MOLECULAR GENETIC STUDIES OF SPORADIC PITUITARY ADENOMAS

By

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A thesis submitted for the degree of
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to the Faculty of Medicine,
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Abstract

Tumour formation may result from the activation of dominant oncogenes or by inactivation of recessive, tumour suppressor genes. The role of such mutations in the development of pituitary tumours has been studied. Tumours from 88 patients, representing the 4 major classes of pituitary adenoma, were investigated. In DNA extracted from matched leucocyte and tumour samples allelic deletions were sought with 15 probes identifying restriction fragment length polymorphisms on chromosomes 1, 5, 10, 11, 13, 17, 20 and 22. Evidence of amplification or re-arrangement of 10 recognised cellular oncogenes (NRAS, MYCL1, MYCN, MYC, HRAS, BCL1, HSTF1, SEA, KRAS2 and FOS) was sought in tumour DNA. Activating dominant mutations of the oncogene Gsα were detected using the polymerase chain reaction to amplify exons 7-10 and hybridising the product to normal and mutant allele specific oligonucleotides.

Allelic deletions on chromosome 11 were identified in 16 tumours (18%) representing all 4 major sub-types. Deletions on other autosomes were observed in less than 6% of tumours. Three adenomas had deletions on multiple autosomes, two of these were aggressive and recurrent. Mutations of Gsα were confirmed to be specific to somatotrophinomas, being identified in 36% of such tumours in this series. No evidence of amplification or re-arrangement of other recognised cellular oncogenes was found.

Inactivation of a recessive oncogene on chromosome 11 is an important and possibly early event in the development of the 4 major types of pituitary adenoma whilst activating mutations of Gsα are confirmed to be specific to somatotrophinomas. Two aggressive tumours were found to have multiple autosomal losses, suggesting a multi-step progression in the development of tumours of this phenotype.

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Finally thanks to my wife and family for their endless tolerance.

1 INTRODUCTION

1.1 PITUITARY ADENOMAS - HISTORICAL AND CLINICAL PERSPECTIVES

1.1.1 Historical background

The existence of the pituitary gland has been recognized for over 2000 years. The origin of the word being in Greek and Latin - to spit/mucus - referring to its proposed function in guiding mucus from the brain to the nose. The first case of tumour of the pituitary are thought to have been reported in the late 17th century (Hirsch, 1952). It was not until 1886 that Marie proposed the link between a pituitary tumour and a clinical syndrome - Acromegaly (Marie, 1886). This initial association focused attention on the pituitary gland and over ensuing years both the clinical syndromes associated with pituitary neoplasms - Cushings disease, acromegaly and hypopituitarism - and the active substances secreted by and controlling the gland were elucidated.

Understanding of the function and actions of the gland has been further expanded by radioimmunoassay - allowing measurement of minute quantities of the relevant hormones - and by advances in pathology and histology in identifying and classifying the cell types in normal and adenomatous pituitary tissue.

Management of these tumours was initially surgical, the first recorded operation for a pituitary adenoma being performed in 1896. Indeed it was the observation of improvement in two patients with acromegaly following resection of adenomas that confirmed their causative role as early as 1908. At around the same time the mainstay of present day surgical management - trans-sphenoidal hypophysectomy - was also pioneered by Schloffer and Hirsch. In the 1950's radiotherapy for pituitary tumours was introduced and over recent years a number of medical therapies

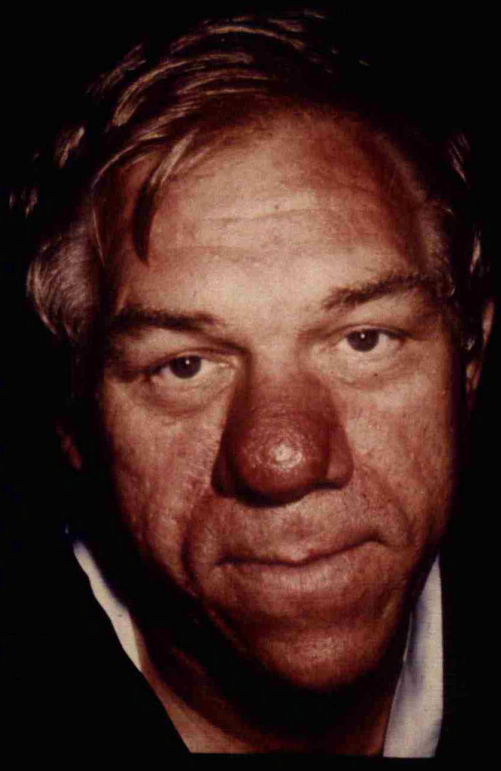
have found their place in the management of these conditions, bromocriptine in acromegaly and prolactinomas, metyrapone in Cushings disease and still more recently the somatostatin analogue octreotide - which may have a role to play in all forms of pituitary neoplasm.

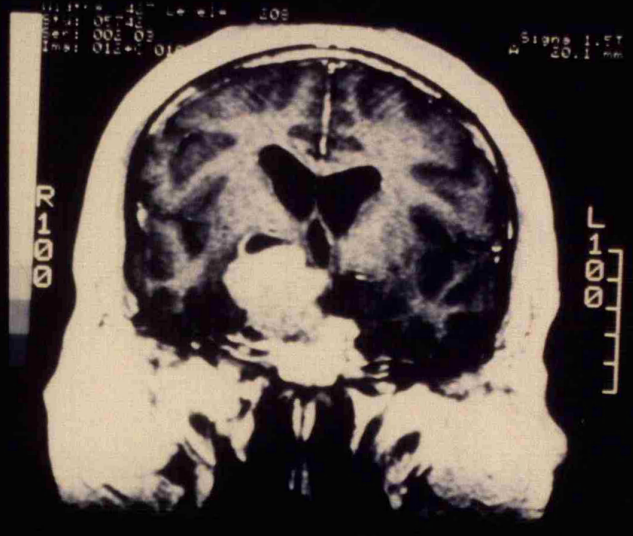
1.1.2 Clinical aspects

Pituitary tumours may present because of hypersecretion of active hormones, by compromising function of the remaining gland or due to the effects of space-occupancy - the tumour expanding in a fixed bony compartment[fig1].

The anterior pituitary gland, site of adenoma formation, secretes six active hormones. Hypersecretion of each of these in conjunction with pituitary adenomas is recognized and three are associated with distinct clinical syndromes. Acromegaly with growth hormone (GH) hypersecretion, Cushings' disease with adrenocorticotrophic hormone (ACTH) and amenorrhoea, galactorrhoea and infertility with hyperprolactinaemia. Hypersecretion of thyroid stimulating hormone (TSH) occurs rarely but is a recognized cause of hyperthyroidism. Hypersecretion of the other glycoprotein hormones (lutenising hormone - LH, follicle stimulating hormone - FSH) usually does not give rise to distinct clinical syndromes and diagnosis may rest on estimation of serum hormone levels or in-vitro studies of tumour tissue. A large group of tumours are said to be 'non-functioning' in terms of hormone secretion, however in recent years it has become apparent that a significant number of these (up to 80%) are in fact secreting inactive sub-units of the glycoprotein hormones (Jameson et al, 1987). These can be measured in serum

Figure 1 - A patient demonstrating the typical facial appearances associated with growth hormone hypersecretion or acromegaly. With magnetic resonance imaging a large pituitary tumour is shown arising from and expanding out of the sella, predominantly to the right (arrowed).




R100
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by radioimmunoassay or demonstrated by immunohistochemical staining of the tumour itself.

Numerous autopsy studies have found pituitary adenomas - almost invariably microadenomas (<10mm diameter) - in between 6 and 20% of patients without known pituitary disease (Costello, 1936. Burrows et al, 1981). This conflicts with the few large epidemiological studies conducted on these tumours. Based on hospital discharge data in the UK between 1970 and 1977 an incidence of 4-8/100,000 patients was calculated (Robinson et al, 1979). More recent data from a study in Northern Italy gave an incidence of 1.55/100,000 and a prevalence of 20 cases per 100,000 (Ambrosi et al, 1991). If autopsy data is correct then though pituitary adenoma formation is common in the general population, the vast majority of such tumours are not manifest during life.

Presentation at all ages is recognized, with the majority of tumours occurring between the ages 20 to 50. Because of more accurate histological assignment of tumour type and improved imaging techniques, allowing earlier (and often incidental) diagnosis of pituitary tumours, figures for the relative frequency of tumour sub-groups have been revised in recent years and will continue to evolve. In a review of 2137 adenomas over the period 1968-1988 the following figures were reported - prolactinomas 50.1%, non-functioning adenoma 23.1%, GH-secreting 21.4%, ACTH-secreting 4.7%, TSH/FSH/LH-secreting 0.4% (Ambrosi et al, 1991). Clearly over the period of this review case definition has been revised and these figures may still not give a true picture of relative incidence.

Histologically these tumours are almost always benign.

Malignant or metastatic pituitary tumours are particularly rare. In the absence of a malignant phenotype many of the clinical manifestations of these tumours are related to the rate of tumour growth. This varies greatly between individual tumours, the majority however grow slowly and may be present for many years before diagnosis is made or intervention required. A sub-group of tumours - prolactin secreting microadenomas - followed in female patients presenting with infertility, were shown to rarely undergo significant expansion and may recede on prolonged follow-up (March et al, 1981. Sisam et al, 1987). Some tumours, of all types, do however demonstrate more aggressive characteristics with rapid tumour expansion, local invasion and early recurrence. There are at present no useful markers to allow early identification of aggressive tumours. Hormonal hypersecretion correlates poorly with tumour size (Landolt, 1982) and is more likely to be a reflection of tumour ultrastructure.

By their expansion in and around the pituitary fossa adenomas may produce a number of symptoms. The most common of these are visual disturbance - due to compression of the optic chiasma or nerves - and headache. In addition compression of the adjacent normal pituitary tissue can impair its function and cause reduced secretion of all pituitary hormones - hypopituitarism.

The aim of management of these tumours is to control both tumour growth and hormonal hypersecretion whilst maintaining adequate residual pituitary function. Treatment of GH and ACTH secreting tumours is generally surgical. Trans-sphenoidal hypophysectomy in experienced centres is safe (mortality <1%) and is generally successful in controlling hypersecretion. Medical treatment may be appropriate prior to surgery to control

hypersecretion and in situations where surgery is contra-indicated. Radiotherapy may be given as adjunctive treatment following surgery or again when surgery is contra-indicated.

For prolactinomas first-line treatment is now medical. The dopamine agonist bromocriptine will rapidly control hyperprolactinaemia in the majority of cases and may induce tumour shrinkage (Vance et al, 1984. Thorner et al, 1980). This is the case even for large tumours in the presence of visual field defects. As stated, long term follow up of prolactin secreting microadenomas suggests that significant expansion of these tumours is rare and a number of such tumours will eventually recede, supporting a conservative approach. Where medical therapy is unsuccessful or poorly tolerated, surgical management is appropriate. Diagnosis and management of hypersecretory pituitary adenomas has been reviewed recently (Klibanski and Zervas, 1991).

For symptomatic, non-functional tumours management is again surgical. Where such tumours are found in the absence of significant symptoms a conservative approach can be taken with assessment of base-line pituitary function and repeated imaging to follow tumour size (Molitch MD and Russell EJ, 1990).

However despite continuing improvement in our understanding of the clinical and pathological features of pituitary tumours and their management, until recent years little was known about the underlying cause of adenomas. What are the stimuli to cell transformation in adenomas and what are the factors promoting expansion of these cells?. It is only with the revolution in molecular genetics over the past decade that significant insights have been gained into the mechanisms underlying formation of

these tumours.

1.2 THE MOLECULAR GENETIC BASIS OF TUMOUR FORMATION

Examination of the genetic basis of the transformed phenotype has identified two major classes of genes involved in the transition from normal to malignant cell. These can be broadly described as cellular oncogenes and tumour-suppressor genes, though as we learn more of their action this distinction is becoming less clear.

1.2.1 Cellular oncogenes

The products of cellular oncogenes, in their non-activated state, serve regulatory cellular functions - growth factors and receptors, nuclear proteins and signal transducers[fig2]. They can be activated to stimulate cellular transformation in a number of ways - point mutations leading to a change in amino acid sequence and thereby protein function; by multiplication of the number of copies of the gene (gene amplification); and by gross re-arrangements of chromosomes or genes bringing the oncogene under the influence of inappropriate control sequences. The mechanisms underlying each of these processes are not fully understood, though by assays of cultured cell transformation following transfection with human DNA fragments and by examination of the genes involved in chromosomal re-arrangements more than 20 such sequences were identified. Many further cellular oncogenes have since been identified by their action in specific tumour types. The activation of a cellular oncogene exerts a positive and dominant influence on cell turnover, 'driving' the cell towards transformation[fig3].

Figure 2 - A schematic representation of the function and sites of action of the recognised classes of cellular oncogenes, with examples in each group.

THE MAJOR FAMILIES OF CELLULAR ONCOGENES AND THEIR SITES OF ACTION

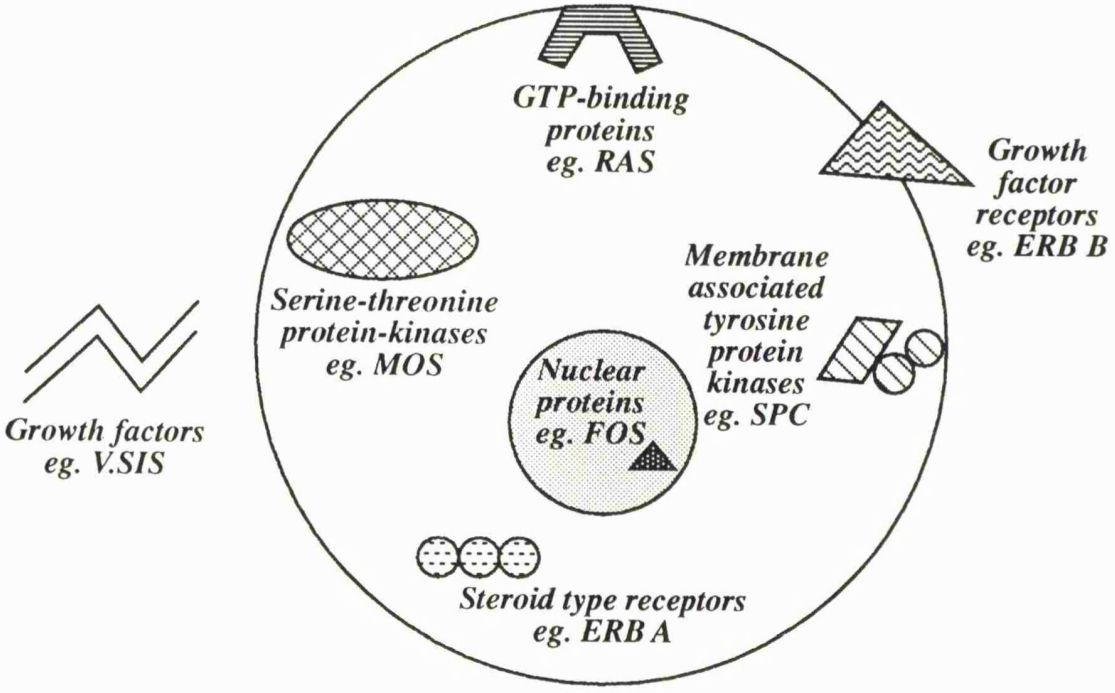
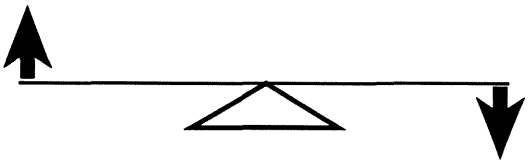


Figure 3 - The balance which regulates cell turnover is controlled by positively and negatively acting genes. As such control may be lost and cellular proliferation may result either from activation of growth stimulating cellular oncogenes or by loss of the protein products of growth constraining genes such as the retinoblastoma gene (RB).

Balanced Cell Turnover

Growth stimulation
- cellular oncogenes -
- ❖ growth factors
- ❖ receptor
- ❖ 2nd messengers
- ❖ nuclear factors



Growth constraint
- tumour suppressor genes -
- ❖ cell cycle regulators (RB)

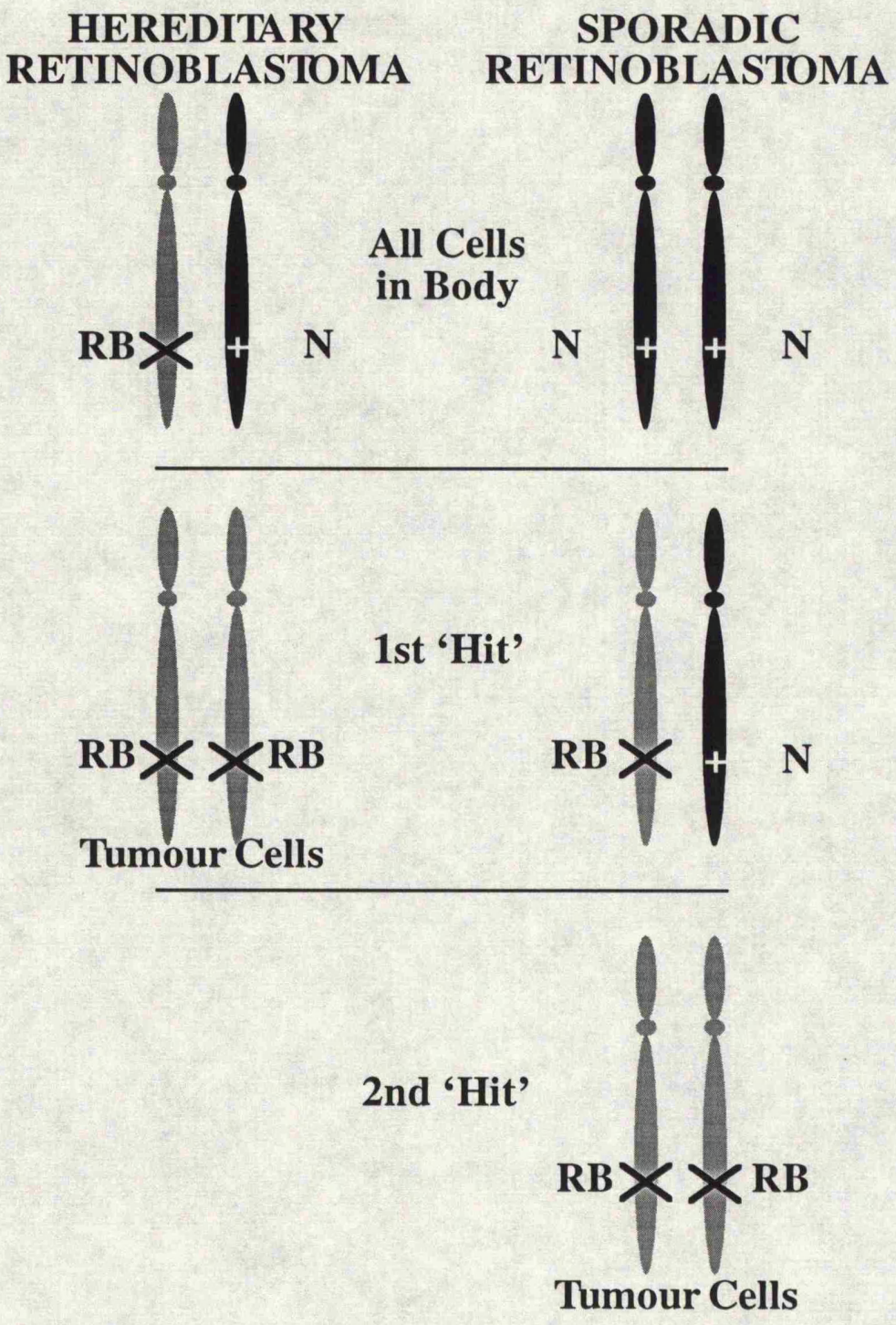
1.2.2 Tumour suppressor genes

The study of tumour-suppressor genes is a more recent development. Their existence was however predicted 20 years ago by Knudson (Knudson, 1971) from his observations on the epidemiology and clinical features of the childhood tumour retinoblastoma. These tumours occur in sporadic and hereditary form. Though histologically identical the inherited form occurs earlier, is often bilateral and shows an association with other tumour types - notably osteosarcoma. Knudson suggested that two recessive mutational events were required in retinoblastoma cells. In the hereditary form the first mutational event is inherited in the germ line. In the rapidly dividing retinal cells the chance of a second mutation occurring in a remaining normal allele are high - explaining the common occurrence of bilateral tumours in these children. By contrast, in the sporadic form, an individual retinal cell must sustain two mutational events involving the retinoblastoma gene - statistically a far less likely occurrence. The nature of such mutational events may vary, ranging from a single base change to deletions of large chromosomal fragments. This 'two-hit' hypothesis[fig4] explained many of the features of these tumours and has since been confirmed by a number of investigators.

Further evidence for the existence of genes which are lost prior to malignant transformation comes from the study of somatic cell hybrids. It has been shown that the fusion of malignant cell lines with normal diploid cells often results in the loss of the malignant phenotype (Harris et al, 1988) and further that this suppression of malignancy depends upon the retention of specific chromosomes by the hybrid cell lines. These chromosomes are

Figure 4 - Knudsons 'two hit' theory accurately predicted the role of tumour suppressor genes in cellular transformation. In inherited tumour syndromes patients inherit only a single active copy of the associated gene in all cells, an individual cell therefore requires only a single 'hit' on this gene to result in tumour formation. In the sporadic form an individual cell must sustain two 'hits' within both copies of the gene.

Knudsons 'Two Hit' Theory



RB = mutant allele
N = normal allele

likely therefore to contain sequences which exert control over cellular transformation.

Confirmation of these theories again comes from the study of retinoblastoma. In a number of retinoblast cell lines a cytogenetically visible deletion of the chromosomal region 13q14 had been noted. Evidence that this was the site of the retinoblastoma gene came from studies of the enzyme esterase D which had been previously assigned to this locus. The inheritance of esterase D alleles in families with the hereditary form of retinoblastoma was found to closely follow the inheritance of tumour predisposition, strongly suggesting that the two were genetically 'linked'. To show this at the molecular level it was necessary to be able to identify the individual alleles on chromosome 13 and demonstrate deletions in tumour tissue. This was achieved by the use of restriction fragmant length polymorphisms (RFLPs).

Restriction enzymes, isolated from bacteria, cut DNA at specific base pair sequences. Digestion of DNA with these enzymes results in a large number of fragments of widely varying length. Because of minor and otherwise inconsequential variations in DNA sequence between individuals - at approximately 1 base in 250 - a number of the sites recognized by these enzymes will be altered. As a result the sizes of some of these fragments will vary between individuals. If we separate the DNA fragments according to size using electrophoresis - we can exploit this with probes which will hybridise DNA fragments known to be variable, or polymorphic, in size. Knowledge of the chromosomal location of the sequences identified by these probes will allow us to differentiate genetic material from a region of both copies

of a particular chromosome. Loss of one copy of a specific chromosomal region, as a consequence of a deletion or by non-disjunction and reduplication of a chromosome carrying the mutant allele, will result in absence of one of the two signals in heterozygous individuals when the DNA is probed with a sequence specific to this region. Such findings being described as 'allele loss' or loss of heterozygosity'[fig5].

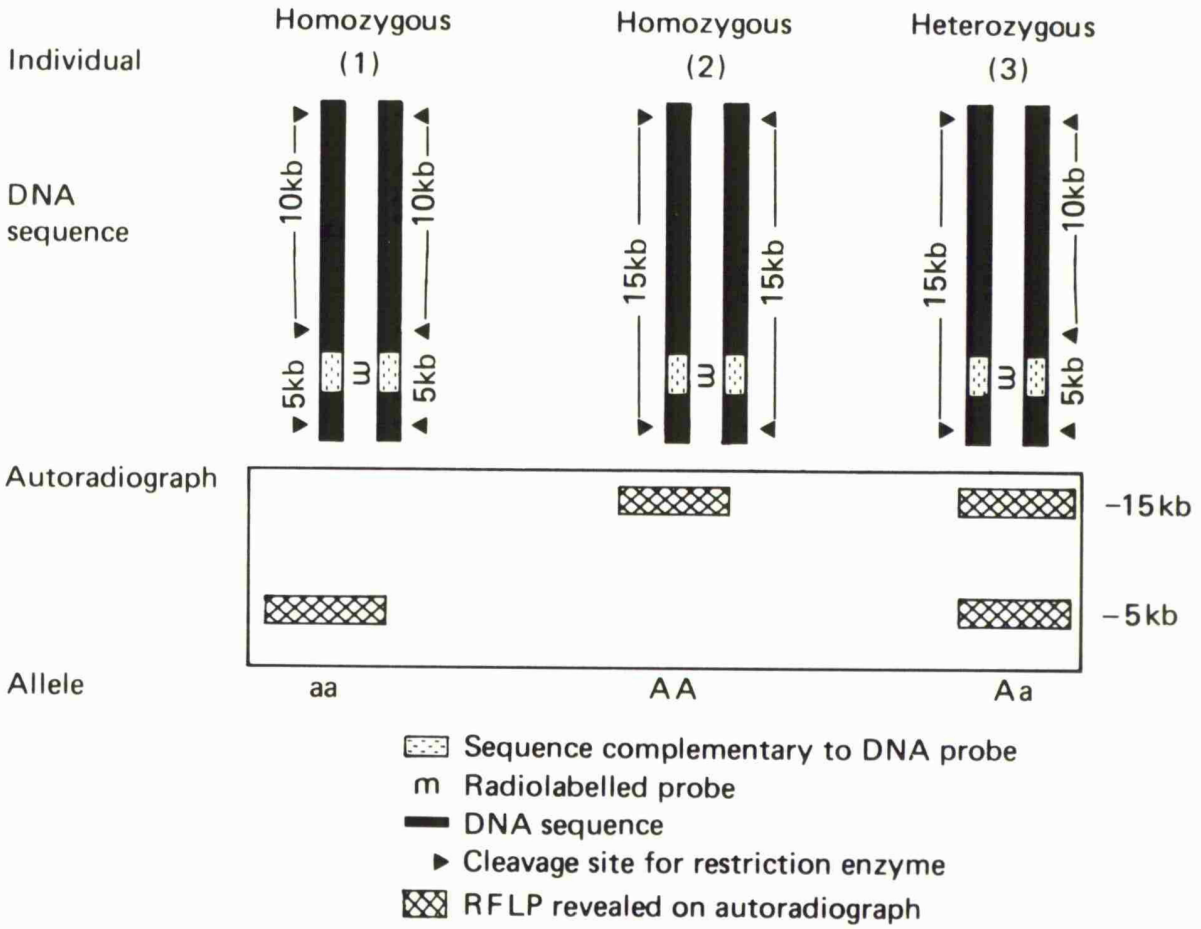
This approach was successful in identifying deletions in retinoblastoma tissue on chromosome 13 thus confirming cytognetic observations (Cavenee et al, 1983). Further work has identified the retinoblastoma gene and its protein product - denoted Rb1 (Friend et al, 1986). As was predicted cell lines showing a deletion of one copy of this gene were found to have mutations in the remaining copy which deleteriously affect production or function of its protein product (Lee et al, 1987. Dunn et al, 1989). Rb1 is a nuclear phosphoprotein which is involved in cell cycle regulation (Hamel et al, 1992).

Following these observations in retinoblastoma presumed tumour-suppressor genes have been localised in a number of other inherited and sporadic tumour types - for example, familial polyposis coli, colorectal carcinoma, breast, bladder and cerebral tumours. In most sporadic tumours studied there is evidence for more than one such gene acting in each tumour type.

1.2.3 Multi-step theories of tumorogenesis

On clinical and histological grounds alone it seemed likely that the development of most tumours was a multi-step process. Increasing evidence suggests that this is in fact the case at the genetic level. The best example of this is in colorectal

Figure 5 - Restriction fragment length polymorphisms (RFLP's) allow identification of DNA fragments resulting from the enzymatic digestion of chromosomal material. Where a pair of chromosomes are heterozygous for the presence of a specific enzyme cutting site a DNA probe identifying a sequence close to the cutting site will reveal a variation insize of the resulting DNA fragments (as in example 3). In such cases if a chromosomal deletion has occurred in tumour tissue this will be revealed as a loss of one band on the autoradiograph when compared to the constitutive genotype - 'loss of heterozygosity' (LOH).


Individual
Homozygous (1)
Homozygous (2)
Heterozygous (3)
DNA sequence
10kb
5kb
15kb
m
Autoradiograph
–15kb
–5kb
Allele
aa
AA
Aa
Sequence complementary to DNA probe
m Radiolabelled probe
DNA sequence
Cleavage site for restriction enzyme
RFLP revealed on autoradiograph

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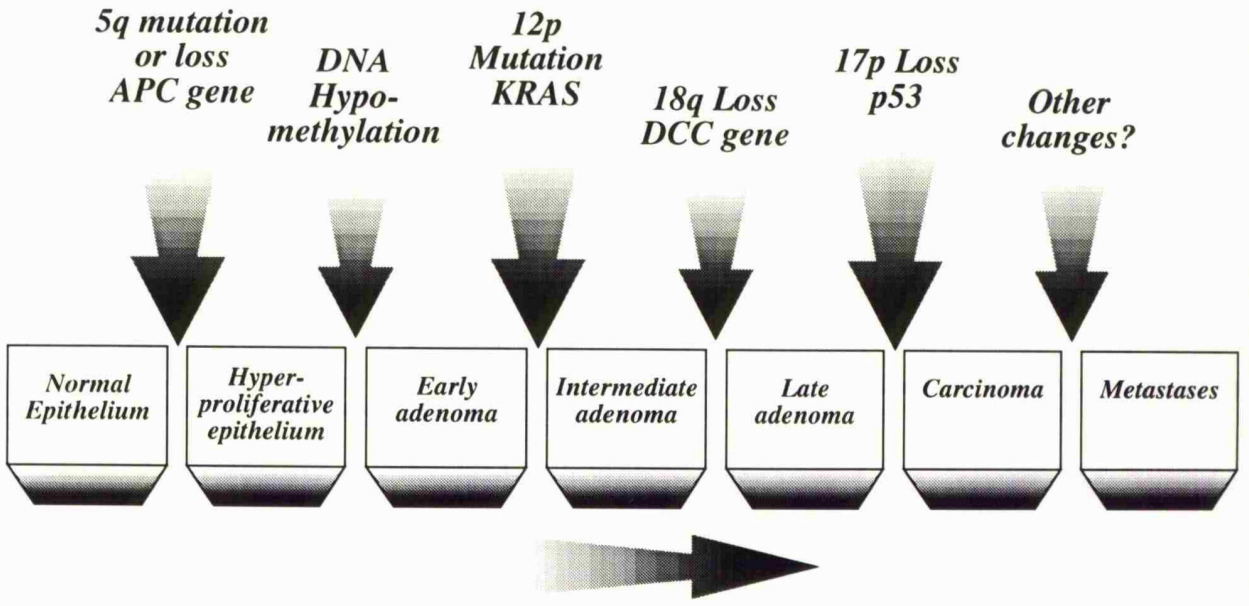
carcinoma where there is a well recognized histological progression from normal through adenomatous to malignant cell types. A number of mutational events have been identified in these tumours (Fearon and Vogelstein, 1990). These include activation of oncogenes - K-ras - and deletions at three or more putative tumour-suppressor loci. These events are seen both in inherited colonic neoplasm (Polyposis Coli - linked to chromosome 5) and the more common sporadic form.

Specific mutational events are noted in tumours at different stages of neoplastic change[fig6]. The emergence of an increasing cellular load of mutational events is a marker for loss of cellular differentiation and increasingly malignant cellular characteristics. Such a pattern is emerging for many tumour types including ovarian (Zheng et al, 1991), gliomas (Chung et al, 1992) and thyroid (Fagin, 1992. Wynford-Thomas, 1993). In tumours arising in the thyroid, aa an endocrine example, to date five genes have been implicated in oncogenesis - ras, gsp, ret, trk and p53. Events at some of these loci are common to many tumour types, particularly in the later stages of malignancy. A particularly common example of this being p53 on chromosome 17 which has documented mutations - over 200 - in a wide range of tumour types. These findings, in inherited and sporadic neoplasms, would suggest an interaction of cellular oncogenes and tumour-suppressor loci, with sequential mutational events leading to cellular transformation.

Pituitary adenomas, being almost universally benign, and with only small quantities of tissue available for study have only recently been subjected to genetic analysis. However several important findings have emerged and the genetic events underlying

Figure 6 - Multiple genetic events may be required to result in tumour formation in some tissues, with an increasing mutational load associated with more aggressive tumour phenotype. Specific events may occur at different stages of neoplasia, a schematic representation of proposed mutational events and their role in the progression of colonic tumours is shown (Fearon and Vogelstein, 1990).

SEQUENTIAL GENETIC CHANGES IN COLORECTAL TUMORIGENESIS



adenoma formation are beginning to be understood.

1.3 STUDIES IN PITUITARY ADENOMAS

1.3.1 Hypothalamic/Pituitary origins - Clonality

Two possible mechanisms have been proposed for the initiation of pituitary adenomas. Firstly an intrinsic pituitary abnormality - a somatic mutation at the level of the individual pituitary cell resulting in growth advantage and a monoclonal cellular expansion - or secondly aberrant regulation of pituitary function with adenoma formation secondary to hyperstimulation or loss of inhibition from hypothalamic control mechanisms.

Evidence has been proposed to support both of these hypotheses, the important points are summarised in table 1. The bulk of this evidence would appear to favour an intrinsic pituitary abnormality, in the majority of cases, possibly with maintainance of tumour growth or secretory dynamics by the hypothalamus. Recently results of molecular genetic studies have given further support to such a mechanism.

Using the technique of X-chromosome inactivation analysis a number of groups have been able to show that in the majority of cases (>90%) pituitary adenomas of all functional types are monoclonal in origin (Herman et al, 1990. Alexander et al 1990. Jacoby et al, 1990. Schulte et al, 1991. Gicquel et al, 1992). These studies are based on the observation that early in female embryogenesis in each cell random and irreversible inactivation of one X-chromosome occurs. Using probes which identify RFLP's on the X-chromosome and methylation sensitive restriction enzymes - which differentiate between transcribed and inactive genes - individual (maternal/paternal) X-chromosomes can be

Table 1 - Factors in favour of hypothalamic vs pituitary aetiology of pituitary tumours

Hypothalamic/Regulatory Abnormality	Intrinsic Pituitary Abnormality
Occurrence of adenomas in cases of end organ failure or in response to excess releasing factors (GHRH/CRH secreting tumours).	Such tumours are very rare, pituitary hyperplasia is more common. TSH-secreting tumours very rarly arise following symptomatic hypothyroidism
There is a significant recurrence rate for pituitary adenomas following apparently successful surgery	The majority do not recur. Recurrence is generally early and probably the result of incomplete removal
Some tumours remain under hypothalamic control, pulsatile GH-secretion maintained in acromegaly	Most hypothalamic controls are lost - i.e. circadian rhythms, paradoxical responses
Hyperplasia rather than a distinct adenoma is found in some operative samples	Absence of hyperplasia around most adenomas, hyperplasia may also support an intrinsic pituitary lesion
	Return of physiological controls and secretory dynamics following tumour removal in most cases
	Occurrence of dual hormone secretion - GH/PRL - in same tumour/cells
	Existence of non-functioning tumours

identified[fig7]. By examining tumour DNA it is possible therefore to reliably differentiate between monoclonal and polyclonal tissue. This technique can clearly only be applied to tumours arising in female patients, there is however no reason to suspect that this is not the case in all patients.

It is therefore likely that the initiating events in pituitary tumorogenesis occur at the level of the individual pituitary cell in the majority of cases. What are these events?

1.3.2 Cytogenetics

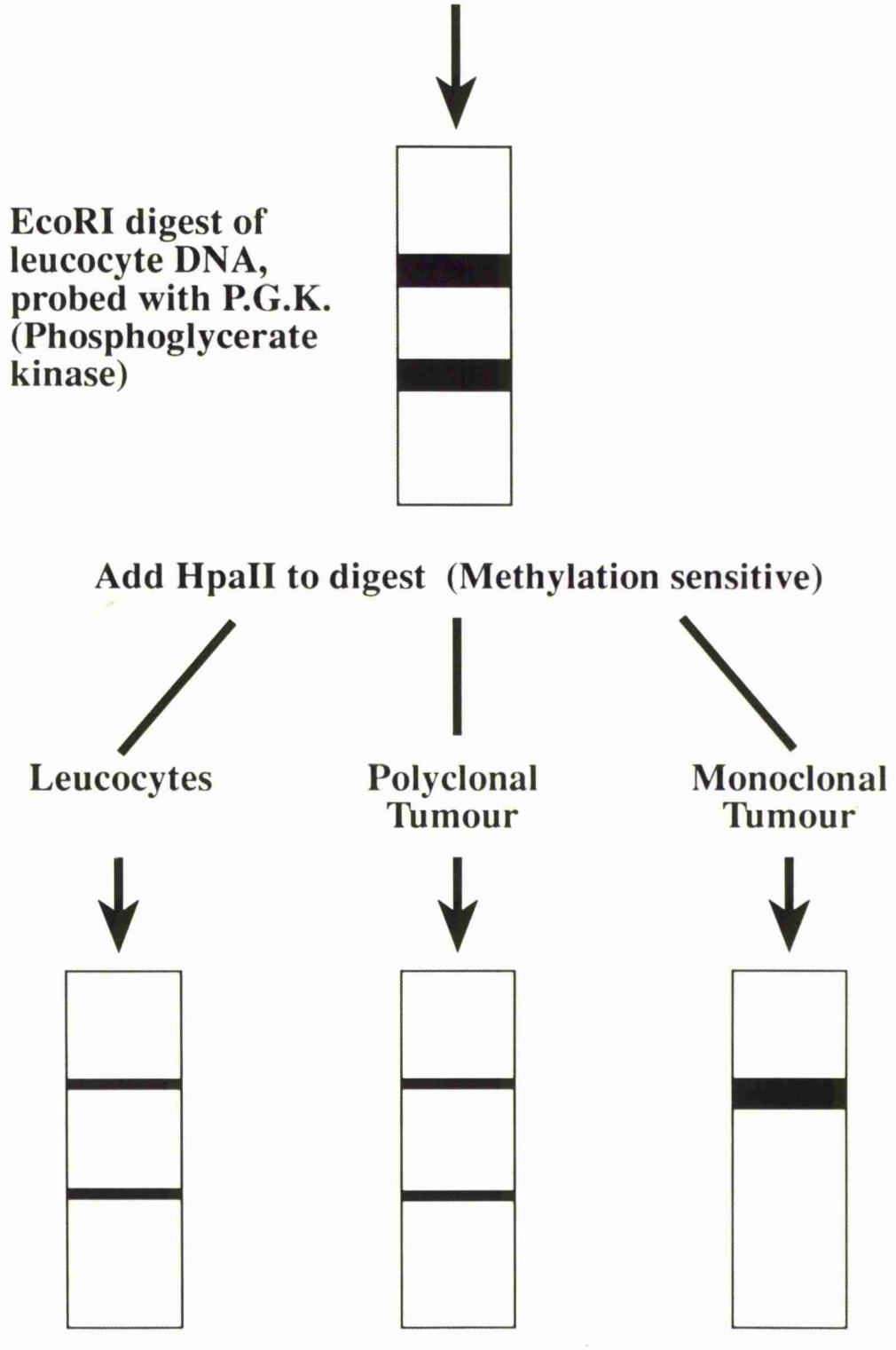
A number of pituitary adenomas have been examined cytogenetically (Mark et al, 1971. Rey et al, 1986). In most a normal or aneuploid chromosomal complement was found. No characteristic chromosomal rearrangements or deletions were identified.

1.3.3 The 'gsp' oncogene

Biochemical studies in GH secreting tumours, based on the cAMP-adenylate cyclase enzyme systems, had suggested that two distinct sub-groups existed, expressing either normal or high basal adenylate cyclase activity (Vallar et al, 1987). The stimulation of somatotroph cells by GHRH results in activation of adenylate cyclase by interaction of hormone/receptor complex with the stimulatory GTP-binding protein G_s. A large family of such proteins (>10) has been identified whose action is to couple receptors to effector molecules (Bourne et al, 1990). G proteins are heterotrimeric, consisting of three polypeptides - α, ß and τ chains. Receptor stimulation results in GTP binding to the α chain and dissociation of ßτ. The GTP/α complex then stimulates

Figure 7 - The study of X-chromosome inactivation in tumour tissue, from female patients, allows differentiation of polyclonal from monoclonal tissue. This involves the use of a probe identifying an RFLP on the X-chromosome followed by a further digestion of DNA by a methylation sensitive enzyme which differentiates between transcribed and inactive genes. In monoclonal tissue, because of embryonic inactivation of one X-chromosome, all cells will transcribe from the same copy of the X-chromosome resulting in complete loss of one of the resulting bands on reprobing and autoradiography.

X Chromosome Inactivation



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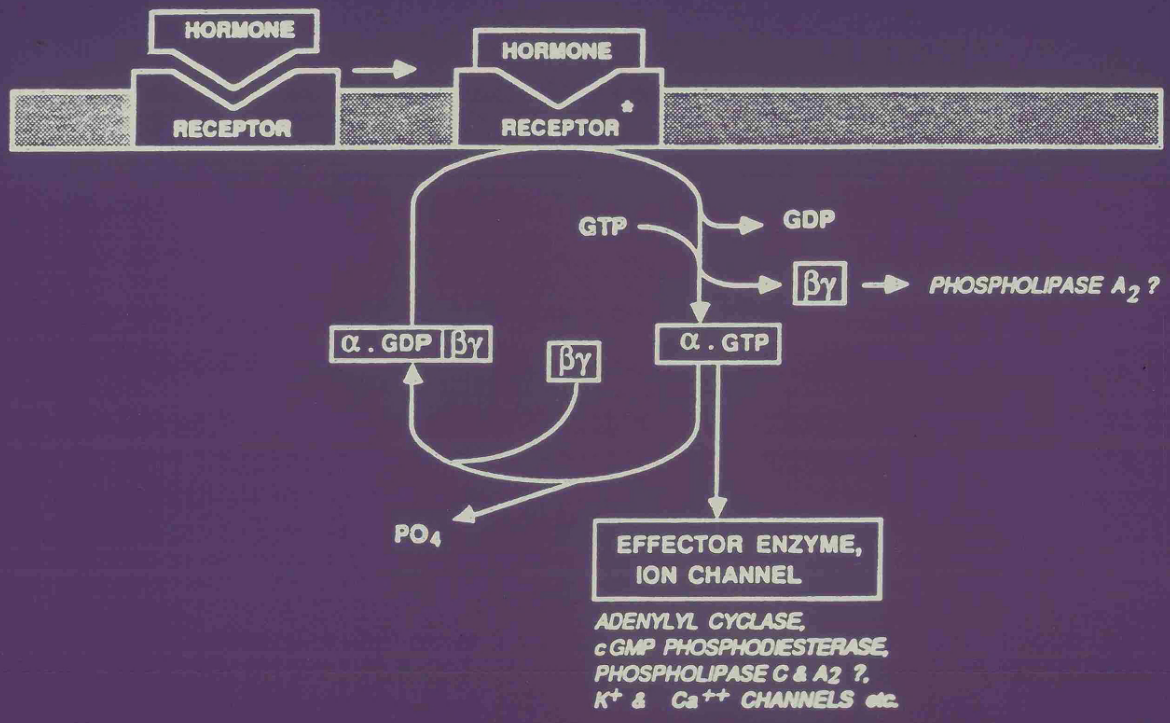
adenylate cyclase-cAMP activity and thereby GH secretion and cellular proliferation (Billestrup et al, 1986). Activity is terminated by virtue of intrinsic GTP-ase activity in the α-chain. On hydrolysis of GTP to GDP dissociation occurs and the heterotrimer reforms and returns to the inactive state[fig8].

Mutations in G-proteins are well recognised in human tumours. The product of the H-ras cellular oncogene (p21) is a member of the G-protein family, mutations of the gene encoding the α-subunit of H-ras result in a protein with reduced ability to hydrolyse GTP. The mutant G-protein is therefore 'locked-in' to the activated state and promotes cellular proliferation (Bourne et al, 1990).

The observation, therefore, that a group of somatotrophinomas (approximately 40%) showed high cAMP/adenylate cyclase activity which was unresponsive to further stimulation (by GHRH) pointed to a possible mutation in the $G_s\alpha$ chain. In a series of somatotrophinomas the cDNA encoding $G_s\alpha$ was therefore sequenced and found to contain mutations at one of two sites - codons 201 and 227 - (Landis et al, 1989). Both mutations result in amino-acid substitutions in the GTP-binding site of the α-subunit and correspond to the site of GTPase catalytic activity in other similar proteins. These mutations were found only in tumours with high basal adenylate cyclase activity and were not present in genomic (leucocyte) DNA suggesting a de-novo mutation in tumour cells.

Clinical and functional studies of these tumours appeared to correlate with these findings, in that those tumours bearing a mutation show more intense secretory activity, were generally smaller at diagnosis and more sensitive to inhibitory factors

Figure 8 - The action of G proteins in coupling hormone-receptor complex to effector enzymes is shown. The reaction is terminated by intrinsic GTP-ase activity of the α-subunit. In somatotrophs the stimulatory G protein G_s couples GH-receptor complex to adenylyl cyclase. In a sub-group of somatotrophinomas mutations resulting in the loss of the intrinsic GTP-ase activity of the α-subunit have been demonstrated. As a result in these tumours this pathway is constitutively activated.


HORMONE
RECEPTOR
HORMONE
RECEPTOR*
GTP
GDP
βγ
PHOSPHOLIPASE A2 ?
α . GDP βγ
βγ
α . GTP
PO4
EFFECTOR ENZYME,
ION CHANNEL
ADENYLYL CYCLASE,
c GMP PHOSPHODIESTERASE,
PHOSPHOLIPASE C & A2 ?,
K+ & Ca++ CHANNELS etc.

(which act via an alternative G-protein) than tumours with wild-type G_s (Spada et al, 1990), though more recent studies have questioned such findings.

The mutated form of $G_s\alpha$ is therefore regarded as an oncogene - designated 'gsp' - and may be implicated in 40% of somatotrophinomas. Studies in other classes of pituitary tumour - albeit in small numbers - have failed to identify such mutations (Lyons et al, 1990) and they would appear therefore to be specific to somatotrophinomas.

1.3.4 Other cellular oncogenes

Studies of other recognised cellular oncogenes in human pituitary adenomas are limited. Examination of the ras oncogenes (K-ras, H-ras and N-ras) for mutations recognised in other human tumours has been performed in 19 pituitary adenomas (Karga et al, 1992). A point mutation at codon 12 of H-ras was identified in a single prolactinoma. Again this is a mutation which inhibits GTP-ase activity of the protein product - p21. The tumour in this patient is reported as being particularly aggressive with rapid recurrence and fatal outcome and the question has therefore been raised whether such mutations may be a marker of aggressive tumour characteristics.

In a study of oncogene expression in two tumours in 1987 a 7-10 fold amplification of the cellular oncogene v-fos - a nuclear protein thought to be involved in gene transcription - is demonstrated in a prolactinoma (U et al, 1988). The clinical course is not reported and the significance of this is not clear. In the same study no evidence of amplification of the oncogenes c-myc, N-myc, v-myc, H-ras and v-sis was found.

Though further novel oncogenes such as 'gsp' may be identified, on the basis of this limited information it is possible that well recognised cellular oncogenes do not play an important role in pituitary adenoma initiation. An alternative approach to identification of candidate genes involved in pituitary adenoma is the application of 'reverse' genetics. This approach relies of the identification of areas of the genome which may be of interest by 'linkage' studies in families with inherited conditions or by classical cytogenetic studies identifying common areas of chromosomal rearrangement or deletions in patients with a specific condition. Fine mapping of such regions may allow identification of candidate genes which can be examined for evidence of mutations and whose protein products can be elucidated. Such an approach has been successful in cystic fibrosis and retinoblastoma.

For this approach to be applied a well defined inherited model is required to allow clear case definition. Though some pituitary tumour families are recognised - where family members develop a particular syndrome such as acromegaly - they are particularly rare (Gardner et al, 1989. Yuasa et al, 1990), insufficient numbers being available to attempt linkage analysis. A much more useful condition is the inherited tumour syndrome Multiple Endocrine Neoplasia Type 1 (MEN 1) where patients may develop tumours of the parathyroid glands, pituitary gland and of pancreatic islet cells.

1.4 MULTIPLE ENDOCRINE NEOPLASIA - TYPE 1 (MEN 1)

The presence of multiple endocrine tumours in a patient was first reported in 1903, a patient with acromegaly was found at

autopsy to have enlargement of the parathyroid glands in addition to an anterior pituitary tumour (Erdheim, 1903). The association of parathyroid, pancreatic and pituitary tumours was reported on several occasions (Cushing and Davidoff, 1927. Lloyd, 1929) and families developing this constellation of tumours were described (Moldawer et al, 1954. Wermer 1954). An autosomal dominant mode of inheritance was proposed by Wermer, whose name the syndrome often bears.

A large number of such families have now been described allowing the frequency of each tumour type and the mode of presentation of the condition to be delineated. Patients can present at all ages, over 80% will manifest one or more tumour type by age 50 (Shepherd, 1985. Caruso et al, 1991). Primary hyperparathyroidism is the most frequent presentation - recognised in 95% of patients. Islet cell neoplasms - gastrinomas, insulinomas and glucagonomas - are found in approximately 60%. Estimations of the frequency of pituitary adenomas vary greatly between series (15-90%), the most recent review - of 72 patients - gave a frequency of 30% (Caruso et al, 1991).

The distribution of pituitary adenoma sub-types seen in MEN 1 is similar to that for sporadic adenomas. Approximately 60% are prolactinomas, 25% GH-secreting, 20% non-functional and 3% ACTH-secreting (Prosser PR 1979, Farid NR 1980, Eberle F 1981).

1.4.1 The genetics of MEN 1

Large family studies confirmed the autosomal dominant mode of inheritance for MEN 1 (Ballard et al, 1964). Attempts to show linkage to classical markers - HLA, leucocyte antigens, serum

proteins - were unsuccessful, as were cytogenetic studies. The first positive localisation of the MEN 1 gene came from studies of allele loss in tumour DNA from two insulinomas from related MEN 1 patients (Larsson et al, 1988). Tumour DNA showed loss of alleles on both the long and short arms of chromosome 11 when compared to leucocyte DNA. Examination with markers from 16 other autosomes failed to show additional deletions. Further studies in MEN 1 families confirmed linkage of the syndrome to markers on chromosome 11 (Larsson et al, 1988. Thakker et al, 1989). Probes for the human muscle phosphorylase gene (PYGM) and the oncogene int-2, both previously localised to the pericentromeric region of the long arm of chromosome 11, were shown to be linked with MEN 1.

These finding suggested that the development of tumours associated with MEN 1 required two mutational events. Firstly an inherited, predisposing mutations on one allele at the MEN 1 locus followed by a somatic mutation involving deletion of the functional (wild-type) allele.

With increasing numbers of informative genetic markers the region of chromosome 11 containing the MEN 1 locus - 11q13 - has been more precisely mapped (Janson et al, 1991; Fujimori et al, 1992) and the order of markers in this region established. However the gene involved in MEN 1 is yet to be identified.

Following the demonstration of chromosome 11 deletions in insulinomas in MEN 1 a number of groups have examined both inherited and sporadic tumours of parathyroid, pituitary and islet-cells for chromosome 11 allele loss (Larsson et al, 1988. Thakker et al, 1989. Freidman et al, 1989. Bystrom et al, 1990. Teh et al 1990. Radford et al, 1990. Bale et al, 1991. Yoshimoto

et al, 1991). The results of these studies are summarised in table 2.

From these figures it is apparent that allele loss on chromosome 11 is associated with all tumour types in MEN 1, allele loss has not been demonstrated on other autosomes. Pituitary adenoma tissue from MEN 1 patients is rarely available and numbers are therefore small.

Table 2 - Loss of heterozygosity on chromosome 11 in inherited and sporadic tumours related to MEN 1.

	Parathyroid	Islet-cell	Pituitary
Inherited	26/38 (68%)	8/10 (80%)	1/2 (50%)
Sporadic	16/58 (27%)	1/9 (11%)	2/30 (7%)

Sporadic and inherited tumours of these tissues cannot be distinguished on clinical or histological grounds. It seemed reasonable to assume therefore that, as is the case with retinoblastoma, at least a proportion of sporadic tumours in these tissues would arise by the same mechanism as postulated for MEN 1. In sporadic tumours this would be the result of two independent somatic mutations at the MEN 1 locus.

The figures for sporadic parathyroid adenomas appear to support this hypothesis. A significant number (27%) do show allele loss on chromosome 11, once again losses on other autosomes have not been identified. The lower incidence of such losses in sporadic tumours (27% vs 68%) would suggest that this may represent only one of a number of genetic mechanisms giving

rise to transformation in parathyroid tissue.

In sporadic pituitary adenomas chromosome 11 deletions have been demonstrated in only 2 cases, both prolactimonas (Bystrom C, 1990). The reasons for this are not clear. As discussed previously on both a histological and functional basis sporadic and MEN 1 associated pituitary adenomas are identical. However, for these studies pituitary adenomas do present several difficulties. Tissue samples are often very small and the techniques utilised in their surgical removal - laser diathermy, suction - may make histological confirmation of tissue type difficult or impossible. The RFLP techniques used to demonstrate allele loss depend upon purity of tumour tissue. Contamination of samples with non-adenomatous tissue, as is common in pituitary samples, will mask allele loss. By contrast, tumours of the parathyroid are generally larger and more accessible and they can often be clearly delineated at surgery, greatly reducing the likelihood of contamination with non-tumour tissue.

Despite the essentially negative findings in pituitary adenomas to date it remains difficult to explain how the MEN 1 locus can play a significant role in the pathogenesis of sporadic parathyroid adenomas yet be uninvolved in the sporadic forms of the other tumour types which form a part of the MEN 1 syndrome.

1.5 AIMS OF THE STUDY

With the exception of a sub-group of somatotrophinomas found to have the Gsα mutation (being only 8% of total sporadic adenomas) no common genetic markers of pituitary adenomatosis have been identified.

The aim of this study therefore is to analyse a large,

representative sample of sporadic pituitary adenomas for evidence of allelic deletion. The study will focus on the locus 11q13, site of the MEN 1 gene, but will also examine a number of other autosomes known to harbour tumour suppressor genes. We hope to define the role of the MEN 1 gene in sporadic pituitary adenomatosis and establish the frequency and distribution of allelic deletions in these tumours. In addition I have examined the frequency and distribution of Gsα mutations and the role of amplification or re-arrangement of recognised cellular oncogenes in all classes of pituitary adenoma. Genetic findings are related to the tumour phenotype.

If specific areas of chromosomal deletion can be identified in these tumours I would proceed to fine mapping of these areas with the long term aim of identifying and characterising the gene or genes predisposing to adenoma formation in these tissues.

Given the limitations of techniques presently available for identification of allelic deletions, with particular regard to the difficulties presented by the quantity and quality of pituitary tissue available, I also aim to develop alternative strategies for identification of deletions, utilising smaller quantities of tumour material and thereby allowing precise histological definition of pituitary tissue.

2 MATERIALS AND GENERAL METHODS

For full details of all solutions and buffers in the text please refer to Appendix 1.

2.1 CLINICAL MATERIAL

2.1.1 Patient Recruitment

The patients in this study were investigated and diagnosed at a number of centres. The contributors were as follows: Professor R.N. Clayton (Stoke-on-Trent, UK), Professor M. Boscaro (Padua, Italy), Dr. W. Burr (Wakefield, UK) and Dr. C. Burke (Oxford, UK). Clinical information was collected from patient records at each centre. Diagnoses were based on clinical and standard biochemical findings (full details in appendix 2). Where immunohistochemical data was not available this was performed on sections taken from the tissue sample received (see section 2.3). To attempt to exclude patients with MEN1 we required that all patients had a negative personal and family history for other tumours associated with the MEN 1 phenotype and were normocalcaemic at the time of surgery.

2.1.2 Sample collection

Blood - 40mls of venous blood was collected from consenting patients pre-operatively to avoid transfusion contamination. Samples were collected into 3mls of 500mM EDTA (pH 7.0) and stored at -20° prior to DNA extraction.

Tissue - pituitary adenoma tissue was obtained at the time of surgery and immediately frozen in liquid nitrogen. Samples were then stored at -70°C until DNA extraction. Where sufficient tissue was available a section was taken immediately prior to

DNA extraction and fixed in 4% formaldehyde. This was then processed for histological assessment to confirm the presence of adenoma tissue.

2.1.3 Samples received

126 blood/tissue pairs were received for study. Of these 38 were excluded, as detailed below. For the remaining 88 samples sufficient, histologically confirmed, tumour tissue was available to proceed with DNA extraction and analysis.

Samples excluded from further analysis;

16 : Insufficient adenoma tissue for extraction
10 : Sample consists of blood clot only
6 : Posterior pituitary tissue only
4 : Connective tissue only
1 : Nelsons syndrome (hyperplasia)
1 : Meningioma

2.1.4 Tumours analysed

Diagnosis	Number
Non-functioning	35
Somatotrophinoma	25
Prolactinoma	17
Corticotrophinoma	7
Gonadotrophinoma	2
Thyrotrophinoma	1
Mixed (GH/PRL) secreting	2

Of the samples excluded the majority were prolactinomas or corticotrophinomas as these were more often microadenomas where insufficient tissue or non-adenomatous tissue was received. As

a result there is a relative excess of non-functional adenomas amongst those analysed.

2.2 DNA PREPARATION

2.2.1 Isolation of genomic DNA from blood

Following thawing, 10mls of blood was removed for extraction. The remaining sample was re-frozen (infrequent freeze/thaw cycles do not appear to effect DNA yield or quality). Five ml of blood was placed into each of two 50ml (Falcon) tubes, to each was added 45mls of lysis solution. Samples were immediately centrifuged at 1000g (2000rpm), 4°C for 10 minutes (MSE, Cool-Spin). The supernatant was decanted off the nuclear pellets. One pellet was re-suspended in 4.5mls of DNA suspension buffer and pooled with the other pellet. Pellets were homogenised with a long glass Pasteur pipette and to the homogenate was added 500ul of freshly prepared digestion buffer. The sample was then incubated at 37°C overnight.

The sample was mixed by gentle inversion with 5mls of phenol (Tris Cl pH 8.0 equilibrated) and centrifuged at 1000g, 25°C for 10 minutes. The upper aqueous layer was removed to a fresh tube with a wide mouthed pipette (to avoid shearing of high molecular weight DNA), taking care to avoid transfer of any proteinaceous material. To the aqueous layer was added an equal volume of chloroform:iso-amyl alcohol (IAA), 24:1, again mixing by inversion. This was centrifuged as previously, the aqueous layer removed to a fresh tube and the chloroform:IAA extraction repeated.

The aqueous layer was removed to a fresh tube and 10% by volume 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute

alcohol added. Inverting the tube gently encourages DNA precipitation and using a glass hook DNA is extracted and placed in 1ml TE buffer. Samples are allowed to dissolve for at least 2 days at 4°C prior to estimation of DNA concentration.

2.2.2 Preparation of DNA from tissue samples

Following removal of tissue for histological assessment samples were placed into liquid nitrogen in a pre-cooled mortar and pestle. The tissue was crushed to a fine powder and poured into a 50ml (Falcon) tube, the liquid nitrogen being allowed to boil off. The resulting powder was re-suspended in 4.5mls STE buffer to which was added 500ul of digestion buffer (fresh) followed by incubation at 37°C overnight.

Phenol/chloroform extraction and precipitation was performed as for blood samples. In most cases precipitated DNA could be lifted out and placed in 500ul of TE buffer, however, when the yield was insufficient to allow this the sample was centrifuged at 1500g, 0°C for 15 minutes. Alcohol was decanted off and the resulting pellet re-suspended in a small volume of TE buffer.

2.2.3 DNA quantitation

15ul of each sample was diluted in 885ul of TE buffer (1:60). Ultraviolet absorption (OD) of the sample was measured (Pye, Unicam SP-500 UV Spectrophotometer) at wavelengths of 260 and 280 nanometeres. from the ratio of OD_{260}/OD_{280} purity of DNA can be estimated. A pure DNA preparation will give an OD of >1.8. Contamination with protein will result in a significantly lower ratio.

From the OD_{260} alone DNA concentration can be estimated. Given

that an OD_{260} of 1.0 corresponds to a DNA concentration of 50ug/ml we can calculate DNA concentration in the original sample using the formula:

$$DNA(ug/ml) = OD_{260} \times 50 \times 60$$

where: 50 is a constant for DNA

: 60 is the dilution factor.

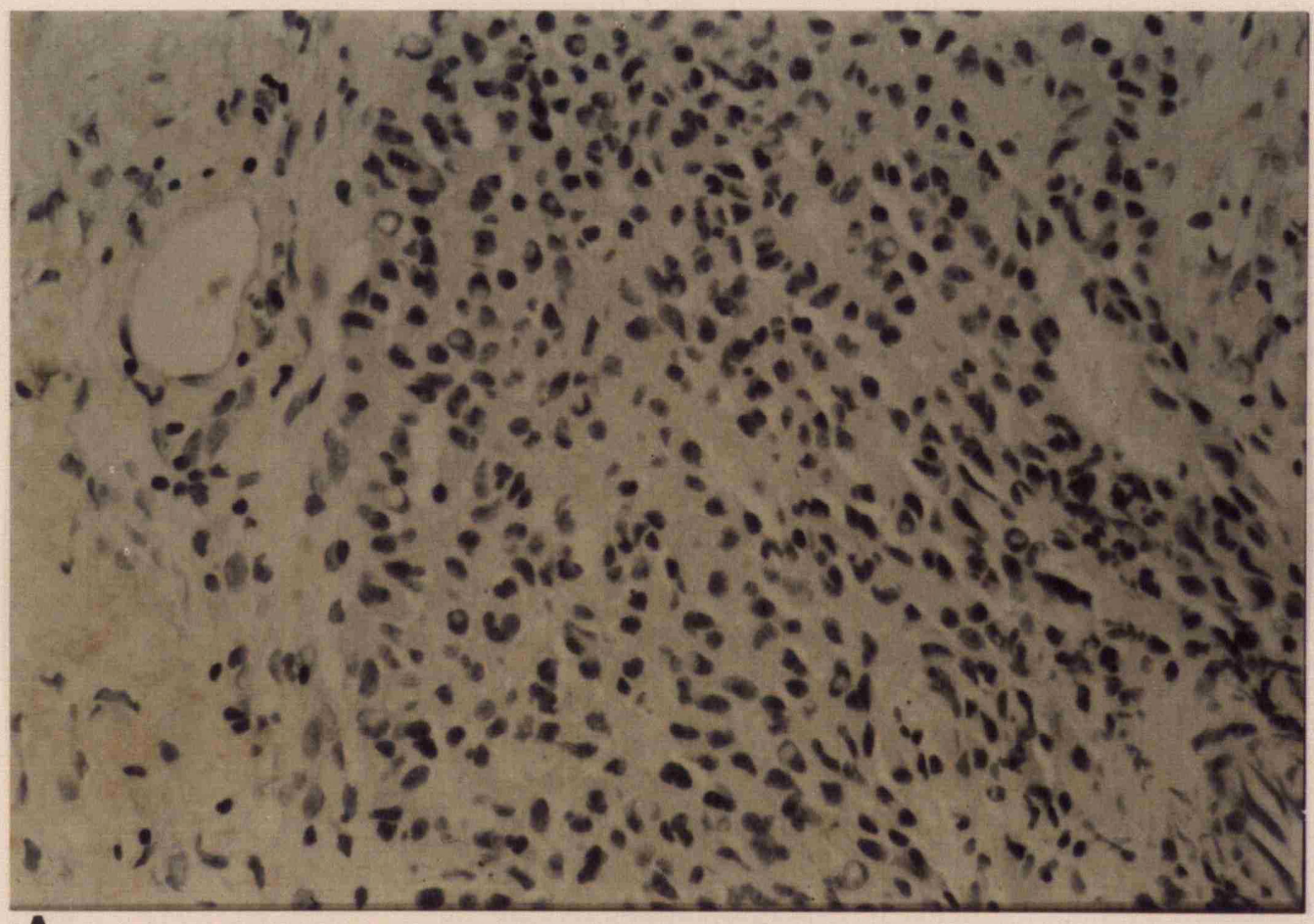
2.3 IMMUNOHISTOCHEMISTRY

For samples where immunohistochemistry was not available from the centre of origin this was performed, on sections taken prior to DNA extraction, by an avidin-biotin complex staining method (Van Noorden et al, 1986). From each fixed paraffin embedded sample eight 5u sections were taken. Sections were deparaffinised in xylene and alcohol and rehydrated in distilled water followed by incubation in 3% hydrogen peroxide for 5 minutes. Sections were then washed in phosphate buffered saline (PBS) and incubated for 20 minutes in 20% normal swine serum (Dako A/S, Denmark).

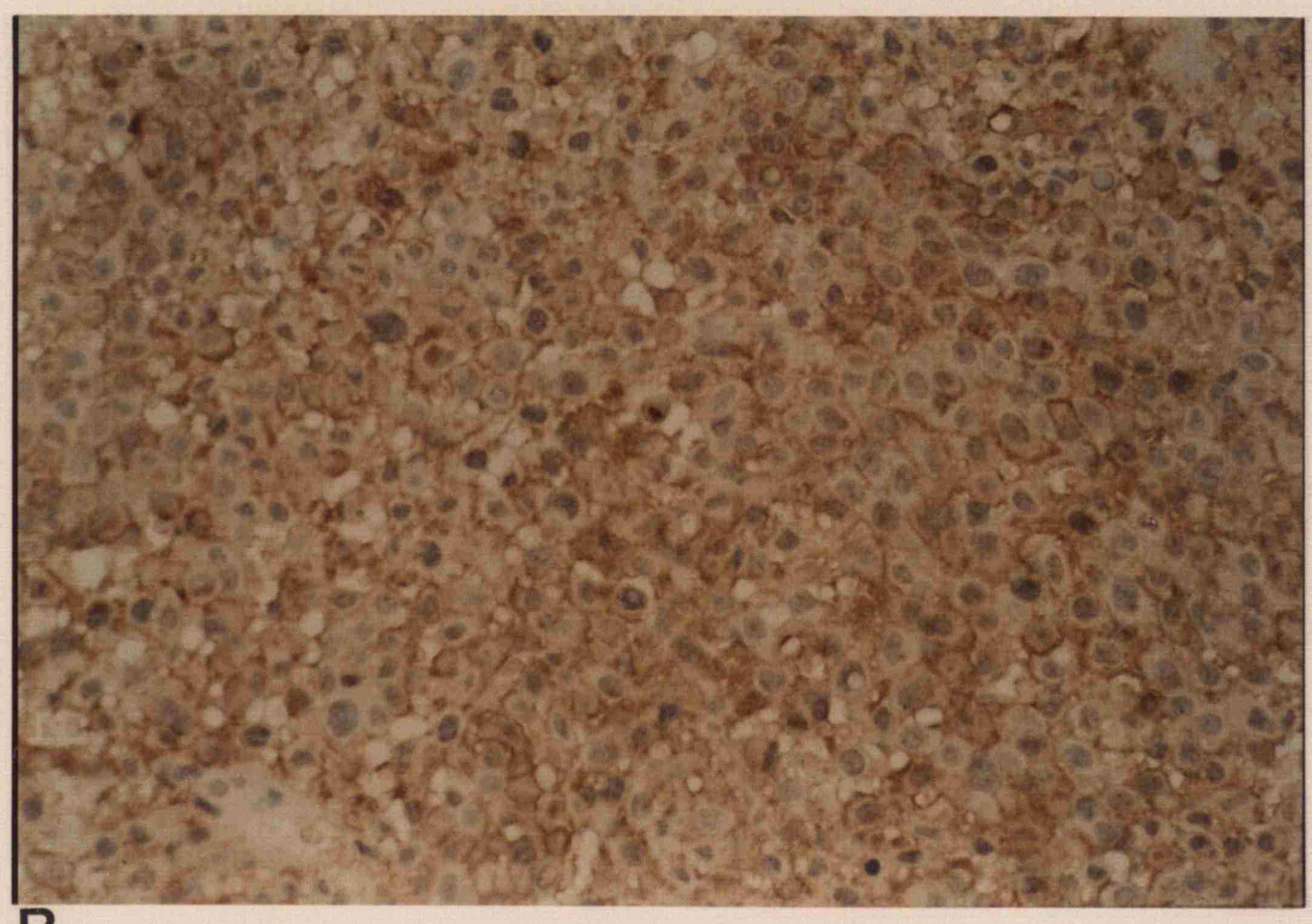
This was followed by incubation of each section in one of seven rabbit anti-sera to anterior pituitary hormones (Biomeda Corporation, USA) - LH, FSH, GH, ACTH, TSH, PRL and α-subunit - for 30 minutes with one section as negative control. Sections were rinsed in PBS and incubated with biotinylated swine anti-rabbit immunoglobulin (Dako A/S, Denmark), 1:200 dilution in PBS, for 30 minutes. After a further PBS rinse sections were incubated with freshly prepared avidin-biotin complex (Dako A/S, Denmark) for 30 minutes and then developed in 3,3-diaminobenzidine tetrahydrochloride (DAB) for approximately 10 minutes. Sections were then rinsed in running water and mounted for viewing[fig1].

Immunohistochemical data is available or 86 of the 88 samples

Figure 1 - Immunohistochemical staining of pituitary tissue from a patient with Cushings syndrome. Panel A - Stained for prolactin; Panel B - Stained for ACTH. In B widespread positive staining (golden-brown) is seen confirming the presence of ACTH within most cells. Staining for prolactin is negative.



A



B

studied, for the remaining two samples insufficient tissue remained to allow this to be performed. Full results are given in appendix 2.

3. ALLELIC DELETIONS IN PITUITARY TUMOURS

3.1 INTRODUCTION

Over recent years allelic deletions involving regions of specific chromosomes have been identified in many tumours (Seizinger et al, 1991). Though deletions involving certain regions appear to be common to many tumours others are specific to individual tumour types or syndromes. The finding of a common area of deletion in a tumour type suggests the presence of a gene or genes in that region whose inactivation promotes tumour formation, such genes have been called recessive oncogenes or tumour-suppressor genes. A number of these genes have now been identified by a 'reverse genetic' approach, most notably the retinoblastoma gene on chromosome 13 (Lee et al, 1987).

In the only study to look for such changes in pituitary tumours deletions were identified in 5 of 27, with no chromosomal region common to the 5 adenomas (Bystrom et al, 1990). However the linkage of the tumour syndrome MEN 1 (parathyroid, pituitary and pancreatic adenomas) to the chromosomal region 11q13 (Larsson et al, 1988. Thakker et al, 1989) and the subsequent demonstration of deletions in this region in MEN 1 and sporadic parathyroid adenomas (Bale et al, 1991) and in an MEN 1 related pituitary adenoma (Yoshimoto et al, 1991) suggest that deletions at the MEN 1 locus may play a role in the development of sporadic pituitary adenomas.

To answer this question and to explore the role of other tumour suppressor loci I have examined a large series of well characterised and histologically confirmed pituitary adenoma samples for allelic deletions with probes from chromosome 11 and

from other autosomes known to harbour tumour suppressor genes.

3.2 METHODS

3.2.1 Source of probes

The probes utilised in this study were obtained from the Human Genome Mapping Project resource centre (Northwick Park Hospital, Harrow, UK) with the exception of pMS51 which was obtained under licence from ICI (Cellmark Diagnostics) and M27ß courtesy of Dr. I.W. Craig (Oxford, UK). Full details of all probes and their originators are give in tables 1 and 2.

Chromosome 11 probes were chosen to span the full length of the chromosome though focusing particularly on the region 11q13, and include the two closest flanking markers to the MEN 1 locus PYGM and D11S146. A schematic representation of chromosome 11, giving the most likely order of these probes based on recent fine mapping of 11q13, is given in figure 1.

The selection of other autosomal probes was based on two factors. Firstly to be as informative as possible, hence the use of variable number tandem repeat (VNTR) probes which are generally highly polymorphic. Secondly for their location in the region of other previously identified tumour suppressor genes, including the RB gene on chromosome 13 and TP53 on chromosome 17.

Probes were all received as purified plasmids. To allow preparation of sufficient probe stocks to complete this study and for future preparations, plasmids were transformed into competent Escherichia Coli.

3.2.2 Preparation of competent E. Coli

Escherichia coli can be made competent to transfection with plasmid DNA by incubation in a cold solution of divalent cations

Table 1 - Chromosome 11 probes

Map Locn	Symbol	Probe Name	Restr. Enzyme	Alleles	Sizes (Kb)	PIC	Originator
11p15	HRAS	pH06TI	BamH1	4	8.2 -6.9	NS	-
11p15	INS	pHins -310	PvuII	3	2.2 -0.6	33%	Dr GI Bell
11p12 -11.2	D11S149	pTHH26	PvuII	2	5.2 -3.2	21%	Dr R White
11q13	PGA	pGA	EcoRI	3	17.8 -13.5	40%	Dr D Porteous
11q12 13.2	PYGM	pMCMP1	Msp1 Taq1	3	2.4 -2.2	46%	Dr Y Nakamura
11q12 -13.2	D11S146	pHB159	Taq 1	2	4.3 -0.8	35%	Dr R White
11q13	D11S97	pMS51	BamH1 Taq1	>10 (VNTR)	2-5	75%	Dr A Jeffries
11q13	INT-2	int-SS6	Msp1	2	8.4 -2.3	35%	Dr C Dickson
11q22	D11S144	pMCT -128.1	Pst1	2	2.9 -2.6	37%	Dr R White
11q23	D11S147	pHBI -18P2		2	5.0 -4.0	30%	Dr R White

PIC - Percentage of chromosome pairs informative for this marker

NS - Not Stated

VNTR - Variable Number Tandem Repeat

Table 2 - Non 11 probes

Map Locn	Symbol	Probe Name	Restr. Enzyme	Alleles	Sizes (Kb)	PIC	Originator
1p35 -32	D1S57	pYNZ2	Pst1	>10 (VNTR)	1 - 3	65%	Dr Y Nakamura
5p	D5S110	pMS621	Pst1	>10 (VNTR)	2 - 5	85%	Dr J Armour
10q26	D10S25	pEFD75	Pst1	7+ (VNTR)	2.0 -3.15	NS	Dr Y Nakamura
13q14	D13S137	pTH162	Bg1II	4+ (VNTR)	8 - 6	60%	Dr R White
17p13	D17S5	pJC -Z16.2	Pst1	>10 (VNTR)	2.0 -3.3	70%	Dr R White
20q	D20S19	pCMM6	Pst1	>10 (VNTR)	2.3 -6.0	76%	Dr Y Nakamura
22	D22S164	pMS619	Pst1	5+ (VNTR)	2 - 4	79%	Dr J Armour
Xp11	DXS255	m27ß	Varies	>10 (VNTR)	3 - 7	70%	Dr I Craig

PIC - Percentage of chromosome pairs informative for this marker

NS - Not Stated

VNTR - Variable Number Tandem Repeat

Figure 1 - Probes used for studies of allele loss on chromosome 11, spanning both 11p and 11q, with a concentration in the region 11q13, locus of the syndrome MEN 1. Also shown are the seven other autosomal probes used in the region of alternative tumour suppressor loci.

Probes Utilised

CHROMOSOME II

HRAS
INS

DIIS149
PYGM
DIIS97
DIIS146
INT2
DIIS144
DIIS147

OTHER AUTOSOMES

CHR 1 : pYNZ2

CHR 5 : pMS621

CHR 10 : pEFD75

CHR 13 : pTHI62

CHR 17 : pJCZ16.2

CHR 20 : pCMM6

CHR 22 : pMS619

(Cohen, 1972), usually Calcium Chloride. To achieve this the following protocol was followed.

Streak plates of the E. Coli strain JM109 were incubated overnight on LB agar at 37°C. A single colony was inoculated into 100mls of LB broth. This was incubated at 37°C with agitation (200 cycles/min) until an OD_{600} of 0.25-0.3 was reached. The culture was then cooled on ice and bacteria collected by centrifugation at 3000rpm (1200g), 4°C for 10 minutes. The resulting pellet was resuspended in 10mls of ice-cold calcium Chloride, 100mM, and incubated on ice for 15 minutes. Centrifugation was repeated and the pellet resuspended in 2mls Calcium Chloride, 100mM. Cells were kept on ice and were now competent for transformation.

3.2.3 Transformation of competent cells

200uL aliquots of competent cell suspension were placed into chilled glass 10ml tubes. Plasmid DNA was added (maximum 50 nanograms per tube) and tubes incubated on ice for 30 minutes. The suspension was heat-shocked in a 42°C water bath for 2 minutes and returned to ice. 2mls of LB broth was added to each tube and incubated at 37°C for 90 minutes to allow plasmid expression. 200ul of suspension was then spread on LB agar plates containing an antibiotic appropriate for the transforming plasmid (generally ampicillin). Plates were incubated at 37°C overnight, scattered colonies of transformed cells were visible after 16 hours incubation. Plates can be stored at 4°C for short periods prior to preparation of plasmid DNA.

3.2.4 Isolation of plasmid DNA

To confirm successful transformation with the appropriate plasmid and to prepare sufficient plasmid DNA for this study the following protocol was used.

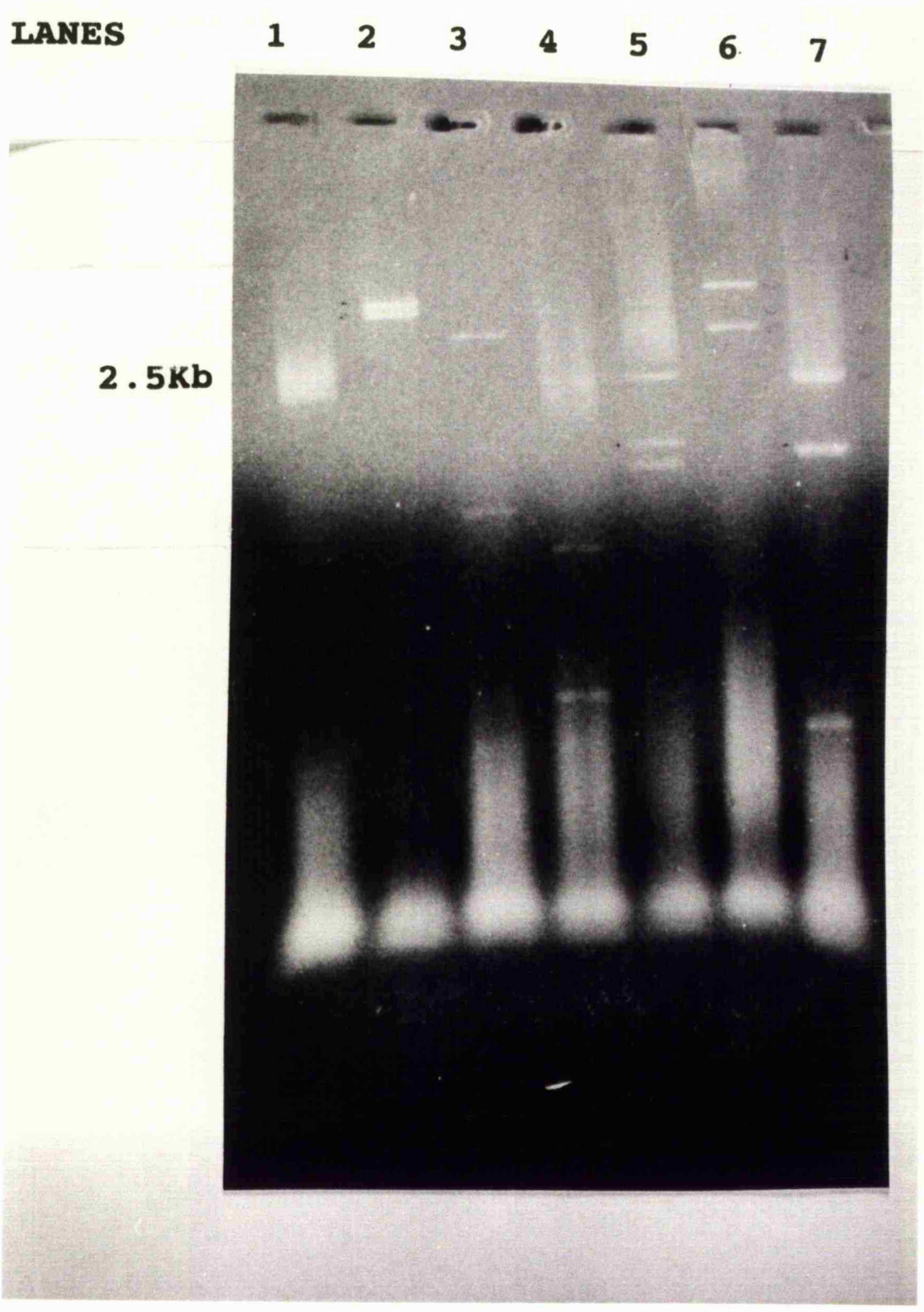
A single transformed colony was inoculated into 10mls of LB broth containing the appropriate antibiotic (ampicillin 100ug/ml). Following overnight incubation at 37°C bacteria were collected by centrifugation at 3000g, 4°C for 5 minutes. The bacterial pellet was resuspended in 200ul 25mM Tris Cl (pH 8.0), 10mM EDTA(pH 8.0), 50mM glucose. To this was added 400ul 0.2MNaOH, 1% SDS. After mixing by inversion this was placed onto ice and 300ul 5M Potassium acetate (pH 4.6) added. The suspension was vigorously mixed and placed on ice for 5 minutes. After centrifugation at 12,000g, 4°C for 5 minutes the supernatant was transferred to a fresh tube. This was then extracted with an equal volume of phenol/chloroform (1:1) and DNA precipitated by addition of 800ul isopropanol. DNA was pelleted by centrifugation at 12,000g, 4°C for 10 minutes and washed with 70% ethanol prior to resuspension in 100ul TE buffer (pH8.0). Typically this method will yield 10-20ug of plasmid DNA which is then stored at -20°C until used.

To confirm the integrity of prepared plasmid 2ul was digested with appropriate restriction enzymes to cut out the full length insert. The resulting DNA fragments were run on 1% agarose gels, with an appropriate size marker, and visualised by ethidium bromide staining[fig2].

3.2.5 Radiolabelling of DNA probes

All probes were labelled in non-linearised form in vectors. Neither linearisation or excision and purification of insert prior to labelling were found to significantly improve

Figure 2 - Agarose gel electrophoresis of digested plasmid preparations to confirm insert size and quality of product. In each case the distal fragment(s) represent the excised insert. For hybridisation probes were labelled within plasmids. Probes are as follows: Lane 1 - pEFD75 (2.5Kb, 2.4Kb), 2 - pHO6T1 (7.6Kb, 6.6Kb), 3 - pMS1-27 (4.0Kb, 1.2Kb), 4 - pHBI59 (2.5Kb, 0.9Kb, 0.7Kb), 5 - pMHZ15 (2.5Kb, 1.8Kb, 1.7Kb), 6 - pTH162 (12.0Kb, 4.0Kb), 7 - pMCMP1 (2.5Kb, 1.7Kb, 0.6Kb).


LANES
1
2
3
4
5
6
7
2.5Kb

hybridisation results. Labelling was performed using the random-primer extension method (Feinberg and Vogelstein, 1983). With this technique hexanucleotides of random sequence are allowed to anneal to denatured DNA and prime DNA synthesis by the Klenow fragment of E. Coli DNA polymerase I. The reaction mixture contains a ^{32}P labelled nucleotide which is incorporated into the synthesised DNA strand.

Reactions were performed using the Random Prime DNA Labelling Kit (Boehringer Annheim, GmbH) and ^{32}PdCTP (Amersham International PLC). Full details of reagents may be found in the booklet which accompanies the Random Prime DNA Labelling kit.

A typical reaction mixture was as follows:

Plasmid DNA (50-100ng)	1-5ul
Hexanucleotide Mixture	2ul
Cold Nucleotides (G,A,T)	3ul
^{32}PdCTP	2.5ul
Klenow fragment (2 units)	1ul
Sterile Water to 20 ul	

This was incubated at 37°C for 1 hour. The reaction was terminated by addition of 2.5ul of loading buffer followed by the addition of sterile 3x SSC to a volume of 100ul. Unincorporated nucleotides were removed using sephadex G-50 spun columns, equilibrated with 3x SSC. Specific activity of the eluted probe was assessed using a 1ul aliquot measured in a beta counter (Beckman LS 1801). Probe ideally should be used immediately but is stable for short periods stored, in an appropriate container, at -20°C.

3.2.6 Restriction enzyme digestion of genomic DNA

DNA probes detect RFLPs because of the presence or absence of specific cutting sites for restriction endonucleases. To detect such polymorphisms DNA must first be digested to completion by a specific endonuclease prior to electrophoresis to fractionate DNA fragments according to size. The appropriate enzyme for each of the probes utilised is given in Tables 1 and 2.

The activity of commercially available endonucleases is expressed in units, where 1 unit will cleave 1ug of lamda DNA in 1 hour. In this study all enzymes were obtained from Pharmacia (P-L Biochemicals Inc, Wisconsin) and reactions performed in standard buffer (One-Phor-All, Pharmacia). Paired leucocyte/tumour DNA samples were digested overnight in 40ul volumes containing 5ug genomic DNA and a 4x excess of restriction endonuclease (20 units) at the appropriate temperature for each enzyme.

3.2.7 Agarose gel electrophoresis

Digested DNA was size fractionated in agarose gels. These are simple to prepare and run and by varying agarose concentration allow resolution of DNA fragments of between 400bp and 20Kb. For probes in this study an agarose concentration of 1% was used, with the exception of PYGM (pMCMP1) where a concentration of 1.4% was used to improve resolution of similarly sized alleles.

Gels were prepared in perspex moulds (volume 300mls), 20 wells/gel. Agarose (Sigma Chemicals, Type 1, low EEO) was boiled in 1x TAE buffer to dissolve, ethidium bromide 0.5ug/ml was added prior to pouring. Once set, gel forming comb and sealing tape were removed and the gel placed into submarine electrophoresis tank containing 1x TAE buffer, 0.5ug/ml ethidium bromide

sufficient to just cover the gel. To each restriction digest 4ul of loading buffer was added and samples applied to the gel. Gels were of 20 lanes, nine paired leucocyte/tumour digests were run on each. In addition a standard size marker was run on each gel - a Hind III digest of lamda DNA - which gives 8 fragments of sizes: 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125bp.

Gels were run at constant voltage, generally 37 volts, for a period of twenty hours. The only exception to this was or the probe pMCMP1 where a prolonged run was performed (40 hours) to improve resolution of similarly sized alleles.

Following completion of electrophoresis each gel was examined under ultraviolet transillumination. Ethidium bromide, which intercalates between the strands of dsDNA, fluoresces in ultraviolet light and this allow a permanent record to be made of the gel by polaroid photography. From this the distance migrated by standard size markers can be recorded and relative DNA load in each lane can be estimated from the intensity of ethidium bromide staining.

3.2.8 Southern Transfer

Prior to blotting gels were denatured for 1 hour in a solution of 1.5M NaCl, 0,5 NaOH with gentle agitation, followed by neutralization in 3.0 NaCl, 0,5M Tris (pH 7.0) for 1 hour. Southern blotting (Southern, 1975) was performed overnight, using 20x SSC as buffer, onto nylon membranes (Hybond N, Amersham). Following transfer membranes were removed and DNA permanently fixed by exposure to UV irradiation on a transillumination box for 5 minutes (optimum exposure previously determined). Gels were re-examined to confirm complete transfer of DNA. Following UV

cross-linking membranes were gently washed in 3x SSC to remove any adherent agarose and allowed to air dry.

3.2.9 Membrane hybridisation

The rate of hybridisation of denatured immobilised DNA and denatured DNA probe sequences is dependant upon a number of factors. Of these the most important are temperature, ionic concentration and pH of buffer, length of probe and the presence of inert polymers and blocking agents in the hybridisation buffer. During early experiments an aqueous hybridisation solution was used, consisting of 4x SSc, 10x Denharts solution (to saturate DNA binding sites on the membrane), 5% dextran sulphate (increasing effective probe concentration), sodium tetrapyrophosphate (to stabilise pH during hybridisation) and an excess of sonicated, denatured salmon sperm DNA, (see appendix 1 for full details of preparation).

Though successful, a number of blots had excessive background activity with use of this buffer and for later experiments a phosphate buffer (see appendix 1) was utilised which gave less background and was both simpler to prepare and had a longer shelf-life.

Hybridisation was performed in double sealed plastic bags, 1-2 blots/bag in a volume of 12-15mls of buffer. All stages, pre-hybridisation, hybridisation and washing were performed at 65°C. Pre-hybridisation was for a minimum of 4 hours at which time buffer was extruded from the bags and fresh, warmed hybridisation solution containing denatured, labelled probe (1-5ng/ml) was added. After re-sealing hybridisation was performed for 16-20 hours. With a number of probes containing repetitive DNA

sequences (pMCMP1, pMS51 and pMCT128.1) an excess of denatured human placental DNA (250ug/ml) was added to the hybridisation buffer to compete out these sequences and reduce background hybridisation (Sealey, 1985).

3.2.10 Membrane washing and autoradiography

To remove non-specifically bound probe blots were washed repeatedly to high-stringency with monitoring of the membranes between washes using a hand held geiga counter. A typical series of washes consisting of 3 in 3x SSC, 0.1% SDS for 20 minutes with moderate agitation followed by 1 wash in 1x SSC, 0.1% SDS for 20 minutes and where necessary a further wash in 0.1% SSC, 0.1% SDS. Membranes were then wrapped in Saran wrap and exposed to X-ray film (Fuji Photographic) in light proof cassettes with intensifying screens at -70°C. Exposure times varied from 2-10 days depending upon the strength of signals obtained.

3.2.11 Removal of probe and repeat hybridisation of membranes

DNA immobilised on nylon membranes may be probed repeatedly (up to 10x) with only a minor decrease in resultant signal intensity. To allow stripping of hybridised probe from blots the membranes must not dry out. To remove probes following autoradiography membranes were incubated in 0.4 NaOH at 45°C for 30 minutes and then transferred into 0.1x SSC, 0.1% SDS, 0.2M Tris-Cl (pH 7.5) or a further 15 minute. Autoradiography was then repeated to confirm removal of probe prior to repeat hybridisation.

3.2.12 Analysis of autoradiographs

Allele loss in tumour DNA was scored when there was absence or

a marked reduction of signal at one of the alleles by visual assessment. Losses were confirmed and quantitated by image analysis. To correct for variations in DNA quantity between lanes blots were stripped and reprobed with the x-linked sequence DXS255, deletions involving the X chromosome have not been identified in solid human tumours (Seizinger et al, 1991).

Image analysis (Confocal R2000/Fenestra, Confocal Technologies) was performed on autoradiographs obtained with both the autosomal probes and DXS255. The ratio of signal intensity from leucocyte and tumour DNA obtained with DXS255 was used to calculate expected signal intensity in tumour DNA with the autosomal probe. From this the percentage loss in tumour allele signal intensity can be calculated. In preliminary studies a visual impression of allele loss was found to correspond to a >60% loss in intensity of signal.

3.3 RESULTS

3.3.1 Chromosome 11 studies

Allelic deletions involving chromosome 11 markers were identified in 16 tumours (18%). Deletions were found in all four major tumour classes (Table 3), in all cases the deletions encompassed the region 11q13[fig3,fig4]. No limiting deletions were identified to allow fine mapping of the common area of deletion to 11q13. In all diagnostic classes tumours sustaining deletions could not be distinguished from those retaining heterozygosity for chromosome 11 markers on the basis of tumour size, biochemical or histological features. Numbers in each sub-type are however relatively small. In each case allele loss was confirmed by image analysis[fig5]. Full results are detailed in

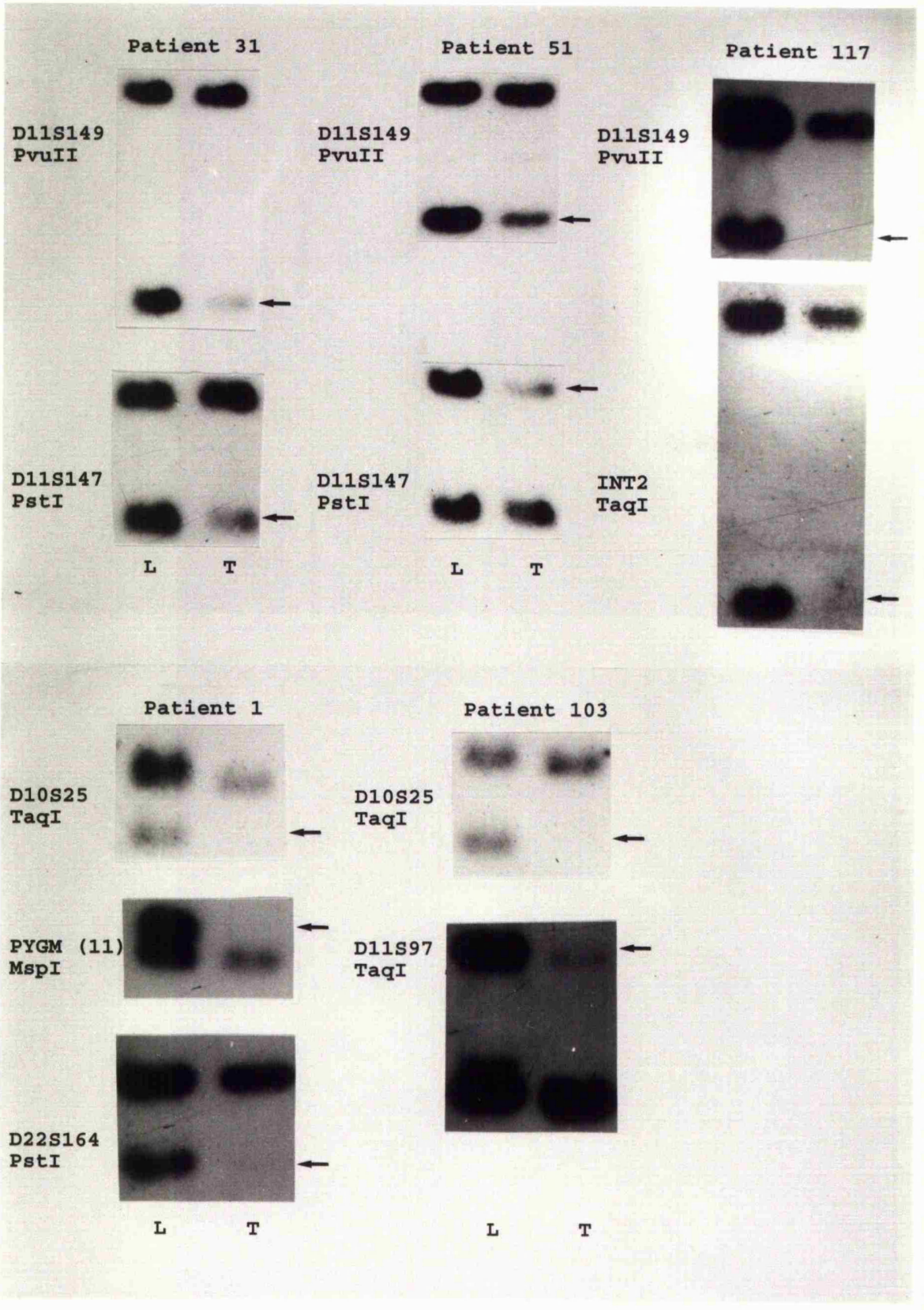
Table 3 - Chromosome 11 deletions by tumour type

TUMOUR TYPE (N)	CHROMOSOME 11 DELETIONS (%)
Non-functioning (35)	7 (20)
Somatotrophinomas (25)	4 (16)
Prolactinoma (17)	2 (12)
Corticotrophinoma (7)	2 (28)
Gonadotrophinoma (1)	0 (-)
Thyrotrophinoma (1)	0 (-)
Mixed GH/PRL (2)	1 (50)

Figure 3 - Schematic representation of alleles on chromosome 11 in 16 patients sustaining deletions. Having established heterozygosity in genomic (leucocyte) DNA, markers in tumour DNA retaining (+) and showing loss (-) of heterozygosity are indicated. o = uninformative. In all tumours the maximum extent of deletion involves the chromosomal region 11q13, locus of the MEN 1 gene. Diagnoses: NF - non-functional, S - somatotrophinoma, P - prolactinoma, C - corticotrophinoma, P/S - mixed (GH/PRL).

PROBE	PATIENT No	1	7	8	31	39	51	58	76	89	103	111	117	121	123	125	126
HRAS		-	•	+	•	•	+	+	+	•	•	+		+		+	+
INS		-	•	+	-	+	•	+	-	•	-	+		•	•	•	
D11S149		•	•	•	-	•	•	•	-	-	-	•	•	•	•	•	
PYGM		-	•	•	•	•	-	•	•	•	•	-		-	•	•	
D11S97		•	-	-	-	•	-	-		-	-			-	•	-	-
D11S146		-	•	-	-	-	-	•	•	•	-	-	-			•	
INT2		-	-	-	-	•	•	•	•	•	•	•	-	•	•	•	
D11S144		•	•	+	•	•	•	+	•	•				+	•	•	
D11S147		•	•	+	-	+	-	•	+	•	-	+	-	•	-	•	
SEX		M	M	M	F	F	M	F	F	M	M	F	M	F	M	F	F
DIAGNOSIS		C	NF	NF	NF	NF	P	NF	C	P	P/S	NF	NF	S	S	S	S

Figure 4 - Matched DNA samples from leucocyte and tumour DNA typed with probes identifying restriction fragment length polymorphisms (RFLP's). Upper panel - Chromosome 11 markers, loss of one allele in tumour DNA is seen at two separate markers on 11q in each patient (arrowed). Patients 31 and 117 - non-functioning adenomas. Patient 51 - prolactin secreting adenoma. The feint residual bands seen in tumour 51 with both markers probably represent contamination with non-adenoma DNA. Lower panel - Two patients with aggressive tumours sustained deletions on multiple autosomes. Patient 1 - a metastatic ACTH-secreting pituitary carcinoma, sustained deletions on chromosomes 10, 11 and 22. Patient 103 - a rapidly recurrent acidophil stem cell adenoma with dual hormone (GH/PRL) secretion, shows losses with markers on chromosomes 10 and 11.


Patient 31
D11S149
PvuII
D11S147
PstI
L
T
Patient 51
D11S149
PvuII
D11S147
PstI
L
T
Patient 117
D11S149
PvuII
INT2
TaqI
Patient 1
D10S25
TaqI
PYGM (11)
MspI
D22S164
PstI
L
T
Patient 103
D10S25
TaqI
D11S97
TaqI
L
T

appendix 3.

3.3.2 Other autosome studies

Examination of other autosomes was complete in 80% of tumours studied, in the remainder the number studied was limited by the quantity of tumour DNA extracted (for full documentation see appendix 2). Each of the VNTR probes used proved highly informative (heterozygosity >65%). Allele loss was found at only low frequency (maximum 6%) on all other autosomes[fig6] (Table 4). Full results are detailed in appendix 3.

3.3.3 Multiple autosomal losses

Three tumours were identified with deletions involving multiple chromosomes, in each case involving chromosome 11 and one or more other autosomes. Two of these tumours were notable for their aggressive growth characteristics. A recurrent, metastatic ACTH secreting tumour was found to have deletions involving chromosomes 10, 11 and 22. The second tumour, a rapidly recurrent acidophil stem-cell tumour with dual (PRL/GH) hormone secretion, had sustained deletions of chromosomes 10 and 11[fig4]. The third, a somatotrophinoma with deletions on chromosomes 1 and 11, though presenting with a high average GH (113 ng/ml, N<5) was not recurrent at 36 months follow-up.

3.4 DISCUSSION

These results suggest that deletions involving chromosome 11 play a role in the development of a proportion of pituitary adenomas of all sub-types. The finding of allele loss in those tumours confirms their monoclonality. Though deletions were

Table 4 - Allelic deletions on other autosomes

PROBE (informative patients)	ALLELIC DELETIONS (%)
pYNZ2, chromosome 1p (48)	3 (6)
pMS621, 5 (65)	0 (-)
pEFD75, 10q (57)	2 (3.5)
pTHI62, 13q (59)	1 (2)
pJCZ16.2, 17p (40)	0 (-)
pCMM6, 20q (56)	1 (2)
pMS619, 22 (51)	3 (6)

Figure 5 - Results of image analysis. 1 - Genomic and tumour DNA from patient 31 probed with the chromosome 11 marker D11S149 and images derived plotted as grey levels. 2 - The same blot reprobed with the X-linked marker M27β.

Signal loss can be calculated by direct comparison of: A/a (using area below each curve). Use of the ratio B/b derived from M27β allows correction for errors in DNA loading in each lane.

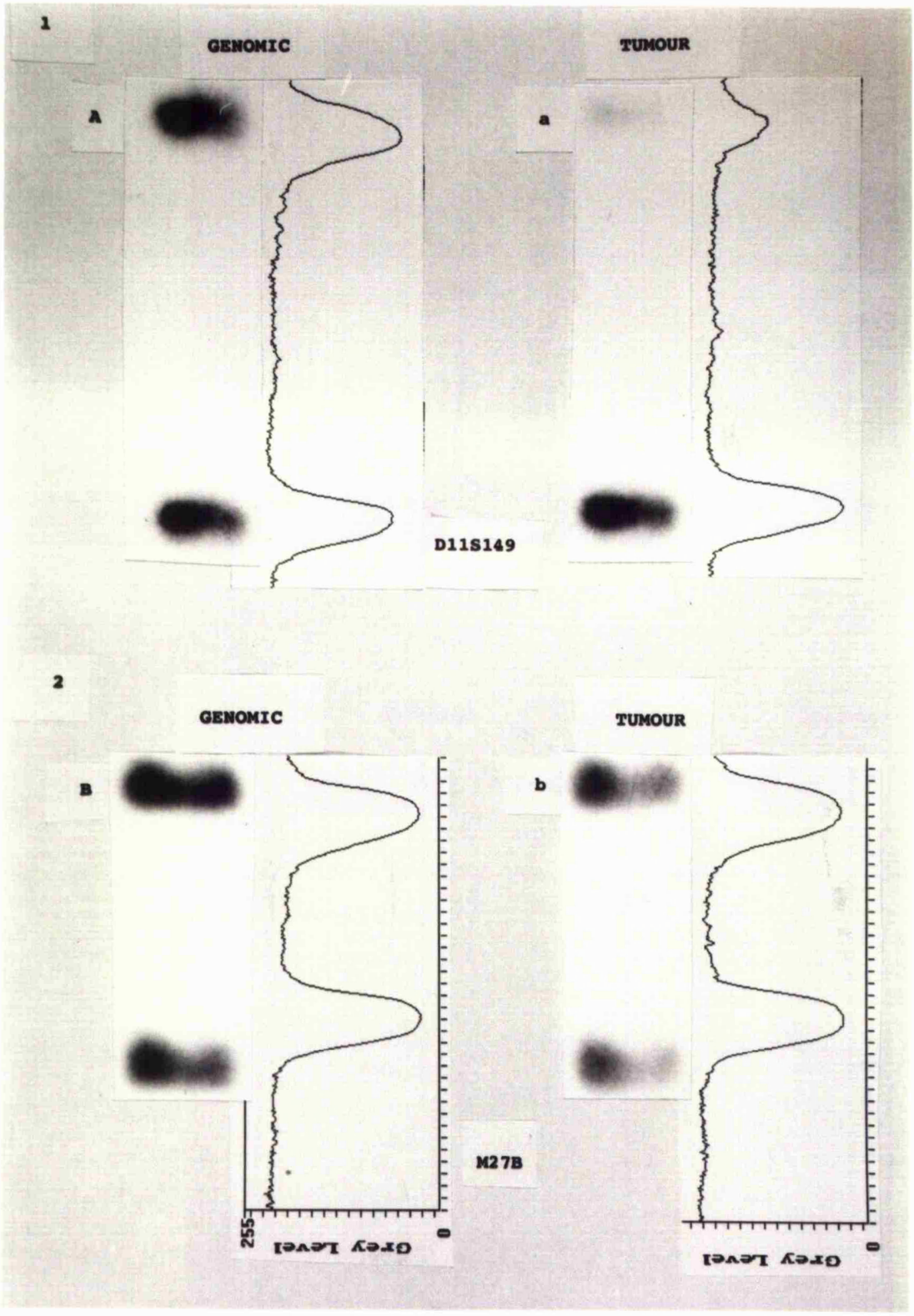
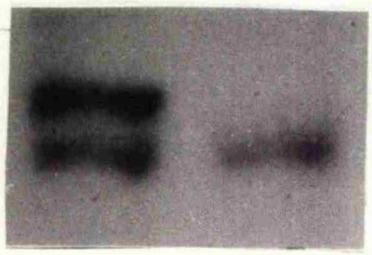

1
GENOMIC
TUMOUR
A
a
D11S149
2
GENOMIC
TUMOUR
B
b
M27B
255
0
Grey Level
Grey Level

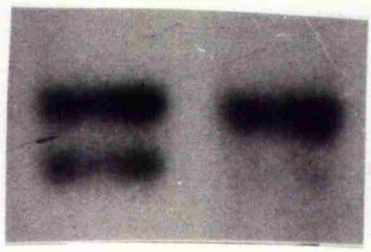
Figure 6 - Allelic deletions demonstrated on autosomes other than chromosome 11. Full details of other autosomal losses are given in appendix 3.

Patient 18



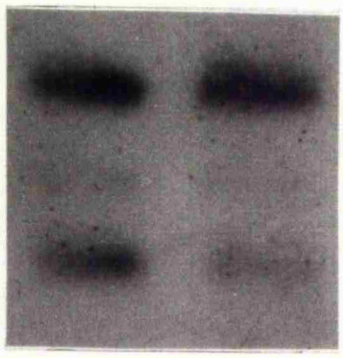
Chromosome 1
(pYNZ2)

Patient 25



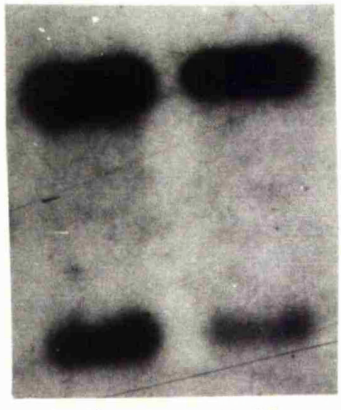
Chromosome 1
(pYNZ2)

Patient 20



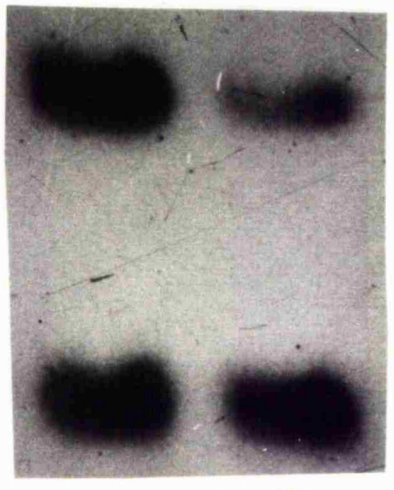
Chromosome 20
(pCMM6)

Patient 11



Chromosome 22
(pMS619)

Patient 57



Chromosome 22
(pMS619)

identified in only a minority of tumours (18%), this is 3x the frequency of deletions observed on other autosomes known to harbour tumour suppressor loci and compares with the frequency of chromosome 11 deletions observed in sporadic parathyroid adenomas (27%). Though chromosome 11 was examined with multiple probes only one of these (D11S97) identifies a highly informative (VNTR) polymorphism, this probe would have identified 14 of the 16 tumours sustaining chromosome 11 deletions. All other autosomes were studied with similarly informative probes.

Our findings differ therefore from the only previous study of sporadic pituitary adenomas where chromosome 11 deletions were found in only 2 of 27 adenomas (9%). The reasons or this are not clear, however the difficulties presented by these tumours both by the size of samples available and their contamination with non-adenomatous tissue can result in allele loss being obscured. By screening all of our samples histologically prior to DNA extraction we hope to have reduced such contamination to a minimum. Despite this a number of our tumours do demonstrate significant residual signals at deleted alleles which are likely to represent non-tumour (connective/normal pituitary) tissue. In addition the use of the probe D11S97, which was not utilised in the previous study, significantly increases the number of patients in whom markers in the region 11q13 are informative. In 3 of the tumours in this series deletions were identified with this probe alone.

No deletions were identified in 72 tumours, all of which were informative at one or more locus on chromosome 11 (average = 4). The failure to detect loss of heterozygosity in these tumours may be because probes presently available do not identify small

deletions in or around a tumour suppressor locus or because tumours may arise by virtue of inactivating point mutations in both copies of a gene. Though our findings may therefore underestimate the role of chromosome 11 mutations in sporadic adenomas it seems likely that pituitary adenomas are heterogeneous at the genetic level. A number of distinct mechanisms may give rise to histologically and functionally similar adenomas (as is apparent with gsp+ and gsp- somatotrophinomas).

Though the MEN 1 gene at 11q13 seems most the likely tumour suppressor locus identified on chromosome 11 to play a role in pituitary adenoma formation, several others have been localised to this autosome. Wilms tumour is associated with deletions at more than one locus on 11p, 11p13 and 11p15 (Mannens et al, 1989. Kouos et al, 1990) and deletions at 11p15.5-15.3 have been reported in a variety of tumours, possibly involving the gene for the Beckwith-Weidemann syndrome (Koufos et al, 1990). Though we are unable to precisely map the common area of deletion in our samples, the probes HRAS and INS at 11p15.5 and 11p15 retain heterozygosity in a number of patients with deletions elsewhere on chromosome 11, suggesting that these loci do not play a role in pituitary tumour formation.

Of the other autosomal probes studied no deletions were detected with a probe in the region of the commonly mutated tumour suppressor gene TP53 (17p13.1) in 40 informative patients. Only 1 of 59 tumours - a prolactinoma - was found to have a deletion in relation to the retinoblastoma gene (RB) at 13q14. Again point mutations or localised deletions in either of these genes as a mechanism for transformation cannot be excluded.

However, in other tumour types where a role or one or other of these genes has been suggested a significant proportion of tumours studied do demonstrate large scale deletions involving closely linked markers.

The identification of two aggressive tumours with multiple chromosomal deletions is of interest. In the previously reported series (Bystrom et al, 1990) a single tumour sustaining deletions on five autosomes (chromosomes 1, 2, 4, 10, 22) but retaining heterozygosity at PYGM and D11S146 on chromosome 11, was described as an 'unusually aggressive' ACTH secreting tumour. Aggressive pituitary tumours, though rare, present difficulties in management and cannot at present be identified at diagnosis on clinical or histological grounds. Early identification of such tumours would be of value in planning patient management in terms of surgical clearance of the pituitary fossa or adjunctive radiotherapy to reduce the risk of tumour recurrence.

Multiple chromosomal deletions are a recognised finding in a number of high grade tumours and the presence of multiple deletions in a small number of aggressive pituitary adenomas is not therefore surprising. However against a low background level of autosomal losses (only 4 of 115 tumours studied show multiple losses) such findings may be a useful marker of tumour phenotype. To assess this further aggressive tumours need to be examined to confirm the association and to determine whether specific chromosomal losses correlate with such characteristics. Deletions involving chromosome 10, sustained by all 3 tumours, have been shown to be associated with the transition from low to high grade astrocytoma - glioblastoma multiforme - as have losses on chromosome 22 (Chung and Seizinger, 1992).

To allow us to study further aggressive tumours, and to attempt to overcome the technical problems presented by pituitary adenoma samples by virtue of their size and heterogeneity, we have examined alternative approaches to identifying allelic deletions. The following chapter describes the development of a method which can demonstrate chromosomal deletions in fresh or archival material from very small tissue samples.

4 IDENTIFICATION OF ALLELIC DELETIONS IN FIXED TUMOUR TISSUE

4.1 Introduction

To allow precise correlation of histological findings with mutational events and to ensure extraction of uncontaminated tumour DNA it was apparent that the methods which we were employing to identify allele loss had to be applied to much smaller tissue samples. It has been demonstrated that DNA can be extracted from paraffin embedded, fixed tissue (Goeltz et al, 1985) and that though not of high molecular weight, it is suitable for analysis by standard molecular biology techniques. However the limited quantities of DNA available from fixed samples are insufficient to proceed with restriction enzyme digestion and DNA hybridisation techniques. If we were to be able to demonstrate allelic deletions from histological samples we required an alternative approach to identification of chromosomal polymorphisms.

Methods to identify allelic deletions utilise inter-chromosomal variations in DNA sequence to separate maternally and paternally derived sequences. Most DNA probes are bi-allelic with variation in allele size determined by the presence or absence of restriction enzyme recognition sites and as such generate limited information. More useful are probes recognising minisatellite repeat elements or variable number tandem repeats (Jeffreys et al, 1985. Nakamura et al, 1987), however these sequences though often highly informative are not evenly distributed along chromosomes and tend to be predominantly telomeric. Recently a further class of polymorphic repeat elements have been identified - termed microsatellites. Short repeat sequences of di- , tri- or tetranucleotides had been

found in a number of sequenced regions of the genome, the commonest of these being the dinucleotide repeat $(CA)^N$ where N is usually between 10 and 40. Where a region had been sequenced by two or more groups it was noted that the number of repeats was variable (Weber and May, 1989). By sequencing the regions flanking these repeats it was possible to design oligonucleotide primers to amplify across them by means of the polymerase chain reaction (PCR). When the products of PCR were separated on polyacrylamide gels they were able to show that these sequences were highly polymorphic and inherited in a Mendelian fashion. Other groups have confirmed this (Litt et al, 1989) and recently by screening a panel of cloned DNA sequences over 600 highly informative (>70%) dinucleotide sequences have been characterised (Weissenbach et al, 1992) and found to be evenly distributed throughout the genome.

The use of PCR means that very small quantities of target DNA are required, hence amplification of DNA extracted from fixed material is feasible to generate polymorphisms. With an informative microsatellite allelic deletions should be identifiable as loss of one PCR product in tumour DNA. To do this required the development of an optimal method for PCR amplification of microsatellites from fixed tissue, there is little published information on this area and each of the available methods had disadvantages for our needs. This chapter describes the development of a method to utilise microsatellite sequences to identify allelic deletions in fixed fresh or archival tissue.

4.2 DEVELOPMENT OF METHODS

4.2.1 DNA extraction from fixed tissue

A number of protocols have been described for the isolation of DNA from fixed tissue (Goeltz et al, 1985. Dubeau et al, 1986. Wright and Manos, 1990. Jackson et al, 1991. Morton et al, 1992). Each of these presented potential difficulties in terms of the size of sample which we wished to analyse and the amount of sample handling required prior to PCR. We have developed a protocol which combines aspects from these methods to give a simple and reliable technique which allows extraction of DNA from a single histological section requiring minimal handling of specimens prior to PCR, thereby reducing the risk of DNA contamination.

This method dos not require the use of a phenol/chloroform extraction step or deparaffinisation of sections, where significant losses of DNA may occur. In parallel experiments omission of deparaffinisation was not found to effect subsequent PCR. A recurrent problem during the development of this method was inhibition of Taq polymerase during PCR (absence of primer-dimer formation). This appeared to be due to the use of the detergent SDS in digestion buffer and was not encountered after this was replaced with the non-ionic detergent Tween-20.

The protocol used was as follows: Ten 5u sections were taken from a paraffin embedded tissue sample, one of these was stained (Haematoxylin/eosin) and examined to identify an area of homogeneous adenoma. This area was removed from the remaining sections with a sterile scalpel blade and placed directly into a 1.5ml microfuge tube. To this was added 100-200ul of freshly prepared digestion buffer; 50 mM Tris Cl (ph8.5), 1m EDTA, 0.5%

Tween 20, 0.2mg/ml proteinase K (Sigma Chemical Company, UK). Samples were incubated at 37°C for 5 days (time can be reduced with a consequent decrease in DNA yield). Samples were spun briefly to remove debris to the bottom of the tube and the supernatant removed to a fresh tube. This was then heated to 95°C for 10 minutes to inactivate the protease and then used directly for PCR (1-10ul/reaction). Samples can be stored at -20°C until required, though some do appear to degrade and become difficult to amplify after a period of 4-6 weeks.

4.2.2 PCR amplification of microsatellites

All groups performing PCR on DNA extracted from fixed material have noted that degradation of nucleic acid during fixation and extraction limits the size of PCR product which can be obtained (Wright and Manos, 1990. Morton et al 1992). Amplification becomes less reliable with products greater than 600bp in length. To avoid this primers were selected to generate products of less than 300bp. From published data informative microsatellite sequences were identified on chromosomes 10, 11 and 22 (Table 1); these being the chromosomes previously found to sustain deletions in two aggressive tumours on which archival material was available.

Primers were synthesised (Applied Biosystems, 391 DNA Synthesiser) and, following deprotection, were precipitated and resuspended in sterile water to give a concentration of 5pmol/ul. PCR was performed in 50ul volumes containing 30pmol of each oligonucleotide primer, 10nmol each of dGTP, dATP, dCTP and dTTP, 50m KCL, 10m Tris Cl (pH 8.3), 1.5m $MgCl_2$, 0.001% gelatin and 0.5 units of Taq polymerase (AmpliTaq, Perkin Elmer Ltd).

Table 1 -Details of microsatellite sequences and PCR primers utilised in this study

Marker	Site	Primers (5'-3)	Hetero zygosity	Allele sizes (BP)	Reference
MFD28	10p	AACACTAGTCACATTATTTTCA AGCTAGGCCTGAAGGCTTCT	80%	142 -156	NAR 1990 18: 4637
MFD28	10q	AAGCTCCCTCGAGATGCACT TTCTTTGCTTTACATGTGGC	67%	115 -125	Marsh - Rel 8
D11S527	11q -13	GCCCCTCTACTTGTCTGGAG ATGCGGCTCCAAGACAAGTTC	88%	142 -166	NAR 1991 19:4790
D11S534	11q -13	ATATGGAAACTCTCGTACT GCAACCATGGAGAGTCTGGA	74%	228 -244	NAR 1991 19: 4308
MFD162	22	GCCTGAAATTATTCCAGCTG AATAGTAGAGTTTGCCTTTC	82%	177 -195	Marsh - Rel 8
MD33	22	AGCCTGGGAGTCAGAGTGA AGCTCCAAATCCAAAGACGT	78%	96 -110	NAR 1990 18:4639

NAR - Nucleic Acids Research

Marsh Rel 8 - Marshfield Markers Primer Sequences (Release 8)

Samples were overlaid with mineral oil and underwent 40 cycles of PCR consisting of 1 minute at 93°C (denaturation), 1 minute at 54-60°C (annealing, temperature dependent upon primers) and 1 minute at 72°C (extension) with a final extension of 5 minutes at 72°C (Hybaid Thermal Reactor, Hybaid Ltd, UK).

4.2.3 Visualisation of PCR products

Low concentrations of DNA can be visualised by a number of methods (Brown TA, 1991). These include radiolabelling of products during PCR, by end labelling one of the primers or including ^{32}PdNTP in the reaction mixture, silver staining of products following gel separation and ethidium bromide staining of gels. Each of these methods has advantages and was assessed during development of this technique. Silver staining adequately visualised products and produced hard copy (by drying down stained gels) but was prolonged and labour intensive. Radiolabelling, by either approach, again allowed visualisation of products but was slow, entailed the risks of handling radioactivity and occasionally products failed to run into polyacrylamide gels. Ethidium bromide, though requiring careful handling, was found to be sufficiently sensitive to visualise products and staining of gels is both rapid and simple. Stained gels can be photographed under ultraviolet illumination to give hard copy and, as results were based on the presence or absence of PCR products, this was an adequate record.

To separate dsDNA of 100-300bp in length a 10% non-denaturing polyacrylamide gel was used (see appendix 1 for preparation). Electrophoresis was performed on a 20cm vertical gel of 1mm thickness (Protean II xi Slab Gel, Bio Rad) in TBE

buffer. Gels were run at constant current (28mA) for 4-5 hours, depending upon product size. Twenty microliters of PCR product was loaded in each lane, after electrophoresis gels were stained in TBE, 0.5ug/ml ethidium bromide for 30-60 minutes. Stained gels were then photographed under ultraviolet transillumination.

4.3 RESULTS

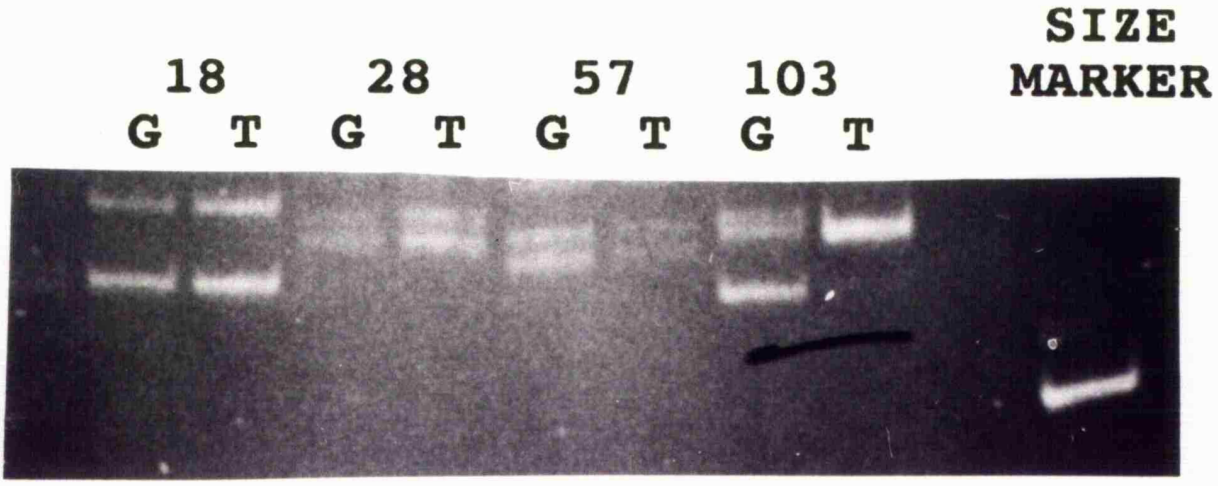
4.3.1 Chromosome 11 deletion demonstrated by PCR

Having established these methods on non-tumour tissue it was necessary to validate the technique with pituitary samples to confirm that this approach would identify allelic deletions. A series of experiments were therefore performed on adenoma samples previously analysed by standard methods. One of the adenomas studied (Patient 103) had sustained deletions of all markers on chromosome 11. Genomic (leucocyte) and slide extracted adenoma DNA was amplified with primers for the 11q13 microsatellites D11S527 and D11S534. Patient 103 was heterozygous at both loci and loss of one PCR product was demonstrated in tumour DNA from this patient[fig1]. All other patients retained heterozygosity in tumour DNA. At D11S527 'mirroring' of PCR products was seen at a higher molecular weight than predicted alleles, this has been found by other groups (Spurr, personal communication) and appears to be primer specific, though the mechanism is unclear.

4.3.2 Demonstration of sequential deletions in serial biopsies

If mutational events can be identified in DNA from archival tissue it is possible to examine the sequence of such events in tumours where serial operative samples are available. For patient 1, found to sustain deletions on chromosomes 10, 11 and 22, three

Figure 1 - Amplification of the microsatellite sequence D11S527 in four matched genomic (G)/slide extracted tumour (T) DNA samples. In patient 103, previously shown to sustain LOH at all markers examined on chromosome 11, loss of one PCR product is seen.


18
28
57
103
SIZE
MARKER
G T G T G T G T

histological samples were available, from 1984, 1986 and 1991. Sections from these were obtained and amplification of genomic and archival tumour DNA was performed with markers on each of these autosomes[fig2]. Genomic DNA was informative with markers on each autosome and the results suggest that a deletion involving chromosome 11 occurred early in this patients clinical course, prior to his first presentation in 1984. This was found with both 11q13 markers. The deletion on chromosome 10 was sustained between 1986 and 1991. With the microsatellites on chromosome 22 the patient was homozygous in genomic DNA with the marker MFD162 and retained heterozygosity in tumour DNA at MFD33. Neither of these markers has been mapped to a region of chromosome 22, nor has the DNA probe pMS619 used for RFLP analysis. As a result we are unable to date the deletion on chromosome 22 and further markers need to be examined in the region of pMS619 when it is precisely mapped. However it is apparent that the deletion of chromosome 22 is partial in this tumour.

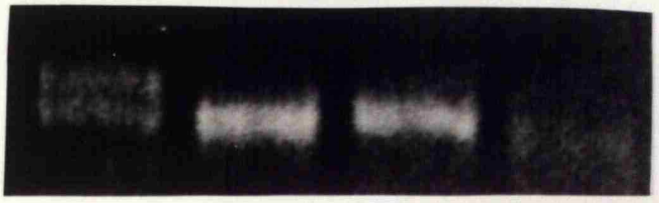
4.4 DISCUSSION

These initial results, in tumours previously examined by standard methods, confirm that a PCR based approach will allow identification of allele loss in mixed, archival tissue. This finding considerably expands the scope of such investigations. It allows correlation of histological and molecular genetic findings at a microscopic level and greatly increases the number and range of specimens suitable for analysis. As a consequence of the relative stability of DNA in fixed tissue any pathological specimens are suitable for analysis, regardless of age.

The major advance of this approach is that, where serial

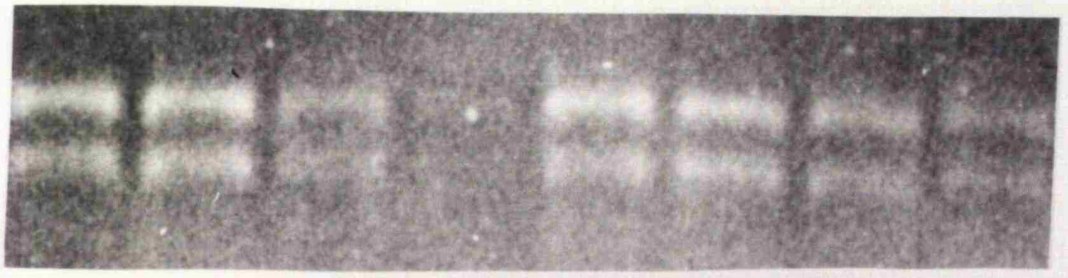
Figure 2 - Microsatellite amplification in serial histological sections from a pituitary carcinoma (patient 1). Operative specimens were available from 1984, 1986 and 1990. A - D11S527, only genomic DNA (G) gives two products, all operative samples give rise to a single product suggesting LOH on chromosome 11 prior to 1984. B - With primers from chromosome 22 (MFD33) no LOH is seen, suggesting that the deletion of chromosome 22 is partial.

A



G 1984 1986 1991

B



G 1984 1986 1991

biopsies are available, it allows the study of sequential genetic events at both dominant and recessive oncogenes in an individual tumour. We have demonstrated such changes in pituitary tumour with multiple autosomal deletions. Clinically this tumour was felt to be non-functioning at diagnosis in 1984 and at recurrence in 1986. Retrospective immunohistochemistry however was positive for ACTH in both specimens suggesting a silent corticotrophinoma. In 1990 the patient represented with florid Cushing's disease in association with a much more aggressive, metastatic, tumour phenotype. Our findings indicate that he sustained a deletion involving chromosome 11 prior to his first presentation in 1984. The deletion of a region of chromosome 10 however occurred only between 1986 and representation in 1990, coincidental with the dramatic change in tumour phenotype and secretory characteristics. This is analogous to the situation in astrocytomas where allelic deletions of chromosome 10 are a feature of the transition from low to high grade tumour (Chung and Seizinger, 1992). Though this association is well established at present no candidate genes for such a 'switch' have been identified.

It has been suggested that tumour progression is related to clonal expansion of cells which have gained a further selective growth advantage over previously transformed surrounding cells (Nowell, 1976). Such progression is implied by models of sequential genetic events in tumour stages (Fearon and Vogelstein, 1991) and is supported in cerebral tumours in relation to the oncoprotein p53 in serial biopsy specimens (Sidransky et al, 1992). In our patient the finding of sequential deletions in a progressive tumour lends this further support,

suggesting that a population of cells acquiring a chromosome 10 deletion have become the dominant cell type as the tumour has progressed and that, though possibly coincidental, this clonal expansion has been associated with the switch from silent to hypersecretory adenoma. To expand upon these findings further aggressive pituitary tumours will need to be examined, both to confirm the association of multiple autosomal losses with tumour phenotype and to define the role of individual autosomes in tumour progression. Though these tumours are not common the use of pathological specimens should make such a study possible. If confirmed it may become feasible to screen for these tumours at the time of initial surgery and adjust patient management accordingly.

At an earlier stage of tumour progression it should be possible to apply this method to the study of the genetic events involved in the transition from pituitary hyperplasia to discreet adenoma formation. This relationship has recently been addressed in parathyroid tissue removed from patients with advanced renal disease (Falchetti et al, 1993), where the transition from hyperplasia to discreet adenoma was found to be associated with loss of chromosome 11 markers in two patients. This question will be of particular interest with respect to ACTH secreting tumours where corticoptroph hyperplasia or no identifiable adenoma is the histological finding in a significant proportion of patients (Burke et al, 1990. Kruse et al, 1992).

In conclusion a PCR based approach to identify allelic deletions expands the scope of such studies. The availability of a large number of mapped and highly informative microsatellite sequences will allow data to be generated from any tissue

specimens chosen for study and should facilitate the fine mapping of these deletions to identify the genes responsible for tumour progression.

5 DISTRIBUTION OF GSP MUTATIONS IN PITUITARY TUMOURS

5.1 INTRODUCTION

It is now established that a sub-group of somatotrophinomas, amounting to a 35-40% of these tumours, express a mutated form of the stimulatory GTP-binding protein Gs (Landis et al, 1989. Spada et al, 1990. Lyons et al, 1990. Clementi et al, 1990). Mutations at two sites; codons 201 and 227 of the Gs α-subunit, result in loss of intrinsic GTPase activity leading to constitutive activation of adenylyl cyclase and the cAMP pathway and thereby, by mechanisms as yet undefined, to GH hypersecretion and somatotroph proliferation (See Chapter 1, figure 8). Though the expression of this mutated protein has been examined in a large number of somatotrophinomas (>100) few pituitary tumours of other sub-types have been studied. Lyons et al identified GSα mutations in 19 of 42 somatotrophinomas but in none of 24 other pituitary adenomas (PRL - 12, ACTH - 7, NON-FUN - 3, TSH - 2).

We have therefore examined the distribution of mutations in GSα in our large series of tumours both to confirm and expand previous findings and to assess the role of these mutations in other tumour types, in particular in non-functional tumours. In addition to those already established we have looked for other possible mutations at these codons, both for amino acid substitutions and 'nonsense' mutations. This chapter describes the results of these experiments and the correlation of findings with biochemical and radiological data.

5.2 METHODS

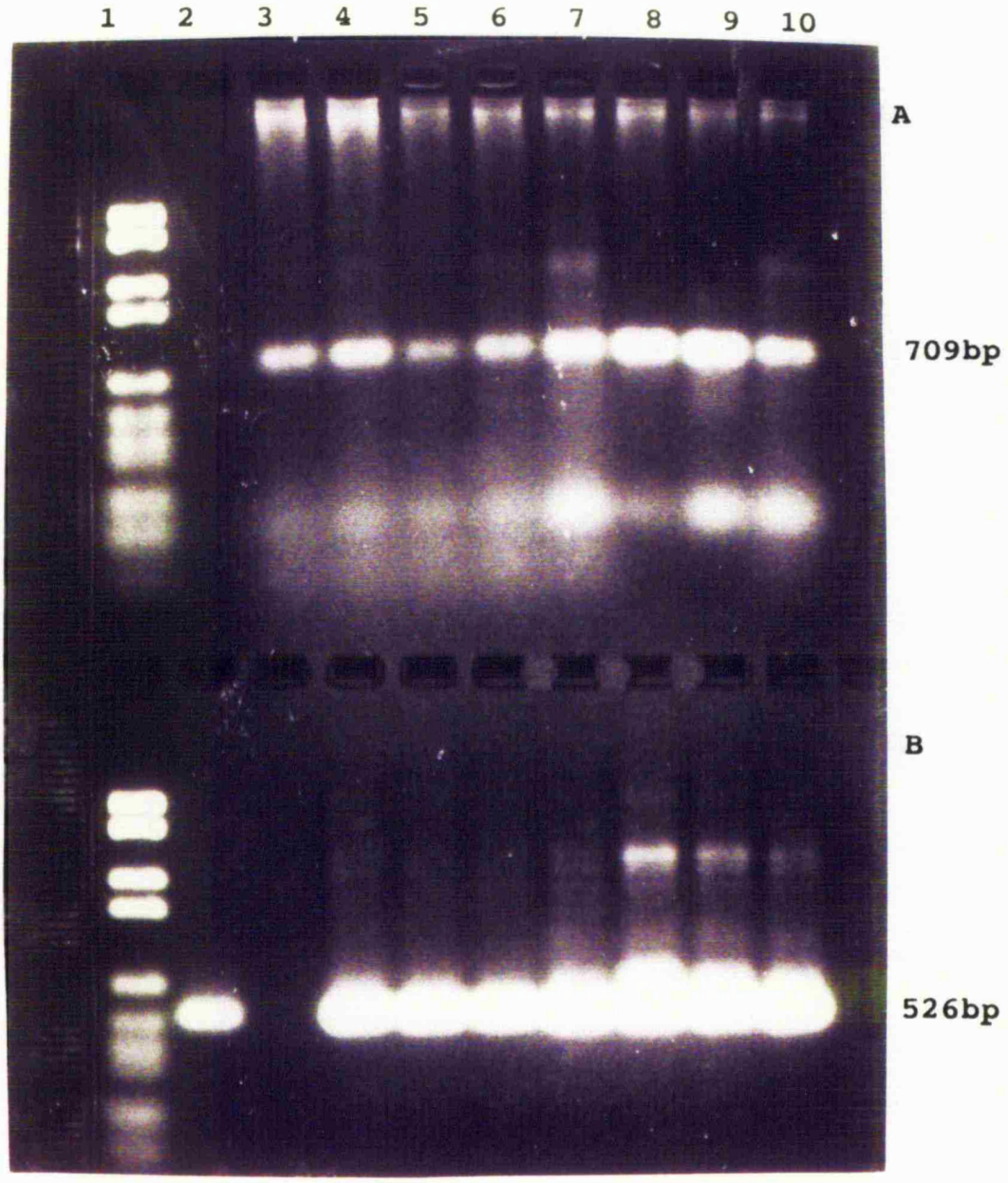
5.2.1 Amplification of Gsα

Exons 7-10 of the gene encoding the α-subunit of Gs were amplified using nested primers to maximize yield and specificity (Lyons et al, 1990). 100ng of tumour DNA and positive control mutant DNA (see Acknowledgements) were amplified with the outer primers 5-GCGCTGTGAAACACCCCACGTGTCT-3 and 5-CGCAGGGGGTGGGCGGTCACTCCA-3 using 30pmol of each per reaction in 50ul of 10nmol dNTPs, 10mM Tris (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin and 0.75 units Taq polymerase (Perkin Elmer Cetus). Amplification cycles consisted of 5 minutes at 95°C, followed by 30 cycles of 1 minute 94°C, 1 minute 50°C and 1 minute 72°C. This resulted in a product of 709bp, 2ul of reaction mix was then subjected to a further amplification with the inner primers 5-GTGATCAGCAGGCTGACTATGTG-3 and 5-GCTGCTGGCCACCACGAAGATGAT-3 under the same conditions. The final product was checked by visualisation of 5ul of reaction mix on agarose gels with ethidium bromide staining[fig1]. The second amplification resulted in a product of 526bp.

5.2.2 Preparation of dot-blots

4ul of product from the second amplification was denatured in 56ul of 0.4M NaOH, 25mM EDTA at room temperature for 10 minutes. A positively charge nylon membrane (Hybond N+, Amersham) was rinsed in a similar denaturing solution and mounted on a dot-blot apparatus. Samples were then spotted onto the filters and allowed to air dry.

Figure 1 - Products of nested PCR amplification of exons 7-10 of Gsα. A - Initial amplification giving a product of 709bp (marker: lamda-HindIII). Lanes 1 - Marker, 2 - Negative control, 3-10 - Amplified tumour DNA samples. B - Second amplification with inner primers resulting in final product of 526bp. Lane 1 - Marker, 3 - Negative control, 2+4-10 - Products of second amplification of tumour samples.


1
2
3
4
5
6
7
8
9
10
A
709bp
B
526bp

5.2.3 Allele specific oligonucleotide hybridisation

Sixteen oligonucleotide probes were synthesised (Applied Biosystems, 391 DNA Synthesiser) specific for the wild-type sequence and all possible amino-acid substitutions or transcription terminating mutations at codons 201 and 227. The oligonucleotides and the mutations they encode are detailed in Table 1.

For hybridisation probes were ^{32}P end-labelled. 10pmol of oligonucleotide was incubated at 37°C for 45 minutes with 2ul buffer (One-Phor-All, Pharmacia), 5ul $\tau^{32}P$-ATP (50uCi), 10u T4 Polynucleotide Kinase (Pharmacia) and 11ul sterile water. The mixture was then heated for 10 minutes at 68°C and DNA precipitated by addition of 40ul sterile water, 240ul 5M sodium acetate, 750ul 100% ethanol and incubation for 2 hours at -20°C. Probe was then centrifuged at 12,000g for 30 minutes and the supernatant decanted off. The resulting pellet was vacuum dried and then resuspended in 100ul TE buffer (pH 7.5).

Filters were prehybridised, in double sealed plastic bags, in 5x SSPE, 0.5% SDS, 1mg/ml denatured salmon sperm DNA at 50°C for 30 minutes. Labelled oligonucleotide was added (10pmol) and incubated for 30 minutes. Filters were then washed twice at room temperature in 2x SSPE, 0.1% SDS for 5 minutes. A final wash in 4M tetramethyl ammonium chloride, 0.2% SDS, 50mM Tris Cl (pH 8.0) was performed for 10 minutes at temperatures specific for each oligonucleotide (Table 1). Filters were then exposed to X-ray film overnight (Fuji Photographic). For subsequent experiments filters were stripped as for Southern hybridisations (see chapter 3.2.11).

Table 1 - Allele specific oligonucleotides specific to codons 201/227 of $G_s\alpha$. Base changes in bold.

Oligonucleotide Sequence	Mutation Encoded	Washing Temparature
TTCGCTGCCGTGTCCTGACT	201, Wild Type	64.5
TTCGCTGC**T**GTGTCCTGACT	201, Cysteine *	"
TTCGCTGC**A**GTGTCCTGACT	201, Serine	"
TTCGCTGC**G**GTGTCCTGACT	201, Glycine	"
TTCGCTGCC**T**TGTCCTGACT	201, Leucine	"
TTCGCTGCC**C**TGTCCTGACT	201, Proline	"
TTCGCTGCC**A**TGTCCTGACT	201, Histidine *	"
GTGGGTGGCCAGCGCGATGA	227, Wild Type	59.0
GTGGGTGGC**T**AGCGCGATGA	227, Stop Codon	"
GTGGGTGGC**A**AGCGCGATGA	227, Lysine	"
GTGGGTGGC**G**AGCGCGATGA	227, Glycine	"
GTGGGTGGCC**T**GCGCGATGA	227, Leucine *	"
GTGGGTGGCC**C**GCGCGATGA	227, Proline	"
GTGGGTGGCC**G**GCGCGATGA	227, Arginine *	"
GTGGGTGGCCA**T**CGCGATGA	227, Histidine	"
GTGGGTGGCCA**C**CGCGATGA	227 Histidine	"

* = Previously reported mutation

5.3 RESULTS

5.3.1 Somatotrophinomas

Gsα mutations were identified in 9 of 25 somatotrophinomas (36%). Mutations were all at codon 201 and consisted of Arg-Cys (201C) in 8 tumours and Arg-His (201H) in a single tumour[fig2]. Group 1 (gsp -) and group 2 (gsp +) tumours were compared on clinical grounds (Table 2). No significant difference in pre-operative growth hormone levels was found between the two groups. Group 2 tumours were however generally radiologically smaller than group 1.

Table 2 - Clinical characteristics of group 1 and 2 somatotrophinomas

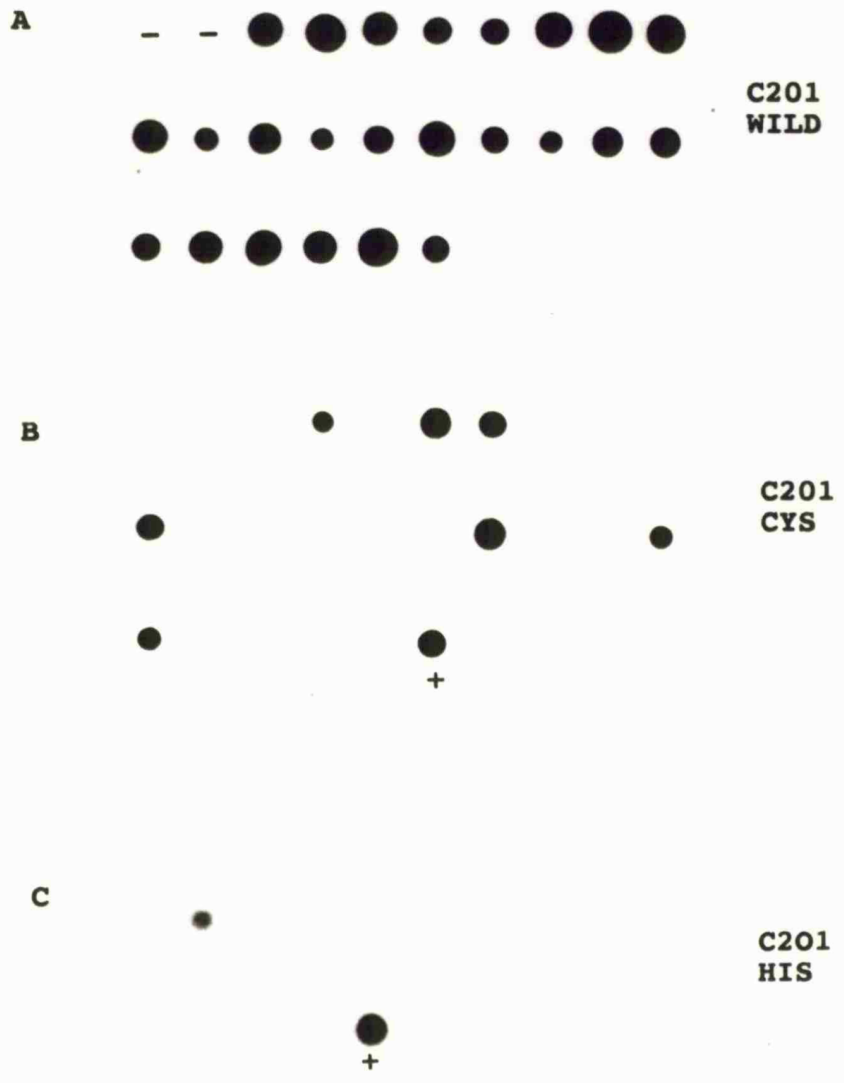
	Average GH Level mU/L (SD)	Tumour Size
Group 1 (gsp -) N = 16	46.2 (17.1)	5 microadenomas 11 macroadenomas
Group 2 (gsp +) N = 9	48.6 (10.4)	5 microadenomas 4 macroadenomas

5.3.2 Other tumour sub-types

In all other tumours examined (N = 63) no mutations at codons 201 or 227 of Gsα were identified with oligonucleotides specific for reported or novel base changes, confirming that Gsp mutations are specific to somatotrophinomas.

Figure 2 - Results of allele specific oligonucleotide hybridisation studies at codon 201 of $G_s\alpha$. Panel A - all samples positive for the wild-type sequence (- = negative control DNA). Panel B - Arg-Cys mutation, 7 tumours positive (+ = positive control DNA). Panel C - Arg-His mutation, 1 tumour positive. Presence of normal DNA sequence in all tumours confirms this to be a dominantly acting mutation.

Allele specific oligonucleotide hybridisation for codon 201 Gsα mutations



5.4 DISCUSSION

The finding of activating mutations of Gsα in 36% of somatotrophinomas and the preponderance of Arg-Cys 201 is in agreement with published figures. The clinical findings in these two sub-types of somatotrophinoma have varied between reported series. It was initially suggested that group 2 tumours were smaller and had significantly lower GH levels (Landis et al, 1990), another group however found that, though group 2 tumours were again generally smaller, they had higher GH levels (Spada et al, 1990). More recently a further study concluded that there was little clinical or biochemical difference between the two groups including assessment of in vitro mitotic activity (Buchfelder et al, 1992).

As an increasing number of these tumours are analysed it is likely that this conclusion is correct, identification of these mutations at present has no bearing on clinical management, though their presence may determine the tumour response to medical therapy as group 2 tumours are more responsive to inhibitory factors (dopamine agonists, somatostatin) which act via alternative G-protein mechanisms (Spada et al, 1990). It is clear that amongst somatotrophinoms genetic heterogeneity must exist, further genetic mechanisms remain to be identified in the majority. Only a single, non-recurrent, group 2 tumour in our series was found to have chromosome 11 allele loss (patient 123), the absence of deletions in the remaining tumours would suggest that these two mechanisms are independent rather than sequential or additive in somatotrophinoms.

The absence of such mutations in a large series of other adenomas confirms their specificity and would appear to exclude

a role for Gsα in non-functional tumours. It seems likely however that mutations with a similar mechanism will be identified in alternative G proteins known to be involved in signalling in other anterior pituitary cell types. The protein Gq is active in gonadotrophs and though hypersecretory gonadotrophinomas are relatively uncommon many non-functional tumours are immunohistochemically positive for LH, FSH or glycoprotein hormone sub-units. Though the mouse Gq sequence has been determined the human gene has not yet been identified.

6 ARE CELLULAR ONCOGENES AMPLIFIED IN PITUITARY TUMOURS?

6.1 INTRODUCTION

Cellular oncogenes may be activated by a number of mechanisms, point mutation (as with Gsα), translocation (Philadelphia chromosome in chronic myeloid leukaemia, t9:22) and amplification of copy-number with consequent over expression of protein product. Of these, amplification, usually of a normal proto-oncogene sequence, is most commonly reported. Though a number of theoretical and experimental mechanisms for gene amplification have been proposed (Windle and Wahl, 1992. Schminke, 1992) the role of each of these in vivo is not yet clear. Despite this, studies of gene amplification have proliferated and amplified cellular oncogene sequences have been described in a range of tumours, both early and late stage.

Epithelial tumours in particular have been shown to amplify a variety of cellular oncogenes (Schwab and Amler, 1990). Studies in pituitary tumours are limited. U et al (1988) examined a series of 6 oncogenes in 2 adenomas and found amplification (7-10X) of c-fos in a single prolactinoma. No clinical details were reported and no further studies have repeated this finding. There are no other reports of oncogene amplification in pituitary tumours. Transforming activity, in mouse cell lines, suggesting the presence of an activated cellular oncogene was reported in studies of DNA from two of five prolactinomas (Gonsky et al, 1991). Transformed cells were found to contain the human gene sequence HST, localised to 11q13, and expression of this gene was confirmed in a prolactinoma. The significance of this finding is unclear, though amplification of the region 11q13, including the

proto-oncogene sequences INT-2, BCL1 and HST, is reported in a number of tumour types including bladder and breast (Gaudray et al, 1992).

To examine the role of oncogene amplification and re-arrangement in pituitary tumour formation I have screened DNA from a representative series of adenomas by Southern blotting and hybridisation with probes identifying 13 recognised cellular oncogenes, including those from 11q13, found to be amplified in other human tumour types.

6.2 METHODS

6.2.1 Probes utilised

For this study probes were obtained from the Human Genome Mapping Resource Centre, Northwick Park Hospital. A list of the oncogenes studied, probes utilised and their originators is give in Table 1. Probes were prepared as described in chapter 3 (3.2.2 - 3.2.5).

6.2.2 Studies of oncogene amplification

Gene amplification was detected by hybridisation of oncogene probes to restriction enzyme digested DNA. Methods for restriction enzyme digestion, electrophoresis, Southern transfer and DNA hybridisation were as previously describe (3.2.5 - 3.2.9). For all probes digestions were performed with the enzyme EcoRI, a frequent cutting enzyme, which gives fragments of suitable size for analysis by these methods.

On each membrane genomic (leucocyte) and normal (early post-mortem) pituitary DNA were included as controls. Each probe was studied in a minimum of 20 tumour samples, representing all

Table 1 - Cellular oncogene probes and their originators

Cellular Oncogene	Map Location	Probe Name	Originator
MYLCL1	1p32	L-myc	Dr JD Minna
MYCN	2p24-23	pNB-1	Dr M Bishop
MYB	6q22-23	c-myb	Dr R Gallo
ERB-B	7p13-12	lamda he-B	Dr NK Spurr
MYC	8q24	c-myc	Dr B Vennstorm
HRAS	11p15.5	pEJ6.6	Dr NK Spurr
BCL1	11q13	p11q13-7	Dr TH Rabbits
HST1	11q13.3	p7RR	Dr OD'Lapeyriere
SEA	11q13	p6.2/HuSEA	Dr MJ Hayman
KRAS2	12p12.1	KRAS2	Dr EH Chang
FOS	14q24.3	p-cFos	Dr I Verma
PDGB	22q12.3-13.1	pC-sis	Dr R Gallo

classes of pituitary tumour and including the two aggressive adenomas found to sustain multiple allelic deletions.

Where possible amplification was identified blots were reprobed with the X-linked sequence DXS255 to correct for DNA loading and subjected to image analysis. A minimum of 2-fold amplification of signal on repeated blots, following correction, was accepted.

6.3 RESULTS

Despite the extensive nature of this study evidence of oncogene amplification was not found in pituitary tumour DNA with any of the probes utilised (Figures 1, 2). In addition no variation in band patterns or sizes was seen between genomic, port-mortem and tumour DNA suggesting that re-arrangement as a mechanism for oncogene amplification was unlikely.

6.4 DISCUSSION

Despite examination of a large number of samples these experiments failed to identify amplification of any of a series of well characterised cellular oncogenes in pituitary tumours. This mechanism is therefore unlikely to play a significant role in cellular transformation in the pituitary. This is perhaps surprising given that tumours of epithelial origin generally overexpress one of a number of these genes. Such changes are however more frequently identified in late stage, malignant tumours. Pituitary adenomas rarely progress to such a phenotype, possibly reflecting the absence of such changes. It has been suggested that tissues which generally develop 'benign' neoplasms may express or preferentially retain genes conferring stability or resistance to metastasis (Sreekantaiah and Sandberg, 1991),

Figure 1 - Normal pituitary and adenoma derived DNA hybridised with a probe specific to the oncogene sequence c-myc. Following EcoR1 digestion of genomic DNA this probe gives two bands at 9.9 and 4.1Kb. No significant variation in signal intensity is seen between samples. Pit - Post-mortem pituitary, Pro - prolactinoma, Cor - corticotrophinoma, Som - somatotrophinoma, NF - non-functioning.

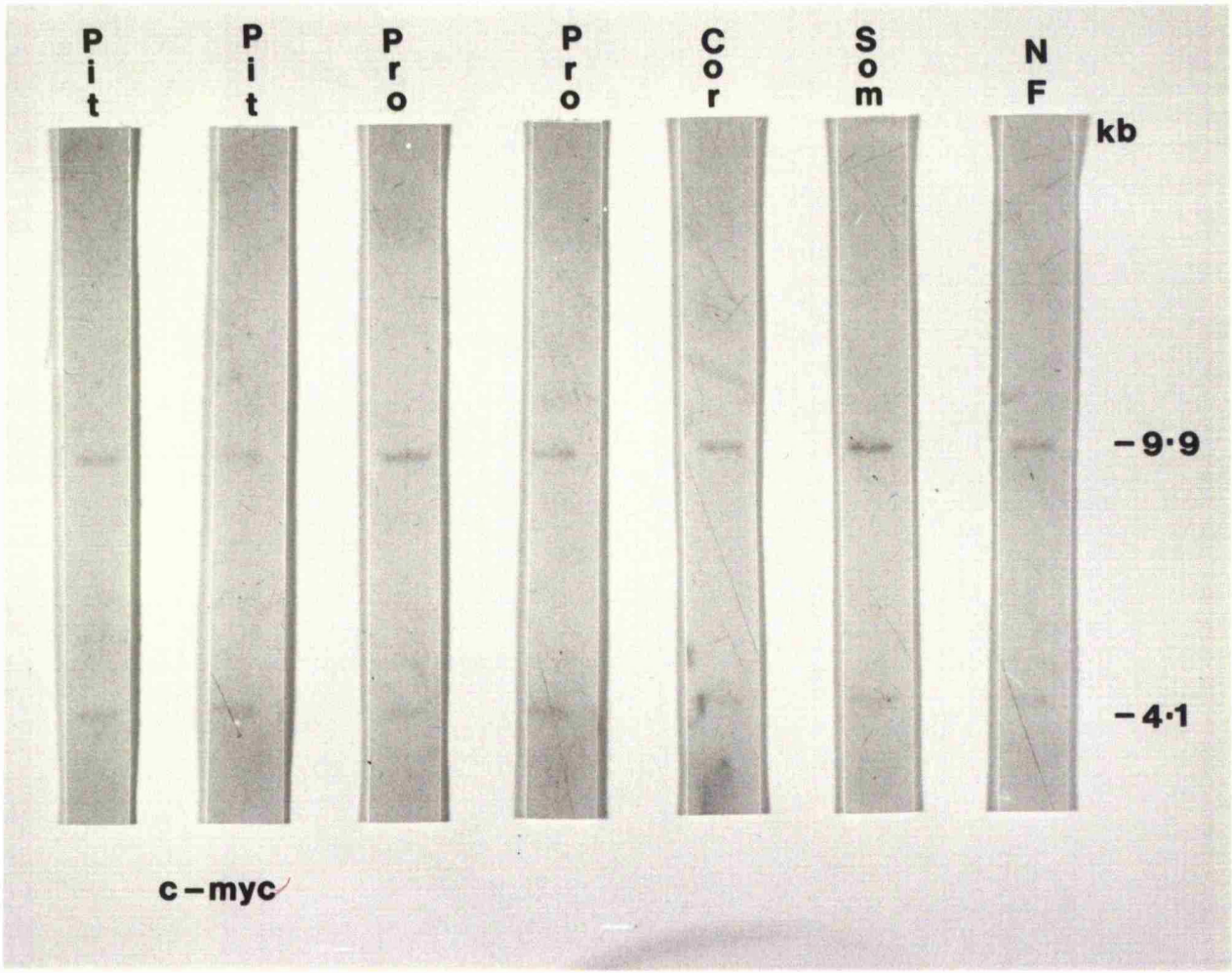
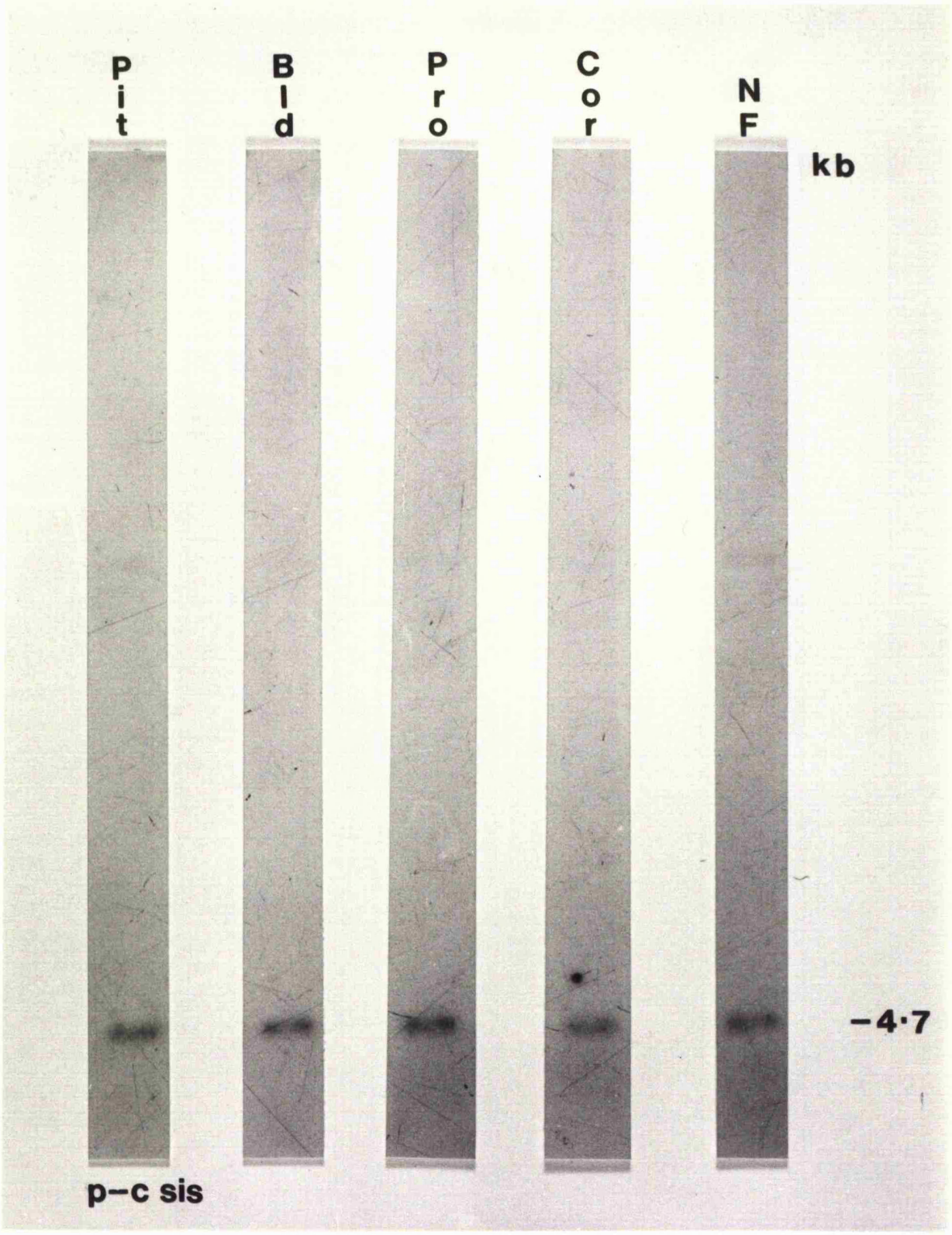

Pit
Pit
Pro
Pro
Cor
Som
NF
kb
-9·9
-4·1
c-myc

Figure 2 - Normal pituitary, leucocyte and pituitary adenoma derived DNA hybridised with probes specific for the sequences of the cellular oncogene c-sis. No significant difference in signal intensity is seen between samples. Bld - Leucocyte, Pro - prolactinoma, Cor - corticotrophinoma, Pit - post-mortem pituitary, NF - non-functional.


Pit
Bld
Pro
Cor
NF
kb
-4·7
p-c sis

alternatively the benign phenotype may reflect patterns of exposure to environmental mutagens. Interestingly neither of the aggressive tumours, previously found to sustain autosomal deletions, demonstrated changes at any of the loci screened, favouring perhaps a 'resistance' to oncogene amplification.

The finding of c-fos amplification in a prolactinoma (U et al, 1988) is therefore an isolated and uncommon event. In addition transforming activity of HST though not addressed by this study does not appear to be related to amplification or rearrangement of this sequence or related sequences within the 11q13 amplicon.

Though excluding a role for amplification or rearrangement of cellular oncogenes in pituitary tumours this approach will not identify activating point mutations in these genes. Such changes have been found in a number of oncogenes, most notably the 'RAS' group where the mechanism of activation was the model for the gsp oncogene. In a study of the RAS gene family (NRAS, KRAS, HRAS) in a series of 19 pituitary tumours an activating mutation in codon 12 HRAS was found in a single prolactinoma. This was described as clinically aggressive and it was suggested that this may be a marker for such a phenotype in the pituitary. Preliminary results in a series of 8 of our tumours, including two of aggressive phenotype, looking for this mutation with allele specific oligonucleotides have been negative.

In summary our findings do not support a significant role for recognised cellular oncogenes in pituitary tumour formation. Further progress seems more likely to come form identification of further 'tumour specific' and novel oncogenes.

7 GENERAL DISCUSSION AND FUTURE DIRECTIONS

It is becoming clear that cellular transformation, in all tissues, arises as a result of accumulated mutational events in genes involved in the regulation of cell turnover and DNA transcription. Such genes may act in either a dominant or recessive manner in relation to balanced cell turnover. An increasing mutational load appears to correlate with more aggressive tumour phenotype and loss of cellular differentiation.

Pituitary tumours are common and histologically and clinically almost invariably benign. They arise from differentiated cells in the anterior pituitary gland whose function is closely regulated by hypothalamic factors. The differentiation in terms of secretory function may not be fixed, there is evidence that cells may alter their secretory characteristics - transdifferentiate - in response to appropriate stimulation even in adult life (Kineman et al, 1992. Horvath et al, 1990). There are also populations of cells - null cells, stem cells - within the pituitary gland which may be pluripotent, tumours of these cells may secrete multiple hormones (Asa et al, 1992).

The majority of tumours however arise in differentiated, functional cells which may hypersecrete intact hormones or in the case of 'non-functional' adenomas glycoprotein hormone sub-units. A minority of tumours secrete more than one product (for example GH/PRL, GH/TSH) though significantly more show evidence of transcription of the genes for multiple hormones (Levy and Lightman, 1993).

Debate over the role of hypothalamic or other extrinsic factors in the initiation of pituitary tumour formation appears to have

been settled by studies of X-chromosome inactivation patterns in adenomas. In the majority of tumours studied to date (>90%), from female patients, a monoclonal pattern of inactivation, implying that an initiating mutation has taken place in an individual pituitary cell with subsequent clonal expansion, suggests an intrinsic pituitary event (Alexander et al, 1990. Herman et al, 1990. Jacoby et al, 1990. Schulte et al, 1991. Gicquel et al, 1992). The finding of loss of heterozygosity in a significant proportion of this tumour series again implies an intrinsic pituitary abnormality. In an attempt to define the nature of such abnormalities this study has examined a number of genetic events which may be involved in cellular transformation in a large series of well characterised pituitary adenomas.

To date the clearest evidence of an intrinsic pituitary defect in the formation of adenomas has come from the finding of the novel gsp oncogene in somatotrophinomas (Landis et al, 1989). Our studies have confirmed the presence of gsp mutations in 35% of such tumours in this series, moreover I have excluded this mechanism in other classes of adenoma in a much larger number of tumours than have been previously reported. Against this a recent study suggests the presence of $G_s\alpha$ mutations in 2 (201C, 227L) of 21 non-functioning tumours and 0 of 4 prolactinomas (Tordjman et al, 1993). Summating our results, these and the only previous study (Lyons et al, 1990) gives a figure of only 2 gsp mutations in 102 non-somatotrophinomas studied.

No other novel oncogenes have been described in pituitary adenomas though another analagous mutation, in the α-chain of G_{i2} - termed gip, has been identified in adrenal and ovarian tumours (Lyons et al, 1990). Increasing data is available on other

recognised cellular oncogenes, however these studies have to date been essentially negative. In screening a panel of 13 such genes for amplification or gross chromosomal rearrangement in a wide variety of tumours this study appears to have excluded such mechanisms in relation to these genes in pituitary tissue. The only previous such study, with the sequence c-fos, detected amplification in a single prolactinoma in a series of 19 tumours (U et al, 1988).

Such results do not address the possibility of activating point mutations or small scale deletions within these genes. Of particular interest are the ras protooncogenes, related to and activated by a mechanism similar to the G-proteins and implicated in a variety of human neoplasms. There are now two reports of molecular screening of the ras gene family in pituitary adenomas (Karga et al, 1992. Herman et al, 1993). In these studies a mutation in codon 12 of H-ras was identified in 1 tumour (a recurrent prolactinoma) of 63 analysed. We have screened a small number of our series, including two aggressive adenomas, for such mutations using allele specific oligonucleotides for codon 12 of H-ras, again with negative results.

The most commonly mutated gene in human tumours is that encoding the protein p53 on the short arm of chromosome 17. This gene may be altered by point mutations, deletions or chromosomal rearrangements (Hollstein M et al, 1991). In a recent paper 44 pituitary adenomas (22 GH, 22 NF) were screened for mutations in exons 5-8 of p53, site of >95% of described mutations, none were found (Herman et al, 1993). Our studies of allele loss involved use of a probe closely linked to p53 (pJCZ16.2, 17p13) and failed to identify any deletions in 40 informative blood/tumour pairs.

In the same studies no alteration in band patterns or sizes generated with this probe were seen in tumour DNA, this would go against any major rearrangement of genes in this region.

A single report suggests the presence of a transforming DNA sequence derived from and transcribed in human prolactinomas (Gonsky et al, 1991). This sequence was identified as the hst-1 gene, a member of the fibroblast growth factor family which is known to be amplified in a number of neoplasms (Gaudray et al, 1992) and which is localised to the region 11q13. We have used a probe recognising this sequence in our studies of cellular oncogenes, no evidence of gene amplification or rearrangement was found though deletions involving this region (11q13), as we have shown, occur in a proportion of pituitary adenomas. The group reporting this finding have recently noted the absence of hst-1 amplification or rearrangement in a further series of 7 prolactinomas (Herman et al, 1993). The significance of the expression and transforming activity of hst-1 in some prolactinomas is therefore uncertain, as is its mechanism.

The role of recessive oncogenes or tumour suppressor genes in human tumours is being increasingly recognised (Seizinger et al, 1991). Many of these genes have been identified initially from their role in inherited tumour syndromes and as such MEN 1 represents an inherited model for pituitary tumour formation. Deletions of the region 11q13 in tumours from MEN 1 patients (Larsson et al, 1988) and the subsequent linkage of the syndrome to this region (Thakker et al, 1989. Bystrom et al, 1990) suggested deletion of a tumour suppressor gene as the underlying mechanism for tumour formation in this condition and hence in sporadic tumours related to MEN 1. In a series of 27 sporadic

pituitary adenomas, 2 prolactinomas sustained deletions of 11q13 (Bystrom et al, 1990). A further study has recently noted a chromosome 11 deletion in 1 of 7 prolactinomas (Herman et al, 1993).

Our finding of such deletions in 18% (16 of 88) of adenomas studied would suggest that this mechanism does indeed play a role in the formation of sporadic adenomas. Identification of the MEN 1 gene is imminent, its eventual localisation and sequencing will allow the extent of its involvement in sporadic tumours related to MEN 1 to be defined. Other autosomes sustain deletions at a low frequency (<6%) as would be expected from the benign phenotype of pituitary neoplasms. As such, numbers of events on other autosomes remain too small to suggest any specific pattern of deletions in pituitary tumours, as compared with patterns now recognised in colorectal carcinoma and other tumours (Fearon and Vogelstein, 1991. Zheng et al, 1991). From our data however it would appear that allele loss on chromosome 11 and gsp mutations are independent events in somatotrophinomas rather than sequential or additive steps in tumour formation.

More recently attention has focused on other tumour suppressor loci in pituitary tumours. Interest in the retinoblastoma gene (RB) was stimulated by the finding that transgenic mice which had been genetically engineered to 'knock-out' one copy of the RB gene in the germ-line developed large, highly malignant and invasive pituitary tumours, which had deleted the remaining RB gene, rather than retinoblastomas (Jacks et al, 1992). Our studies identified a deletion in relation to RB (with the probe PTHI62) in a single, non-aggressive, prolactinoma. A further study in 20 macroadenomas, some of which were rapidly recurrent,

failed to find any abnormalities at the RB locus (Cryns et al, 1993). More recently data has been presented suggesting that allele loss at the RB locus (exon 20) is a feature of very aggressive pituitary tumours. In the single true pituitary carcinoma in our series (patient 1) no loss was found with PTHI62.

Future Directions

This study identifies mutational events in 50% of somatotrophinomas and 20% of other pituitary tumour sub-types. Given that pituitary tumours are monoclonal in origin further somatic mutations therefore remain to be elucidated. Screening of recognised cellular oncogenes, though incomplete, has been essentially negative suggesting that progress is likely to come from identification of other novel oncogenes amongst the receptors and signalling proteins involved in regulation of hormone production and cell proliferation.

Increasing interest has focused on study of aggressive pituitary tumours in an attempt to identify the events governing the switch from benign to aggressive phenotype, the development of PCR based methods in this study represent a move in this direction. Such studies are more likely to be fruitful given the higher incidence of mutational events in aggressive tumours and the presence of common mutations in a wide variety of anaplastic tumours of all origins.

Identification of events common to aggressive pituitary tumours may represent a useful early marker of tumour phenotype and could possibly guide clinical management. Candidates for such a role at present would appear to be RB gene mutations or deletions, as

detailed earlier, or deletions of other as yet unidentified tumour suppressor loci. The finding of LOH on chromosomes 10 and 22 in two aggressive tumours would suggest that these are candidate autosomes for such loci. A further possibility is suggested by a recent study, in four invasive adenomas (2 GH, 1 ACTH, 1 NF), of α-protein kinase-C (Alvaro et al, 1993). Increased expression and activity of this ubiquitous kinase had previously been demonstrated in pituitary tumours (Alvaro et al, 1992). This study found a three-fold increase in α-protein kinase-C (PKC) expression in radiologically invasive tumours when compared to non-invasive and goes on to demonstrate a point mutation in PKC common to all four invasive tumours. The authors speculate on possible effects of this mutated kinase, further studies are required both to confirm the findings and to elucidate the role of PKC in these tumours.

Though these studies will have important implications they are unlikely to have any significant bearing on the pathogenesis of the majority of pituitary adenomas. Both gsp mutations and chromosome 11 LOH appear to be early mutational events associated with tumours of benign phenotype. The full role of the MEN 1 locus in these tumours cannot be defined until it is identified, sequenced and examined in a series of tumours retaining heterozygosity with chromosome 11 markers.

Alternative mechanisms are now being examined, the complex intra-cellular signalling pathways involved in hormone secretion and cell proliferation give rise to a number of candidate loci. The finding of Gsα mutations has focused attention on other G proteins, in particular the Gq family which activate phospholipase-C dependant pathways (Smrcka et al, 1991. Taylor

et al, 1991). These pathways are thought to be active in gonadotrophs though to date the human Gq sequence has not been cloned. G proteins also regulate prolactin secretion, to date however the identities of the G proteins involved (Go, Gi or Gs) and their effector molecules are uncertain (Bouvier et al, 1991).

The activation of G proteins is linked via specific pathways to nuclear proteins which regulate gene transcription, such proteins present a further set of candidate loci. In the case of somatotrophs activated Gs stimulates cAMP synthesis via adenylate cyclase which in turn causes a cAMP dependant protein kinase to activate CREB (cAMP response element-binding protein). Activated CREB binds to the promotor of a nuclear transcription factor - GHF1 or Pit 1 - and positively regulates its transcription.

Pit 1 is a 'tissue-specific' transcription factor which regulates the function of the GH, PRL and TSH genes. Inactivating mutations of the Pit 1 gene in mice result in hypoplasia of these pituitary cell types and dwarfism (Li et al, 1990), a similar mechanism has been shown to cause hypopituitarism in humans (Radovick et al, 1992. Pfaffle et al, 1992). Available evidence suggests that a functional Pit 1 gene is required for both the establishment and maintenance of the differentiated GH, PRL and TSH cell lines (Castrillo et al, 1991).

The role of this important protein in pituitary tumours is presently being investigated. In rat pituitary cell lines two groups have identified alternatively spliced variants of Pit 1, one of which appears to enhance its ability to activate a GH-promotor (Konzak and Moore, 1992. Haugen et al, 1993). Such findings clearly raise the question of activating mutations of Pit 1 in human adenomas and in recent reports this possibility

is beginning to be addressed. Selective expression of Pit 1 has been demonstrated in human adenomas containing GH, PRL or TSH (Asa et al, 1993) though a further report suggests that a small proportion of non-functional tumours also express Pit 1 (Friend et al, 1993). This group also identified a single tumour containing a truncated Pit 1 mRNA transcript which they suggest may be tumour specific.

In conclusion, this study has more clearly defined the role of a number of genetic mechanisms in pituitary adenoma formation. From the further development of these findings, and from the progress of a number of other groups, it seems likely that a much clearer understanding of cellular transformation in the anterior pituitary will emerge in the near future. How this understanding will evolve into both treatment strategies and improved tumour management remains to be seen. If our management is to be influenced by molecular findings then from these and other studies it seems likely that the first step may be early identification of those tumours with a high risk of recurrence for perhaps more radical surgery or early adjunctive radiotherapy. In the longer term however attention seems more likely to focus on the complex strategies required for direct or perhaps indirect replacement of deleted tumour-suppressor genes.

REFERENCES

Alexander JM, Biller BMK, Bikkal NT, Zervas A, Arnold A and Klibanski A. Clinically non-functioning tumours are monoclonal in origin. J Clin Investigation. 1990. 86: 336-340.

Alvaro V, Touraine Ph, Raisman Vozari R, Bai-Grenier F, Birman P and Joubert D. Protein kinase C activity and expression in normal and adenomatous human pituitaries. Int J Cancer. 1992. 50: 724-730.

Alvaro V, Levy L, Dubray C, Roche A, Peillon F, Querat B and Joubert D. Invasive human pituitary tumours express a point-mutated α-protein kinase-C. J Clin Endocrinol Metab. 1993. 77: 1125-1129.

Ambrosi B and Faglia G. Epidemiology of pituitary tumours. in; Pituitary adenomas: New trends in basic and clinical research. Faglia G et al, eds. 1991. pp159-168. Elsevier Science Publishers.

Asa SL, Cheng Z, Raymar L, Singer W, Kovacs K, Smyth HS and Muller P. Human pituitary null cell adenomas and oncocytomas in vitro: Effects of adenohypophysiotropic hormones and gonadal steroids on hormone secretion and tumour cell morphology. J Clin Endocrinol Metab. 1992. 74: 1128-1134.

Asa SL, Puy LA, Lew AM, Sundmark VC and Elsholtz HP. Cell type-specific expression of the pituitary transcription activator Pit 1 in the human pituitary and pituitary adenomas. J Clin Endocrinol Metab. 1993. 77: 1275-1280.

Ballard HS, Frame B and Hartstock RJ. Familial multiple endocrine adenoma-peptic ulcer complex. Medicine. 1964. 43: 481-515.

Bale AE, Norton JA, Wong EL, Fryberg JS, Maton PN, Oldfield EH, Streeten E, Aurbach GD, Brandi ML and Friedman E. Allelic loss on chromosome 11 in hereditary and sporadic tumours related to familial MEN 1. Cancer Research. 1991. 51: 1154-1157.

Billestrup N, Swanson LW and Vale W. Growth hormone releasing factor stimulates cell proliferation of somatotrophs in vitro. Proc Natn Acad Sci (USA). 1986. 83: 6854-6857.

Bourne HR, Sanders DA and McCormick F. The GTPase superfamily: a conserved switch for diverse cell functions. Nature. 1990. 348: 125-132.

Bouvier C, Forget H, Lagace G, Drews R, Sinnett D, Labuda D and Collu R. G proteins in normal rat pituitaries and in prolactin secreting rat pituitary tumours. Mol Cell Endocrinol. 1991. 78: 33-44.

Buchfelder M, Adams EF, Brockmeier S, Friedman E and Fahlsbuch R. Clinical and biochemical characteristics of acromegalics harbouring the gsp oncogene. J Endocrinol. 1992. 135: 38 (supp).

Burke CW, Adams CBT, Esiri MM, Morris C and Bevan JS. Transsphenoidal surgery for Cushing's disease: does what is removed determine the endocrine outcome? Clin Endocrinol. 1990. 33: 525-537.

Burrow GN, Wortzman G and Rewcastle NB. Microadenomas of the pituitary and abnormal sellar tomograms in an unselected autopsy series. N Eng J Med. 1981. 304: 156-158.

Bystrom C, Larsson C, Blomberg K, Sandelin U, Falkmer B, Skogseid B, Oberg K, Werner S and Nordenskjold M. Localisation of the MEN 1 gene to a small region of chromosome 11q13 by deletion mapping in tumours. Proc Natl Acad Sci (USA). 1990. 87: 1968-1972.

Caruso DR. Multiple endocrine neoplasia. Curr Opinion in Oncol. 1991. 3: 103-108.

Castrillo JL, Theill LE and Karin M. Function of the homeodomain protein GHF1 in pituitary cell proliferation. Science. 1991. 253: 197-199.

Cavanee WK, Dryja TP and Phillips RA. Expression of recessive

alleles by chromosomal mechanisms in retinoblastoma. Nature. 1983. 305: 779-784.

Chung RY and Seizinger BR. Molecular genetics of neurological tumours. J Med Genet. 1992. 29: 361-367.

Clementi E, Malgaretti N, Meldolesi J and Tarmellini R. A new constitutively activating mutation of the Gs protein α subunit-gsp oncogene is found in pituitary tumours. Oncogene. 1990: 1059-1061.

Cohen SN, Chang ACY and Hsu L. Non-chromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA. Proc Natl Acad Sci (USA). 1972. 69: 2110-2114.

Costello RJ. Subclinical adenoma of the pituitary gland. Am J Pathol. 1936. 12: 205-215.

Cryns VL, Alexander JM, Klibanski A and Arnold A. The retinoblastoma gene in human pituitary tumours. J Clin Endocrinol Metab. 1993. 77: 644-646.

Cushing H and Davidoff LM. The pathological findings in four autopsied cases of acromegaly with discussion of their significance. Monograph 22. 1927. The Rockerfeller Institute for Medical Research, New York.

Dunn JM, Phillips RA, Zhu X, Becker A and Gallie BL. Mutations

in the retinoblastoma gene and their effects on transcription. Mol Cell Biol. 1989. 9: 4596-4604.

Eberle F and Grun R. MEN 1. Ergebnisse der Innern Medizin und Kinderheilkunde. 1981. 46: 76-149.

Erdheim J. Zur normalen und pathologischen histologie der glandula thyreoidea, parathyreoidea und hypophysis. Beitrage zur Pathologischen Anatomie und Allegemeinem Pathologie. 1903. 33: 158-236.

Fagin JA. Molecular defects in thyroid gland neoplasia. J Clin Endocrinol Metab. 1992. 75: 1398-1400.

Falchetti A, Bale AE, Amorosi A, Bordi C, Cicchi P, Bandini S, Marx SJ and Brandi ML. Progression of uraemic hyperparathyroidism involves allelic loss on chromosome 11. J Clin Endocrinol Metab. 1993. 76: 139-144.

Farid NR, Buchler S, Russell NA, Maroun FB, Allerdoce P and Smyth HS. Prolactinomas in familial MEN 1: Relationship to HLA and carcinoid tumours. Am J Medicine. 1980. 59: 874-880.

Fearon ER and Vogelstein B. A genetic model for colorectal tumorogenesis. Cell. 1990. 61: 751-767.

Feinberg AP and Vogelstein B. A technique for radiolabelling DNA restriction endonuclease fragments to high specific

activity. Analytical Biochemistry. 1983. 132: 6-13.

Fraser NJ, Boyd Y, Brownlee GG and Craig IW. Multi-allelic RFLP for M27β, an anonymous single copy genomic clone at Xp11.3-Xcen (DXS255). Nuc Acid Res. 1987. 15: 9616.

Freidman E, Sakaguchi K, Bale AE, Falchetti A, Streeten E, Zimering MB, Weinstein LS, Norton JA, Aurbach GD, Spiegel AM and Marx SJ. Clonality of parathyroid tumours in familial MEN 1. NEJM. 1989. 321: 313-318.

Friend KE, Chiou Y, Laws ER, Lopes BS and Shupnik MA. Pit-1 messenger ribonucleic acid is differentially expressed in human pituitary adenomas. J Clin Endocrinol Metab. 1993. 77: 1281-1286.

Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM and Dryja TP. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature. 1986. 323: 643-645.

Fujimori M, Wells SA and Nakamura Y. Fine scale mapping of the gene responsible for MEN 1. Am J of Hum Genet. 1992. 50:399-403.

Gardner DF, Barlascini C, Downs RW and Sahni KS. Cushings disease in two sisters. Am J Med Sci. 1989. 297: 387-389.

Gicquel C, Le-Bouc Y, Luton JP, Girard F and Bertagna X. Monoclonality of corticotroph macroadenomas in Cushings disease. J Clin Endocrinol Metab. 1992. 75: 472-475.

Goeltz SE, Hamilton SR and Vogelstein B. Purification of DNA from formaldehyde fixed and paraffin embedded tissue. Biochem Biophys Res Comm. 1985. 130: 118-126.

Gonsky R, Herman V, Melmed S and Fagin J. Transforming DNA sequences present in human prolactin-secreting pituitary tumours. Mol Endocrinol. 1991. 5:1687-1695.

Gupta SK, Gallego C and Johnson GL. Mitogenic pathways regulated by G protein oncogenes. Mol Biol Cell. 1992. 3: 123-128.

Hamel PA, Gallie BL and Phillips RA. The retinoblastoma protein and cell cycle regulation. TIG. 1992. 8: 180-185.

Harris H. The analysis of malignancy by cell fusion: the position in 1988. Cancer Res. 1988. 48: 3302-3306.

Haugen BR, Gordon DF, Wood WM, Sarapura VD and Ridgway EC. A novel thyrotrophe-specific variant of Pit 1 stimulates the TSHB promotor. 75th Annual Meet of The Endocrine Soc. 1993.

Herman V, Fagin J, Gonsky E, Erzin C and Kovaks K. Clonal

origin of pituitary adenomas. J Clin Endocrinol Metab. 1990. 71: 1427-1433.

Herman V, Drazin NZ, Gonsky R and Melmed S. Molecular screening of pituitary adenomas for gene mutations and rearrangements. J Clin Endocrinol Metab. 1993. 77: 50-55.

Hirsch O. Symptoms and treatment of pituitary disease. Arch Otolaryngol. 1952. 55: 268-280.

Hollstein M, Sidransky D, Vogelstein B and Harris CC. p53 mutations in human cancers. Science. 1991. 253: 49-53.

Horvath E, Lloyd RV and Kovacs K. PTU induced hypothyroidism results in reversible transdifferentiation of somatotrophs into thyroidectomy cells. A morphological study of the rat pituitary including electron microscopy. Lab Invest. 1990. 63: 511-520

Jacks T, Fazeli A, Schmitt EM, Bronson RT, Goodell MA and Weinberg RA. Effects of an RB mutation in the mouse. Nature. 1992. 359:295-300.

Jackson DP, Hayden JD and Quirke P. Extraction of nucleic acid from fresh and archival material, in; PCR: a practical approach. 1991. Oxford University Press. pp 33-40.

Jacoby LB, Hedley-Whyte ET, Pulaski K, Seizinger BR and

Martuza RL. Clonal origin of pituitary adenomas. J Neurosurgery. 1990. 73: 731-735.

Jameson JL, Klibansky A, Black PM, Zervas NT, Lindell CM, Hsu DW, Ridgway EC and Habener JF. Glycoprotein hormone genes are expressed in clinically non-functioning pituitary adenomas. J Clin Investigation. 1987. 80: 1472-1478.

Janson M, Larsson C, Werelius B, Jones C, Glaser T, Nakamura Y, Jones CP and Nordenskjold M. Detailed physical map of human chromosomal region 11q12-13 shows high meiotic recombination rate around the MEN 1 locus. Proc Natl Acad Sci (USA). 1991. 88: 10609-10613.

Jeffreys AJ, Wilson V and Thein SL. Hypervariable 'minisatellite' regions in human DNA. Nature. 1985. 314: 67-73.

Karga HJ, Alexander JM, Hedley-Whyte ET, Klibanski A and Jameson JL. Ras mutations in human pituitary tumours. J Clin Endocrinol Metab. 1992. 74: 914-919.

Kineman RD, Faught WJ and Frawley LS. Steroids can modulate transdifferentiation of prolactin and growth hormone cells in bovine pituitary cultures. Endocrinology. 1992. 130: 3289-3294.

Klibanski A and Zervas NT. Diagnosis and management of hormone

secreting pituitary adenomas. New Eng J Med. 1991. 324: 822-831.

Knudson AG. Mutations and cancer: A statistical study of retinoblastoma. PNAS (USA). 1971. 68: 820-828.

Koufos A, Grundy P and Morgan K. Familial Weidemann-Beckwith syndrome and a second Wilms tumour locus both map to 11p15.3. Am J Hum Genet. 1989. 44: 711-719.

Konzak KE and Moore DD. Functional isoforms of Pit 1 generated by alternative messenger RNA splicing. Mol Endocrinol. 1992. 6: 241-247.

Kruse A, Klinken L, Holck S and Lindholm J. Pituitary histology in Cushing's disease. Clin Endocrinol. 1992. 37: 254-259.

Landis CA, Masters SB, Spada A, Pace AM, Bourne HR and Vallar L. GTPase inhibiting mutations activate the α chain of Gs and stimulate adenylate cyclase in human pituitary tumours. Nature. 1989. 340: 692-696.

Landolt AM. Cerebrospinal fluid rhinorrhea: A complication of therapy for invasive prolactinomas. Neurosurgery. 1982. 11: 395-401.

Levy A and Lightman SL. The pathogenesis of pituitary adenomas. Clin Endocrinol. 1993. 38: 559-570.

Li S, Crenshaw EB, Rawson EJ, Simmons DM, Swanson LW and Rosenfeld MG. Dwarf locus mutants lacking three pituitary cell types result from mutations in the PUO-domain gene Pit 1. Nature. 1990. 347: 528-533.

Litt M and Luty JA. A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. Am J Hum Genet. 1989. 44: 397-401.

Lloyd PC. A case of hypophysed tumour with associated tumour like enlargement of the parathyroids and islands of Langerhans. Bulletin of the John Hopkins Hospital. 1929. 45: 1-14.

Larsson C, Skogseld B, Oberg K, Nakamura Y and Nordenskjold M. The MEN 1 gene maps to chromosome 11 and is lost in insulinoma. Nature. 1988. 332: 85-87.

Lee WH, Bookstein R, Hong F, Yoing LJ, Shew JH and Lee EYHP. The human retinoblastoma susceptibility gene: cloning, identification and sequence. Science. 1987. 235: 1394-1399.

Lyons J, Landis CA, Harsh G, Vallar L, Grunewald K, Feichtinger H, Quan-Yang D, Clark OH, Kawasaki E, Bourne HR and McCormick F. Two G-protein oncogenes in human endocrine tumours. Science. 1990. 249: 655-659.

Mannens M, Devilee P and Bliek J. Loss of heterozygosity in Wilms tumour, studied for 6 putative tumour suppressor loci, is limited to chromosome 11. Cancer Res. 1990. 50: 3279-3283.

March CM. Longitudinal evaluation of patients with untreated prolactin secreting pituitary adenomas. Am J Obs Gynae. 1981. 139: 835-844.

Moldawer MP, Nardi GL and Raker JW. Concomitance of multiple adenomas of the parathyroids and pancreatic islet cells with tumours of the pituitary: A syndrome with familial incidence. Am J Med Sci. 1954. 228: 190-206.

Molitch ME, Elton RL, Blackwell RE, Caldwell B, Chang RJ, Jaffe R, Joplin G, Robbins RJ, Tyson J, Thorner MO and the bromocriptine study group. Bromocriptine as primary therapy for prolactin secreting macroadenomas: results of a prospective study. J Clin Endocrinol Metab. 1985. 60: 698-705.

Molitch ME and Russell EJ. The pituitary "incidentaloma". Ann Int Med. 1990. 112: 925-931.

Morton DG, Macdonald F, Cachon-Gonzales MB, Rindl PM, Neoptolomos AH, Keighley MR, Delhanty JP, McKeown CM, Kilpatrick M and Hulten MA. The use of DNA from paraffin wax preserved tissue for predictive diagnosis in familial adenomatous polyposis. J Med Genet. 1992. 29:

571-573.

Nakamura Y, Leppert M, O'Connell P, Wolff R, Holm T, Culver M, Martin C, Fujimoto B, Hoff M, Kumlin E et al. Variable number of tandem repeat (VNTR) markers for human gene mapping. Science. 1987. 235: 1616-1622.

Nowell P. The clonal evolution of tumour cell populations. Science. 1976. 194: 23-28.

Pfaffle RW, DiMattia GE and Parks JS. Mutations of the PUO-specific domain of Pit 1 and hypopituitarism without pituitary hypoplasia. Science. 1992. 257: 1118-1121.

Prosser PR, Karem JH, Townsend JJ and Forsham PH. Prolactin secreting pituitary tumours in MEN 1. Ann Int Med. 1979. 91: 41-44.

Radford DM, Ashley SW, Wells SA and Gerhard DS. Loss of heterozygosity of markers on chromosome 11 in tumours from patients with MEN 1. Cancer Res. 1990. 50: 6529-6533.

Radovick S, Nations M, Du Y, Berg LA, Weintraub BD and Wondisford FE. A mutation in the PUO-homeodomain of Pit 1 responsible for combined pituitary hormone deficiency. Science. 1992. 257: 1115-1118.

Rey JA. A case of pituitary adenoma with 58 chromosomes.

Cancer Gen Cytogenet. 1986. 23: 171-174.

Robinson N, Beral V, Ashley JSA. Incidence of pituitary adenoma. Lancet. 1979. 22: 630.

Schulte HM, Oldfield EH, Allolio B, Katz DA, Berkman RA and Ali IU. Clonal composition of pituitary adenomas in patients with Cushings disease: Determination by X-chromosome inactivation analysis. JCEM. 1991. 73: 1302-1308.

Sealey PG, Whittaker PA and Southern EM. Removal of repeated sequences from hybridisation probes. Nuc Acid Res. 1985. 13: 1905-1922.

Seizinger BR, Klinger HP, Junien C, Nakamura Y, Le Beau M, Cavanee W, Emmanuel B, Ponder B et al. Report of the committee on chromosome and gene loss in human neoplasia. Cytogenet Cell Genet. 1991. 58: 1080-1096

Shepherd JJ. Latent familial multiple endocrine neoplasia in Tasmania. Med J Aust. 1985. 142: 395-397.

Sidransky D, Mikkelsen T, Schnelhheimer K, Rosenblum ML, Cavanee W and Vogelstein B. Clonal expansion of p53 mutant is associated with brain tumour progression. Nature. 1992. 355: 846-847.

Sisam DA, Sheehan JP and Sheeler LR. The natural history of

untreated microprolactinomas. Fertil Steril. 1987. 48: 67-71.

Smrcka AV, Hepler JR, Brown KO and Sternweis PC. Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. Science. 1991. 251: 804-807.

Southern EM. Detection of specific sequences among DNA fragments separated by agarose gel electrophoresis. J Mol Biol. 1975. 98: 503-515.

Spada A, Arosio M, Bochicchio D, Bazzoni N, Vallar L, Bassetti M and Faglia G. Clinical, biochemical and morphological correlates in patients bearing GH-secreting pituitary tumours with or without constitutively activated adenylate cyclase. J Clin End Metab. 1990. 71: 1421-1426.

Sreekantaiah C and Sandberg AA. Clustering of aberrations to specific chromosomal regions in benign neoplasms. Int J Cancer. 1991. 48: 194-198.

Taylor SJ, Chae HZ, Rhee SG and Exton JH. Activation of the B1 isoenzyme of phospholipase C by α subunits of the Gq class of G proteins. Nature. 1991. 350: 516-518.

Teh BT, Hayward NK, Wilkinson S, Woods GM, Cameron D and Shepherd JJ. Clonal loss of int-2 alleles in sporadic and familial pancreatic endocrine tumours. Brit J

Cancer. 1990. 62: 253-254.

Thakker RV, Bouloux P, Wooding C, Chotai K, Broad PM, Spurr NK, Besser GM and O'Riordan JLH. Association of parathyroid tumours in MEN 1 with loss of alleles on chromosome 11. New Eng J Med. 1989. 321: 218-223.

Tordjman K, Stern N, Ouaknine G, Yossiphov Y, Razon N, Nordenskjold M and Friedman E. Activating mutations of the Gs α-gene in nonfunctioning pituitary tumours. J Clin Endocrinol Metab. 1993. 77: 765-769.

U HS, Kelley P and Lee W-H. Abnormalities of the human GH gene and protooncogenes in some human pituitary adenomas. Mol Endocrinol. 1988. 2: 85-89.

Vallar L, Spada A and Giannattasio G. Altered Gs and adenylate cyclase activity in human GH-secreting pituitary adenomas. Nature. 1987. 330: 566-568.

Vance ML, Evans WS and Thorner MO. Bromocriptine. Ann Int Med. 1984. 100: 78-91.

Van Noorden S, Stuart MC, Cheung A, Adams EF and Polak JM. Localisation of pituitary hormones by multiple immunoenzyme staining procedures using monoclonal and polyclonal antibodies. J Histochem and Cytochem. 1986. 34: 287.

Weber JL and May PE. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet. 1989. 44: 388-396.

Weissenbach J, Gyapay G, Dib C, Vignal A, Morissette J, Millasseau P, Vayesseix G and Lathrop M. A second generation linkage map of the human genome. Nature. 1992. 359: 794-801.

Wermer P. Genetic aspects of adenomatosis in endocrine glands. Am J Med. 1954. 16: 363-371.

Windle BE and Wahl GM. Molecular dissection of mammalian gene amplification: New mechanistic insights revealed by analysis of very early events. Mutation Res. 1992. 276: 199-224.

Wright DK and Manos MM. Sample preparation from paraffin embedded tissues, in: PCR protocols: A guide to methods and applications. 1990. Academic Press, San Diego. pp 153-158.

Wynford-Thomas D. Molecular genetics of thyroid cancer. Trends Endocrinol Metab. 1993. 4: 224-232.

Yuasa H. Familial pituitary adenoma: Report of four cases from two unrelated families. Neurologica Medico-Chirugica. 1990. 30: 1016-1019.

Yoshimoto K, Iwanha H, Kubo K, Saito S and Itakura M. Allele loss on chromosome 11 in a pituitary tumour from a patient with MEN 1. Jp J Cancer Res. 1991. 82: 886-889.

Zheng JP, Robinson WR, Ehlen T, Yu MC and Dubeau L. Distinction of low grade from high grade human ovarian carcinomas on the basis of losses of heterozygosity on chromosomes 3, 6 and 11 and her-2/neu gene amplification. Cancer Res. 1991. 51: 4045-4051.

APPENDIX 1 - BUFFERS AND SOLUTIONS

Denaturing solution

0.5M NaOH

1.5M NaCl

Denhart's solution (100x)

20g Polyvinyl pyrolidine

20g Ficoll 400

20g Bovine serum albumin (Fraction V)

To 1 litre with water, filter sterilise, store at -20°C.

Dextran sulphate (25%)

50g Dextran sulphate

Dissolve in 200mls sterile water at 68°C in shaking waterbath.

Digestion buffer (for DNA extraction)

2.5mls, 20% Sodium dodecyl sulphate

20mg Proteinase K

Sterile water to 10mls, use immediately.

DNA Suspension buffer

0.075 M NaCl (37.5mls, 2M NaCl solution)

0.024 M EDTA (48mls, 0.5 M solution)

Water to 1 litre, sterilise by autoclaving.

0.5 M EDTA (pH 8.0)

186.1g disodium ethylene diamine tetra-acetate.$2H_20$ in

800mls water, vigorous stirring. Adjust pH to 8.0 with NaOH (approximately 18g). Adjust volume to 1 litre, sterilise by autoclaving.

Ethidium bromide (10mg/ml)

100mg ethidium bromide

Dissolve in 10mls water by vigorous stirring. Store at 4°C, wrapped in aluminium foil. Handle with care.

Hybridisation solution (aqueous)

200mls 20X SSC

100mls 100X Denhart's solution

10mls 10% sodium tetrapyrophosphate

5mls 10mg/ml salmon sperm (denatured/sheared)

200mls 25% dextran sulphate

485mls sterile distilled water

Store at 4°C, use within 4-6 weeks.

LB Broth

10g Bacto tryptone

5g Bacto yeast extract

10g NaCl

Water to 1 litre, adjust pH to 7.5 with NaOH, sterilise by autoclaving.

(for LB agar, add 15g/L Bacto agar prior to autoclaving)

Loading buffer

20mg Bromophenol blue

5mls, 2x TE buffer

5mls Glycerol

Mix well, sterilise by autoclaving.

Lysis solution

109g Sucrose

10mls Tris Cl, 1M

1.0g Magnesium chloride

10mls Triton-X100

To 1 litre with sterile water.

1M magnesium chloride

203.3g $MgCl_2.6H_2O$

Dissolve in 1 litre water, sterilise by autoclaving.

2M NaCl

116.9g NaCl

To 1 litre with water, sterilise by autoclaving.

Neutralising solution

1.5M NaCl

0.5M Tris-Cl pH 8.0

Phenol preparation

Make up volume as required. To aqueous phenol add 8-hydroxyquinoline to a concentration of 0.1%. Extract phenol with an equal volume of buffer (1M Tris-Cl pH 8.0, followed by 0.1M Tris-Cl pH 8.0). Repeat until pH of the aqueous phase is >7.6. Store phenol under 10% by volume of buffer and 0.2% mercaptoethanol at 4°C, wrap

bottle in aluminium foil to exclude light.

0.01M phosphate buffered saline

0.71g Na_2HPO_4

0.78g $NaH_2PO_4.2H_2O$

8.76g NaCl

0.5g sodium azide

Dissolve in 800mls water, adjust pH to 7.6 with NaOH, make up to 1 litre.

Phosphate hybridisation buffer

9.6g $NaH_2PO_4.2H_2O$

59.6g Na_2HPO_4

2mls 0.5M EDTA (Ph 8.0)

70g sodium dodecyl sulphate

Make up to 1 litre with sterile water. Store at room temperature, shelf-life approximately six weeks. Before use add 100ug/ml denatured, sonicated salmon sperm.

Polyacrylamide gel (10%)

5.76g polyacrylamide

0.24g bis-acrylamide

6mls TBE buffer (10x)

Dissolve in 60mls with water. To this add:

600ul ammonium persulphate

60ul TEMED, swirl to mix and pour immediately.

Salmon sperm DNA (10mg/ml)

500mg Salmon sperm (DNA, sodium salt, type II)

Dissolve in 50mls sterile water, sonicate for 10

minutes, freeze for storage at -20°C. Prior to use thaw, boil for 10 minutes to denature and shear through fine bore (orange) needle.

Sephadex G-50

30g Sephadex G-50 (medium)
Add to 250mls TE buffer (pH 7.5). Dissolves slowly, heat to 65°C for 1-2 hours. Store at 4°C.

3M sodium acetate (pH 5.2)

408.1g sodium acetate.$3H_2O$
Dissolve in 800mls water, adjust pH to 5.2 with glacial acetic acid, make up to 1 litre. Sterilise by autoclaving.

Sodium dodecyl sulphate (20%)

200g sodium dodecyl sulphate
Dissolve in 800mls water at 68°C, adjust pH to 7.2 with HCl, adjust volume to 1 litre with water.

Sodium tetrapyrophosphate (10%)

10g sodium tetrapyrophosphate
Dissolve in 100mls sterile water. Store at -20°C, thaw thoroughly prior to use.

SSC (20X)

175.3g NaCl
88.2g sodium citrate
Dissolve in 800mls water, adjust pH to 7.0 with NaOH,

make up to 1 litre. Sterilise by autoclaving.

SSPE (20X)

174g NaCl

27.6g $NaH_2PO_4.H_2O$

7.4g EDTA

Dissolve in 800mls water, adjust pH to 7.4. Make up to 1 litre, sterilise by autoclaving.

STE

10mM Tris-Cl (pH 8.0)

100mM NaCl

1mM EDTA (pH 8.0)

TAE (20x)

96.8g Tris

22.8mls glacial acetic acid

40mls 0.5M EDTA (pH 8.0)

Make up to 1 litre with water.

TE buffer

10mM Tris-Cl

1mM EDTA

The pH is varied by adjusting the pH of the Tris.

1M Tris-Cl

121.1g Tris base

Dissolve in 800mls water, adjust pH to desired value by addition of concentrated HCl. Make up to 1 litre.

Sterilise by autoclaving.

APPENDIX 2 - Clinical Data

Pt No	Diag	Macro	24UC nM24	FSH mU/L	GH mU/L	LH mU/L	PRL mU/L	TSH mU/L	Imm (+)
1	COR	Y	3400						ACTH
2	NF	Y					2000		-
3	SOM	Y			73				GH
4	COR	N	2396						ACTH
5	NF	Y							-
6	COR	N	3011						ACTH
7	NF	Y							-
8	NF	Y							-
9	NF/P	Y					2600		PRL weak
10	COR	Y	1307						ACTH
11	PRL	N					7600		PRL
12	NF	Y							-
14	PRL	Y					36400		PRL
15	SOM	N			56				GH PRL
16	SOM	Y			38				GH
18	SOM	Y			48				-
19	NF	Y							FSH weak
20	SOM	Y			43				GH PRL
23	SOM	N			19				GH PRL

Pt Num	Diag	Macro	24UC nm24	FSH mU/L	GH mU/L	LH mU/L	PRL mU/L	TSH mU/L	Imm (+)
24	PRL	N					4390		-
25	NF	Y					760		-
26	NF	Y							αSUB
28	SOM	Y			47				GH,LH FSH
29	PRL	Y					19700		PRL
30	NF	Y							-
31	NF	Y							GH weak
32	COR	N	1690						ACTH weak
38	NF	Y							-
39	NF	Y							NA
40	NF	Y							LH
41	PRL	N					8600		PRL LH
42	NF	Y							-
43	NF	N							-
44	SOM	N			18				GH PRL
48	SOM	Y			17				GH
50	PRL	Y					31000		PRL weak
51	PRL	Y					22300		PRL
52	SOM	N			38				GH,LH PRL

Pt Num	Diag	Macro	24UC nm24	FSH mU/L	GH mU/L	LH mU/L	PRL mU/L	TSH mU/L	Imm (+)
56	PRL	N					9500		PRL LH
57	NF	Y							LH FSH
58	NF	Y							-
61	SOM	Y			28				GH
62	SOM	Y			36				GH PRL
63	GON	Y		21.5		21			LH, α FSH
64	PRL	Y					13000		PRL
65	SOM	N			>150				GH PRL
66	PRL	Y					6000		PRL GH-wk
70	NF	Y							-
71	NF	Y							-
72	SOM	N				46			GH
73	SOM	N				35			GH PRL
75	NF	Y					1700		-
76	COR	N	ACTH 17ngL						ACTH
79	COR	N	1032						ACTH
80	NF	Y							LH FSH
81	NF	Y							LH
82	SOM	Y				30			GH

Pt Num	Diag	Macro	24UC nm24	FSH mU/L	GH mU/L	LH mU/L	PRL mU/L	TSH mU/L	Imm (+)
83	NF	Y							GH weak
86	PRL	Y					18000		PRL
88	NF	Y							LH αSUB
89	PRL	Y					14500		PRL weak
92	MIXED	Y			45		6680		GH-wk PRL
93	NF	Y							GH
94	NF	Y							-
95	NF	Y							-
96	NF	Y							-
97	NF	Y							αSUB
98	NF	Y							-
99	SOM	Y			51				-
100	NF	Y					600		-
101	SOM	Y			41				GH
102	NF	Y							LH,α FSH
103	MIXED	Y			9		23700		PRL GH
107	TSH	Y						53	TSH LH,α
108	PRL	Y					19700		PRL
111	NF	Y							TSH L/FSH

Pt Num	Diag	Macro	24UC nm24	FSH mU/L	GH mU/L	LH mU/L	PRL mU/L	TSH mU/L	Imm (+)
113	PRL	Y					18700		PRL L/FSH
115	PRL	Y					1800		PRL
116	NF	Y							FSH weak
117	NF	N							ACTH
119	PRL	Y					22000		PRL
120	SOM	Y			40				GH
121	SOM	Y			70				GH
122	SOM	Y			>50				GH
123	SOM	N			73				GH
124	SOM	Y			24				GH PRL
125	SOM	Y			113				GH
126	SOM	N			>50				NA

SOM = Somatotrophinoma

COR = Corticotrophinoma

PRL = Prolactinoma

GON = Gonadotrophinoma

NF = Non-functional adenoma

MIXED = Dual secreting adenoma

MACRO = Macroadenoma (>10mm diameter)

NA = Not available

APPENDIX 3 - FULL DATA ON RFLP STUDIES

Chromosome 11 probes: 1 - HRAS
2 - INS
3 - D11S149
4 - PYGM
5 - D11S97
6 - D11S146
7 - INT2
8 - D11S144
9 - D11S147

Other autosomes: 10 - Chromosome 1
11 - Chromosome 5
12 - Chromosome 10
13 - Chromosome 13
14 - Chromosome 17
15 - Chromosome 20
16 - Chromosome 22

\+ = Retains heterozygosity
\- = Loss of heterozygosity
. = Uninformative
N = Normal sequence
M = Mutated sequence

	Chromosome 11 probes									Other autosomes							
pt no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Gs
1	−	−	.	−	.	−	−	.	.	.	+	−	+	+	+	−	N
2	.	.	+	.	+	+	+	+	+	.	+	+	−	+	+	+	N
3	+	+	.	.	+	+	+	+	+	+	+	+	+	+	+	+	N
4	+	+	.	.	.	.	+	+	.	+	+	+	+	+	.	+	N
5	.	.	.	+		+	.	.	.	.	+	+	+		.	+	N
6	+	.	+	.		.	+	.	+	+	+	.	+		.	+	N
7	.	.	.	.	−	.	−	.	.	.	+	+	+	+	+	.	N
8	+	+	.	.	−	−	−	+	+	+	+	+	.	+	+	+	N
9	+	.	.	+	+	.	.	+	.	.	+	.	+	+	+	.	N
10	+	+	.	.	+	+	.	.	+	.	.	+	.	+	.	.	N
11	+	.	.	+	+	+	+	.	.	+	+	+	+	+	.	−	N
12	.	.	.	+	+	.	.	+	.	+	+	+	+	+	+	.	N
14	+	.	.	.	+	+	.	.	.	+	+	.	+	.	+	+	N
15	.	.	.	+	+	.	.	+	+	+	+	+	.	+	.	.	M
16	.	.	.	.	+	.	.	+	+	+	+	.	+	+	+	.	M
18	.	+	+	+		+	.	.	.	−	+	.	+		+	+	M
19	.	.	.	.	+	+	+	+	+	+	+	+	+	+	+	+	N
20	+	.	.	+	.	+	.	+	.	+	+	.	+	+	−	+	N

	Chromosome 11 probes									Other autosomes							
pt no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Gs
23	.	+	.	.	+	.	.	+	.	+	+	+	+	+	+	+	N
24	+	+	+	+	+	+	.	.	.	+	+	.	+		+	.	N
25	+	+	.	.	+	.	+	.	+	-	.	+	+	+	.	.	N
26	+	.	.	.	.	.	+	.	+	+	+	+	+	.	.	+	N
28	.	.	.	.	+	+	+	+	.	+	.	.	+	+	.	+	M
29	.	.	.	.	+	+	.	.	+	.	+	+	.	.	.	+	N
30	.	+	.	+	+	+	.	.	+	+	+	+	.	.	+	+	N
31	.	-	-	.	-	-	-	.	-	+	+	+	+	+	.	.	N
32	+	+	.	.	.	.	.	+	.	+	+	.	+	+	.	.	N
38	+	.	.	+		+	.	.	+	.	+	+	+	+	+	+	N
39	.	+	.	.	-	-	.	.	+	.	+	+	+	.	+	+	N
40	.	.	.	+		+	+	.	.	+	+	+	+		+	.	N
41	.	.	.	.	+	.	+	.	.	+	+	+	+	.	.	+	N
42	.	+	.	.	+	.	.	.	.	+	+	.	+	+	+	.	N
43	.	.	+	.		.	+	+	.								N
44	+	+	.	+	+	.	+	.	+								N
48	.	.	.	.	.	.	+	+	.	+	.	+	+	+	+	.	N
50	.	.	.	.	+	.	+	.	.	+	+	+	+	.	+	+	N

	Chromosome 11 probes									Other autosomes							
pt no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Gs
51	+	.	.	−	−	−	.	.	−	.	+	+	+	+	+	+	N
52	.	+	.	+		+	.	.	+	+	+	.	.		+	.	M
56	+	.	.	.	+	+	+	.	.	+	+	.	+	+	+	+	N
57	.	+	.	.	+	.	+	+	.	.	+	.	+	+	+	−	N
58	+	+	.	.	−	.	.	+	.	+	+	.	.	+	+	+	N
61	.	+	.	+		+	+	+	.	+	+	.	+		+	+	N
62	+	.	.	+		+	+	.	.								N
63	+	+	.	+	.	.	.	.	.	+	+	.	.	.	+	+	N
64	+	.	+	.	+	+	+	.	.	.	+	+	+	.	+	+	N
65	.	+	.	.	+	.	+	.	.	+	.	+	+	+	.	+	N
66	+	.	+	+	+	.	.	.	+	+	+	+	+	+	+	+	N
70	.	.	.			+	+	.	.								N
71	.	.	.	.		+	.	+	.	+	+	+	+		+	.	N
72	.	+	.	.	+	.	+	.	+	+	+	+	+	.	+	+	M
73	.	.	.	.	+	+	.	+	.	.	+	+	.	.	+	+	M
75	.	.	.	+		+	+	.	.	.	+	+	+		+	.	N
76	+	−	−	.		.	.	.	+	+	+	.	+	+	.	.	N
79	.	.	.	+	+		+	+	.	.	+	.	.	.	+	.	N

	Chromosome 11 probes									Other autosomes							
pt no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Gs
80	+	+	+	.	+	.	.	+	.	+	+	+	+	+	.	.	N
81	.	.	.	+	.		+	+	.	.	+	+	+	.	+	+	N
82	+	.	.	.	+	+	+	+	.	+	+	+	+	+	+	+	M
83	.	.	.	+	+	.	+	+	.	.	+	.	+	+	+	.	N
86	.	+	.	.	.	.	+	+	+	+	+	.	.	+	+	.	N
88	+	.	.	.	+	.	.	.	+	−	+	.	+	.	+	.	N
89	.	.	−	.	−	.	.	.	.	+	+	.	+	+	+	.	N
92	+	.	.	+		+	+	+	.	.	+	+	+		+	+	N
93	+	+	.	.	+	.	+	.	.	.	.	+	+	+	+	+	N
94	+	.	.	+		+	.		.								N
95	+	+	.	+		.	+	+	.	+	+	.	+		+	.	N
96	+	.	+	.		+	+		.	+	+	+	+		+	+	N
97	+	.	.	.	+	.	.	.	+	.	+	.	+		+	+	N
98		.	.	.		+	+		.	.	.	.	.		+	+	N
99	+		.	+	+	+	.	.	+	+	+	.	+		+	+	N
100	.	.	.		+	+	+	.	.			+		.		.	N
101	.	.	.		+	.	+	.	+	+	+	+	+	.	+	.	N
102	+	+	.			+	+		.	+	+	+	+	+		.	N

	Chromosome 11 probes									Other autosomes							
pt no	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Gs
103	.	–	–		–	–	.	.	–	.	+	–	+	.	+	+	N
107	+	.	.		+	+	+	.	+								N
108	+	+	.	+		+	+	.	.			+		+		+	N
111	+	+	.	–		–	.		+			+		.		+	N
113	+	.	.		+	.	+		.			+		+		.	N
115	.	+	+		+	+	+		.			+		+		+	N
116	+	+	.	+		+	.		+			+		.		+	N
117			.			–	–		–			+				+	N
119	.	.	.			+	+		+			+		.		.	N
120	.	.	.		+	+	+		.			+		+		+	M
121	+	.	.	–	–		.	+	.								N
122	.	.	.	+	+	+	.	.	.								N
123			.		.	.	.	.	–								M
124	+	+	.	.	+	+	.	.	+								N
125	+	.	.	.	–	.	.	.	.								N
126	+	.			–												N