA* **83**, 2934-2938.

Ponder, B. (1988) Gene losses in human tumours. *Nature* **335**, 400-402.

Ponder, B.A.J. 1990 *Inherited predisposition to cancer*. **6**, 213-218 Elsevier Science.

Pringle, J.H., Homer, C.E., Warford, A., Kendall, C.H. and Lauder, I. (1987) *In situ* hybridization: alkaline phosphatase visualization of biotinylated probes in cryostat and paraffin sections. *Histochem. J.* **19**, 488-496.

R

Rao, V.N., Huebner, K., Isobe, M., ar-Rushdi, A., Croce, C.M. and Reddy, E.S.P. (1989) *elk*, tissue-specific *ets*-related genes on chromosomes X and 14 near translocation breakpoints. *Science* **244**, 66-70.

Rappold, G.A. and Lehrach, H. (1988) A long range map of the pseudoautosomal region by partial digest PFGE analysis from the telomere. *Nucl. Acids Res.* **16**, 5361-5377.

Richards, F.M., Maher, E.R., Latif, F., Phipps, M.E., Tory, K., Lush, M., Crossey, P.A., Oostra, B., Gustavson, K.H., Green, J., Turner, G., Yates, J.R.W., Linehan, W.M., Affara, N.A., Lerman, M., Zbar, B. and Ferguson-Smith, M.A. (1993) Detailed genetic mapping of the von Hippel-Lindau disease tumour suppressor gene. *J. Med. Genet.* **30**, 104-107.

Rocchi, M., Baldini, A., Archidiacono, N., Lainala, S., Miller, O.J. and Miller, D.A. (1990) Chromosome-specific subsets of human aliphoid DNA identified by a chromosome 2-derived clone. *Genomics* 8, 705-709.

Rodgers, C.S., Hill, S.M. and Hultén, M.A. (1984) Cytogenetic analysis of human breast carcinoma. I. Nine cases in the diploid range investigated using direct preparations. *Cancer Genet. Cytogenet.* 13, 95-119.

Roelofs, H., Schuurin, E., Wiegant, J., Michalides, R. and Giphart-Gassler, M. (1993) Amplification of the 11q13 region in human carcinoma cell lines: a mechanistic view. *Genes Chrom. Cancer* 7, 74-84.

Ron, D., Tronick, S.R., Aaronson, S.A. and Eva, A. (1988) Molecular cloning and characterization of the human *dbl* proto-oncogene: evidence that its overexpression is sufficient to transform NIH/3T3 cells. *EMBO J.* 7, 2465-2473.

Rose, E.A., Glaser, T., Jones, C., Smith, C.L., Lewis, W.H., Call, K.M., Minden, M., Champagne, E., Bonetta, L., Yeger, H. and Housman, D.E. (1990) Complete physical map of the WAGR region of 11p13 localizes a candidate Wilms' tumor gene. *Cell* 60, 495-508.

Rouyer, F., Simmler, M.-C., Johnsson, C., Vergnaud, G., Cooke, H.J. and Weissenbach, J. (1986) A gradient of sex linkage in the pseudoautosomal region of the human sex chromosomes. *Nature* 319, 291-295.

Rowley, J.D. (1984) Biological implications of consistent chromosome rearrangements in leukemia and lymphoma. *Cancer Res.* 44, 3159-3168.

Rowley, J.D. (1990) Molecular cytogenetics: Rosetta Stone for understanding cancer. *Cancer Res.* 50, 3816-3825.

Royle, N.J., Armour, J.A.L., Crosier, M. and Jeffreys, A.J. (1993) Abnormal segregation of alleles in CEPH pedigree DNAs arising from allele loss in lymphoblastoid DNA. *Genomics* 15, 119-122.

S

Sager, R. (1989) Tumor suppressor genes: the puzzle and the promise. *Science* 246, 1406-1412.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning. A laboratory manual.* 2nd edition. Cold Spring Harbor Press. New York.

Sandberg, A.A. (1983) The X chromosome in human neoplasia, including sex chromatin and congenital conditions with X-chromosome anomalies. In: *Cytogenetics of the mammalian X chromosome*, B. Ed. Sandberg, A.A. 459-498. Alan R. Liss. New York.

Sandberg, A.A., Cohen, M.M., Rimm, A.A. and Levin, M.L. (1967) Aneuploidy and age in a population survey. *Am. J. Hum. Genet.* 19, 633-643.

Sandberg, A.A., Turc-Carel, C. and Gemmill, R.M. (1988) Chromosomes in solid tumors and beyond. *Cancer Res.* 48, 1049-1059.

Sano, T., Tsujino, T., Yoshida, K., Nakayama, H., Haruma, K., Ito, H., Nakamura, Y., Kajiyama, G. and Tahara, E. (1991) Frequent loss of heterozygosity on chromosomes 1q, 5q, and 17p in human gastric carcinomas. *Cancer Res.* 51, 2926-2931.

Sasaki, M., Okamoto, M., Sato, C., Sugio, K., Soejima, J.-I., Iwama, T., Ikeuchi, T., Tonomura, A., Miyaki, M. and Sasazuki, T. (1989) Loss of constitutional heterozygosity in colorectal tumors from patients with familial polyposis coli and those with nonpolyposis colorectal carcinoma. *Cancer Res.* 49, 4402-4406.

Sasi, R., Hoo, J.J., Samuel, I.P., Tainaka, T., Shiferaw, S. and Lin, C.C. (1991) Chromosome aberrations and oncogene alterations in two new breast tumor cell lines. *Cancer Genet. Cytogenet.* **51**, 239-254.

Sato, T., Akiyama, F., Sakamoto, G., Kasumi, F. and Nakamura, Y. (1991a) Accumulation of genetic alterations and progression of primary breast cancer. *Cancer Res.* **51**, 5794-5799.

Sato, T., Saito, H., Morita, R., Koi, S., Lee, J.H. and Nakamura, Y. (1991b) Allelotype of human ovarian cancer. *Cancer Res.* **51**, 5118-5122.

Sato, T., Tanigami, A., Yamakawa, K., Akiyama, F., Kasumi, F., Sakamoto, G. and Nakamura, Y. (1990) Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. *Cancer Res.* **50**, 7184-7189.

Satya-Prakash, K.L., Pathak, S., Hsu, T.C., Olivé, M. and Cailleau, R. (1981) Cytogenetic analysis on eight human breast tumor cell lines: high frequencies of 1q, 11q and HeLa-like marker chromosomes. *Cancer Genet. Cytogenet.* **3**, 61-73.

Saunders, P.T.K., Padayachi, T., Tincello, D.G., Shalet, S.M. and Wu, F.C.W. (1992) Point mutations detected in the androgen receptor gene of three men with partial androgen insensitivity syndrome. *Clin. Endocrinol.* **37**, 214-220.

Savino, A. and Koss, L.G. (1971) The evaluation of sex chromatin as a prognostic factor in carcinoma of the breast. *Acta Cytol.* **15**, 372-374.

Scharf, S.J., Bowcock, A.M., McClure, G., Klitz, W., Yandell, D.W. and Erlich, H.A. (1992) Amplification and characterization of the retinoblastoma gene VNTR by PCR. *Am. J. Hum. Genet.* **50**, 371-381.

Schiebel, K., Weiss, B., Wöhrle, D. and Rappold, G. (1993) A human pseudoautosomal gene, ADP/ATP translocase, escapes X-inactivation whereas a homologue on Xq is subject to X-inactivation. *Nat. Genet.* **3**, 82-87.

Schlessinger, D., Willard, H.F. and Mandel, J.-L. (1993) Report of the fourth international workshop on human X chromosome mapping 1993. *Cytogenet. Cell Genet.* **64**, 147-194.

Schmidt, M.E., Miller, W.V., van Peenan, H.J. and Lucas, F.V. (1966) Changes in sex chromatin pattern during the menstrual cycle. *Am. J. Obstet. Gynecol.* **94**, 422-424.

Seizinger, B.R., Klinger, H.P., Junien, C., Nakamura, Y., Le Beau, M., Cavenee, W., Emanuel, B., Ponder, B., Naylor, S., Mitelman, F., Louis, D., Menon, A., Newsham, I., Decker, J., Kaelbling, M., Henry, I. and von Deimling, A. (1991) Report of the committee on chromosome and gene loss in human neoplasia. *Cytogenet. Cell Genet.* **58**, 1080-1096.

Shimizu, M., Yokota, J., Mori, N., Shuin, T., Shinoda, M., Terada, M. and Oshimura, M. (1990) Introduction of normal chromosome 3p modulates the tumorigenicity of a human renal cell carcinoma cell line YCR. *Oncogene* **5**, 185-194.

Simmler, M.C., Johnsson, C., Petit, C., Rouyer, F., Vergnaud, G. and Weissenbach, J. (1987) Two highly polymorphic minisatellites from the pseudoautosomal region of the human sex chromosomes. *EMBO J.* **6**, 963-969.

Simmler, M.-C., Rouyer, F., Vergnaud, G., Nyström-Lahti, M., Yen Ngo, K., de la Chapelle, A. and Weissenbach, J. (1985) Pseudoautosomal DNA sequences in the pairing region of the human sex chromosomes. *Nature* **317**, 692-697.

Slim, R., Levilliers, J., Lüdecke, H.-J., Claussen, U., Nguyen, V.C., Gough, N.M., Horsthemke, B. and Petit, C. (1993) A human pseudoautosomal gene encodes the ANT3 ADP/ATP translocase and escapes X-inactivation. *Genomics* **16**, 26-33.

- Smith, M.J., Goodfellow, P.J. and Goodfellow, P.N. (1993) The genomic organisation of the human pseudoautosomal gene *MIC2* and the detection of a related locus. *Hum. Mol. Genet.* **2**, 417-422.
- Smith, S.A., Easton, D.F., Evans, D.G.R. and Ponder, B.A.J. (1992) Allele losses in the region 17q12-21 in familial breast and ovarian cancer involve the wild-type chromosome. *Nat. Genet.* **2**, 128-131.
- Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503-517.
- Sreekantaiah, C. and Sandberg, A.A. (1991) Clustering of aberrations to specific chromosome regions in benign neoplasms. *Int. J. Cancer* **48**, 194-198.
- Stallings, R.L., Ford, A.F., Nelson, D., Torney, D.C., Hildebrand, C.E. and Moyzis, R.K. (1991) Evolution and distribution of (GT)_n repetitive sequences in mammalian genomes. *Genomics* **10**, 807-815.
- Stanbridge, E.J. (1976) Suppression of malignancy in human cells. *Nature* **260**, 17-20.
- Stanbridge, E.J. (1990) Human tumor suppressor genes. *Ann. Rev. Genet.* **24**, 615-657.
- Steiner, M.G., Harlow, S.P., Colombo, E. and Bauer, K.D. (1993) Chromosomes 8, 12, and 17 copy number in Astler-Coller stage C colon cancer in relation to proliferative activity and DNA ploidy. *Cancer Res.* **53**, 681-686.
- Stoler, A. and Bouck, N. (1985) Identification of a single chromosome in the normal human genome essential for suppression of hamster cell transformation. *Proc. Natl. Acad. Sci. USA* **82**, 570-574.
- Straub, D.C., Lucas, L.A., McMahon, N.J., Pellet, O.L. and Teplitz, R.L. (1969) Apparent reversal of X-condensation mechanism in tumors of the female. *Cancer Res.* **29**, 1233-1243.
- Sugawara, O., Oshimura, M., Koi, M., Annab, L.A. and Barrett, J.C. (1990) Induction of cellular senescence in immortalized cells by human chromosome 1. *Science* **247**, 707-710.
- Sundaresan, V., Ganly, P., Hasleton, P., Bleehen, N.M. and Rabbitts, P. (1993) Paraffin wax-embedded material as a source of DNA for the detection of somatic genetic changes. *J. Pathol.* **169**, 43-52.

T

- T'Ang, A., Varley, J.M., Chakraborty, S., Murphree, A.L. and Fung, Y.-K.T. (1988) Structural rearrangement of the retinoblastoma gene in human breast carcinoma. *Science* **242**, 263-266.
- Takayama, H., Suzuki, T., Mugishima, H., Fujisawa, T., Ookuni, M., Schwab, M., Gehring, M., Nakamura, Y., Sugimura, T., Terada, M. and Yokota, J. (1992) Deletion mapping of chromosome 14q and 1p in human neuroblastoma. *Oncogene* **7**, 1185-1189.
- Tamura, H.-O., Kohchi, C., Yamada, R., Ikeda, T., Koiwai, O., Patterson, E., Keene, J.D., Okada, K., Kjeldsen, E., Nishikawa, K. and Andoh, T. (1991) Molecular cloning of a cDNA of a camptothecin-resistant human DNA topoisomerase I and identification of mutation sites. *Nucl. Acids Res* **19**, 69-75.
- Tanaka, K. and Testa, J.R. (1987) Assessment of methods for the cytogenetic analysis of human solid tumors. *J. Natl. Can. Inst.* **79**, 1287-1293.
- Tanaka, K., Yanoshita, R., Konishi, M., Oshimura, M., Maeda, Y., Mori, T. and Miyaki, M. (1993) Suppression of tumorigenicity in human colon carcinoma cells by introduction of normal chromosome 1p36 region. *Oncogene* **8**, 2253-2258.

Teyssier, J.R. (1989) The chromosomal analysis of human solid tumors. A triple challenge. *Cancer Genet. Cytogenet.* 37, 103-125.

Theillet, C., Lidereau, R., Escot, C., Hutzell, P., Brunet, M., Gest, J., Schlom, J. and Callahan, R. (1986) Loss of a c-H-ras-1 allele and aggressive human primary breast carcinomas. *Cancer Res.* 46, 4776-4781.

Thibodeau, S.N., Bren, G. and Schaid, D. (1993) Microsatellite instability in cancer of the proximal colon. *Science* 260, 816-819.

Thompson, A.M., Steel, C.M., Chetty, U., Hawkins, R.A., Miller, W.R., Carter, D.C., Forrest, A.P.M. and Evans, H.J. (1990) p53 gene mRNA expression and chromosome 17p allele loss in breast cancer. *Br. J. Cancer* 61, 74-78.

Tlsty, T.D. 1990 Normal diploid human and rodent cells lack a detectable frequency of gene amplification. *Proc. Natl. Acad. Sci. USA* 87, 3132-3136.

Tlsty, T.D., Margolin, B. and Lum, K. 1989 Differences in the rates of gene amplification in nontumorigenic and tumorigenic cell lines as measured by Luria-Delbrueck fluctuation analysis. *Proc. Natl. Acad. Sci. USA* 86, 9441-9445.

Tlsty, T.D., White, A. and Sanchez, J. 1992 Suppression of gene amplification in human cell hybrids. *Science* 255, 1425-1427.

Tomlinson, G.E., Nisen, P.D., Timmons, C.F. and Schneider, N.R. (1991) Cytogenetics of a renal cell carcinoma in a 17-month-old child. Evidence for Xp11.2 as a recurring breakpoint. *Cancer Genet. Cytogenet.* 57, 11-17.

Tronick, S.R., McBride, O.W., Popescu, N.C. and Eva, A. (1989) Chromosomal location of DBL oncogene sequences. *Genomics* 5, 546-553.

Tsai, Y.C., Nichols, P.W., Hiti, A.L., Williams, Z., Skinner, D.G. and Jones, P.A. (1990) Allelic losses of chromosomes 9, 11, and 17 in human bladder cancer. *Cancer Res.* 50, 44-47.

Tsuchiya, E., Nakamura, Y., Weng, S.-Y., Nakagawa, K., Tsuchiya, S., Sugano, H. and Kitagawa, T. (1992) Allelotype of non-small cell lung carcinoma - comparison between loss of heterozygosity in squamous cell carcinoma and adenocarcinoma. *Cancer Res.* 52, 2478-2481.

Tsutsumi, M., Yokota, J., Kakizoe, T., Koiso, K., Sugimura, T. and Terada, M. (1989) Loss of heterozygosity on chromosomes 1p and 11p in sporadic pheochromocytoma. *J. Natl. Can. Inst.* 81, 367-380.

Turc-Carel, C., Dal Cin, P., Limon, J., Rao, U., Li, F.P., Corson, J.M., Zimmerman, R., Parry, D.M., Cowan, J.M. and Sandberg, A.A. (1987) Involvement of chromosome X in primary cytogenetic change in human neoplasia: nonrandom translocation in synovial sarcoma. *Proc. Natl. Acad. Sci. USA* 84, 1981-1985.

V

van Dekken, H., Hagenbeek, A. and Bauman, J.G.J. (1989) Detection of host cells following sex-mismatched bone marrow transplantation by fluorescent in situ hybridization with a Y-chromosome specific probe. *Leukemia* 3, 724-728.

van Dekken, H., Kerstens, H.M.J., Tersteeg, T.A., Verhofstad, A.A.J. and Vooijs, G.P. (1992) Histological preservation after *in situ* hybridization to archival solid tumour sections allows discrimination of cells bearing numerical chromosome changes. *J. Pathol.* 168, 317-324.

Varley, J.M., Armour, J., Swallow, J.E., Jeffreys, A.J., Ponder, B.A.J., T'Ang, A., Fung, Y.-K.T., Brammar, W.J. and Walker, R.A. (1989) The retinoblastoma gene is frequently altered leading to loss of expression in primary breast tumours. *Oncogene* 4, 725-729.

Varley, J.M., Brammar, W.J., Lane, D.P., Swallow, J.E., Dolan, C. and Walker, R.A. (1991) Loss of chromosome 17p13 sequences and mutation of p53 in human breast carcinomas. *Oncogene* 6, 413-421.

Varley, J.M., Swallow, J.E., Brammar, W.J., Whittaker, J.L. and Walker, R.A. (1987) Alterations to either *c-erb-2 (neu)* or *c-myc* proto-oncogenes in breast carcinomas correlate with poor short-term prognosis. *Oncogene* 1, 423-430.

Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flintner, F., Hammarström, L., Kinnon, C., Levinsky, R., Bobrow, M., Smith, C.I.E. and Bentley, D.R. (1993) The gene involved in X-linked agammaglobulinaemia is a member of the *src* family of protein-tyrosine kinases. *Nature* 361, 226-233.

Viegas-Péquignot, E., Jeanpierre, M., Dutrillaux, A.M., Gerbault-Seureau, M., Muleris, M. and Dutrillaux, B. (1989) Detection of 1q polysomy in interphase nuclei of human solid tumors with a biotinylated probe. *Hum. Genet.* 81, 311-314.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M.M. and Bos, J.L. (1988) Genetic alterations during colorectal-tumor development. *New Engl. J. Med.* 319, 525-532.

Vogelstein, B., Fearon, E.R., Kern, S.E., Hamilton, S.R., Preisinger, A.C., Nakamura, Y. and White, R. (1989) Allelotype of colorectal carcinomas. *Science* 244, 207-211.

W

Wang, J.C., Passage, M.B., Ellison, J., Becker, M.A., Yen, P.H., Shapiro, L.J. and Mohandas, T.K. (1992) Physical mapping of loci in the distal half of the short arm of the human X chromosome: implications for the spreading of X-chromosome inactivation. *Somat. Cell Mol. Genet.* 18, 195-200.

Wang, N., Cedrone, E., Skuse, G.R., Insel, R. and Dry, J. (1990) Two identical active X chromosomes in human mammary carcinoma cells. *Cancer Genet. Cytogenet.* 46, 271-280.

Weber, J.L. and May, P.E. (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am. J. Hum. Genet.* 44, 388-396.

Weinberg, R.A. 1991 Tumor suppressor genes. *Science* 254, 1138-1146.

Weith, A., Martinsson, T., Cziepluch, C., Brüderlein, S., Amler, L.C., Berthold, F. and Schwab, M. (1989) Neuroblastoma consensus deletion maps to 1p36.1-2. *Genes Chrom. Cancer* 1, 159-166.

White, P.S., Kaufman, B.A., Marshall, H.N. and Brodeur, G.M. (1993) Use of the single-strand conformation polymorphism technique to detect loss of heterozygosity in neuroblastoma. *Genes Chrom. Cancer* 7, 102-108.

Wong, Z., Wilson, V., Patel, I., Povey, S. and Jeffreys, A.J. (1987) Characterization of a panel of highly variable minisatellites cloned from human DNA. *Ann. Hum. Genet.* 51, 269-288.

Wooster, R., Mangion, J., Eeles, R., Smith, S., Dowsett, M., Averill, D., Barrett-Lee, P., Easton, D.F., Ponder, B.A.J. and Stratton, M.R. (1992) A germline mutation in the androgen receptor gene in two brothers with breast cancer and Reifenshtein syndrome. *Nat. Genet.* 2, 132-134.

Y

Yamada, H., Horikawa, I., Hashiba, H. and Oshimura, M. (1990a) Normal human chromosome 1 carries suppressor activity for various phenotypes of a Kirsten murine sarcoma virus-transformed NIH/3T3 cell line. *Jpn. J. Cancer Res.* **81**, 1095-1100.

Yamada, H., Wake, N., Fujimoto, S.-I., Barrett, J.C. and Oshimura, M. (1990b) Multiple chromosomes carrying tumor suppressor activity for a uterine endometrial carcinoma cell line identified by microcell-mediated chromosome transfer. *Oncogene* **5**, 1141-1147.

Yang-Feng, T.L., Han, H., Chen, K.-C., Li, S.-B., Claus, E.B., Carcangiu, M.L., Chambers, S.K., Chambers, J.T. and Schwartz, P.E. (1993) Allelic loss in ovarian cancer. *Int. J. Cancer* **54**, 546-551.

Yang-Feng, T.L., Li, S., Han, H. and Schwartz, P.E. (1992) Frequent loss of heterozygosity on chromosomes Xp and 13q in human ovarian cancer. *Int. J. Cancer* **52**, 575-580.

Yokota, J., Akiyama, T., Fung, Y.-K.T., Benedict, W.F., Namba, Y., Hanaoka, M., Wada, M., Terasaki, T., Shimosato, Y., Sugimura, T. and Terada, M. (1988) Altered expression of the retinoblastoma (RB) gene in small-cell carcinoma of the lung. *Oncogene* **3**, 471-475.

Yokota, J., Mori, N., Akiyama, T., Shimosato, Y., Sugimura, T. and Terada, M. (1990) Multiple genetic alterations in small-cell lung carcinoma. In: *Genetic basis for carcinogenesis: tumor suppressor genes and oncogenes*. Eds. Knudson, A.G., Stanbridge, E.J., Sugimura, T., Terada, M. and Watanabe, S. 43-48. Taylor and Francis Ltd. London.

Yokota, J., Wada, M., Shimosato, Y., Terada, M. and Sugimura, T. (1987) Loss of heterozygosity on chromosomes 3, 13, and 17 in small-cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. *Proc. Natl. Acad. Sci. USA* **84**, 9252-9256.

Z

Zhang, R., Wiley, J., Howard, S.P., Meisner, L.F. and Gould, M.N. (1989) Rare clonal karyotypic variants in primary cultures of human breast carcinoma cells. *Cancer Res.* **49**, 444-449.