THE PREVALENCE OF HUMAN PAPILLOMA VIRUSES IN LARYNGEAL SQUAMOUS CELL CARCINOMA

A POLYMERASE CHAIN REACTION INVESTIGATION

SUBMITTED BY

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To my parents

Vernon L and Naomi A Nunez

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ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
bcl	b cell lymphoma gene
B-globin	beta globin gene
bp	basepairs
BSA	bovine serum albumin
CD4,CD8	leucocyte sub-populations based on surface antigens
CIN	cervical intraepithelial neoplasia
DNA	deoxyribonucleic acid
DNAadducts	DNA chemical addition products
dNTP	deoxynucleotide-phosphate
E1-6	Early coding regions of the Human papilloma virus genome
EBNA	Epstein Barr nuclear antigen
GI	gastro-intestinal
HHV	human herpes virus
HLA	human leucocyte antigen
HPV	human papilloma virus
HPV6,11 etc	different human papilloma virus subtypes
IAA	isoamyl alcohol

Igg	immunoglobulin
IgG,A,M,E,D	different immunoglobulin subtypes
IMS	industrial methylated spirits
L1-L2	late coding regions of the Human papilloma virus genome
MHC	major histocompatibility complex
NOCs	n-nitroso compounds
NDMA	n,n-dimethyl amine
P450	cytochrome monoxygenase enzyme pathway
P53	a tumour suppressor gene on the short arm of chromosome 6
PCR	polymerase chain reaction
RHL	recurrent herpes labialis
RRP	recurrent respiratory papillomatosis
SCC	squamous cell carcinoma
STD	sexually transmitted disease
Taq	thermophilus aquaticus
T1-4	different tumour stages
TNM	tumour, node, metastasis staging system
TRIS	-hydroxymethylaminomethane
UICC	International union against cancer
UV	ultraviolet light

UVA,UVB	subtypes of ultraviolet light

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CHAPTER 1

THE LARYNX

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1.Larynx Anatomy

.1 Definition

The larynx is that structure which occupies the transition between the shared upper respiratory and digestive tracts and the exclusive respiratory tract or trachea.

.11 Site

The larynx in the adult is readily surface marked by the thyroid prominence. The superior point of the angulated junction of the two lateral plates of the thyroid cartilage. The angle is more acute in males than females hence the Adam's apple. The cricoid cartilage which marks the inferior limit of the larynx is also easily palpated. The skin of the neck, strap muscles and the thyroid gland(isthmus and lobes) form the important anterior relationships. The carotid sheath and its attendant structures are the important lateral relationships of the larynx. The cervical vertebrae, prevertebral fascia, prevertebral muscles and the pharynx lie posteriorly.

.111 Structure

The larynx has an outer cartilaginous framework consisting of the keel shaped thyroid cartilage, and the signet ring shaped cricoid cartilage. The latter is the only

complete cartilage ring in the upper respiratory tract. The area between the cartilages is bridged by fascial condensations forming membranes, the cricothyroid membrane between the cricoid and thyroid cartilages, the crico-vocal membrane extending from the cricoid cartilage to the vocal ligament on each side. The vocal ligament occupies the free edge of the membranous vocal cord. The epiglottis, arytenoids and lesser corniculate and cuneiform cartilages lie within the outer cartilage skeleton. The arytenoid is the cartilage of the vocal cord.

Intrinsic laryngeal muscles are attached to the cartilages and alter the tension and position of the vocal cords or control the opening of the laryngeal inlet. The intrinsic muscles controlling the vocal cord are the posterior cricoarytenoid, lateral cricoarytenoid, thyroarytenoid and the overlapping interarytenoid. Extrinsic muscles, the strap muscles of the neck and the cricothyroid which are attached to the outer cartilage framework also contribute to laryngeal position and vocal cord tension. The aryepiglottic and thyroepiglottic muscles control the laryngeal inlet(Last RJ, 1978; Ellis H, 1976).

Histology

The internal surface of the larynx is lined by pseudostratified ciliated columnar respiratory and stratified squamous epithelium. The latter is restricted mostly to the vocal cords and the transition between the larynx and pharynx(Copenhaver WM,

Bunge RP, Bunge MB, 1971). There are mucous and serous glands in the mucosa except at the vocal cords. The lamina propria consist of a superficial amorphous layer(Reinke's space) and an intermediate to deep layer of elastic and collagen fibres (Last RJ, 1978; Hirano M, 1991).

Blood supply and innervation

The muscles of the larynx are innervated by the recurrent laryngeal nerve with the exception of the cricothyroid muscle, which is supplied by the external branch of the superior laryngeal nerve. The sensory and secretomotor supply of the larynx above the level of the vocal cords is by the internal branch of the superior laryngeal nerve and below by the recurrent laryngeal nerve.

The main vascular supply is derived from the superior thyroid and inferior thyroid arteries with corresponding venous drainage to the internal jugular and subclavian veins.

.1V Divisions

The shape of the larynx in coronal section is as shown(illustration 1.1). Note the out-pouching of the ventricle between the vestibular and vocal folds. The larynx is divided into 3 main regions. The supraglottis which includes all areas of the larynx above the level of the true vocal cords. The glottis is the region of the true vocal

Illustration 1.1

CORONAL SECTION OF LARYNX SHOWING REGIONS

Regions	Specific Sites
A supraglottis	X.vestibular bands(false cords)
B glottis	Y.vocal(true) cords
C subglottis	

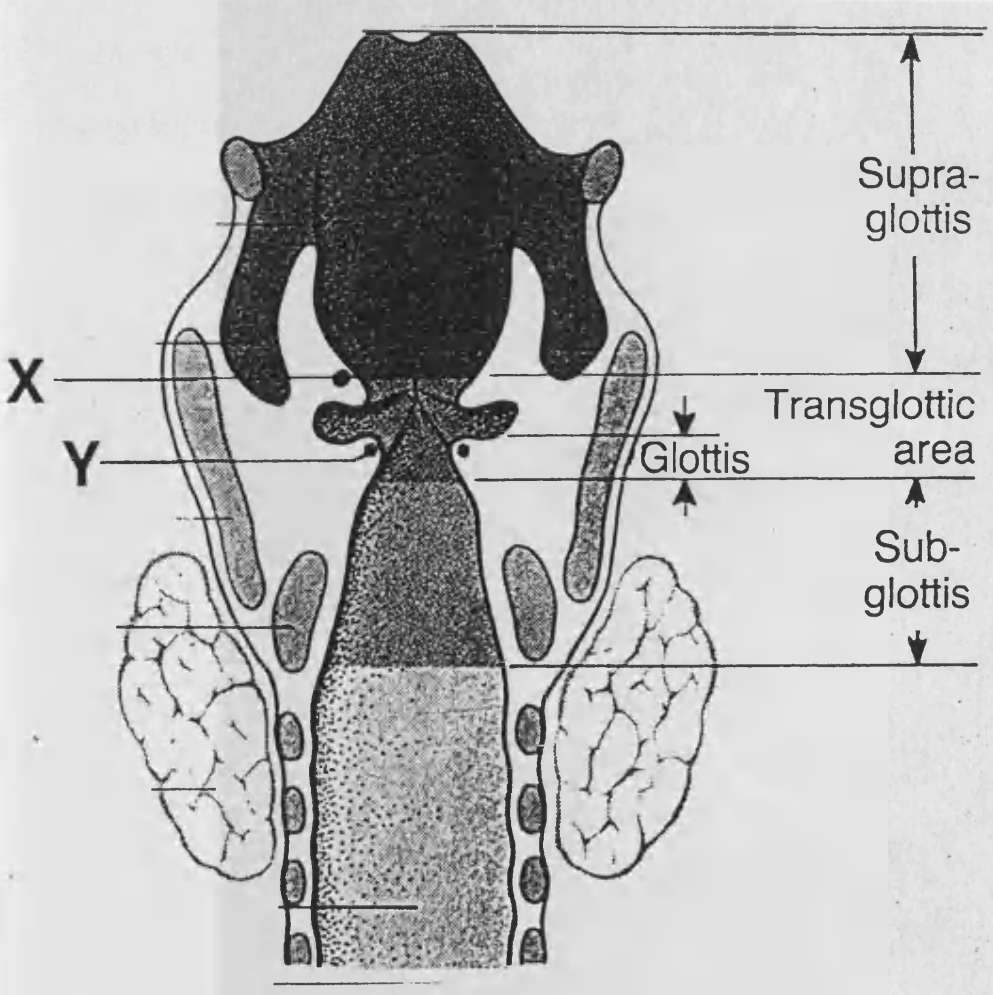
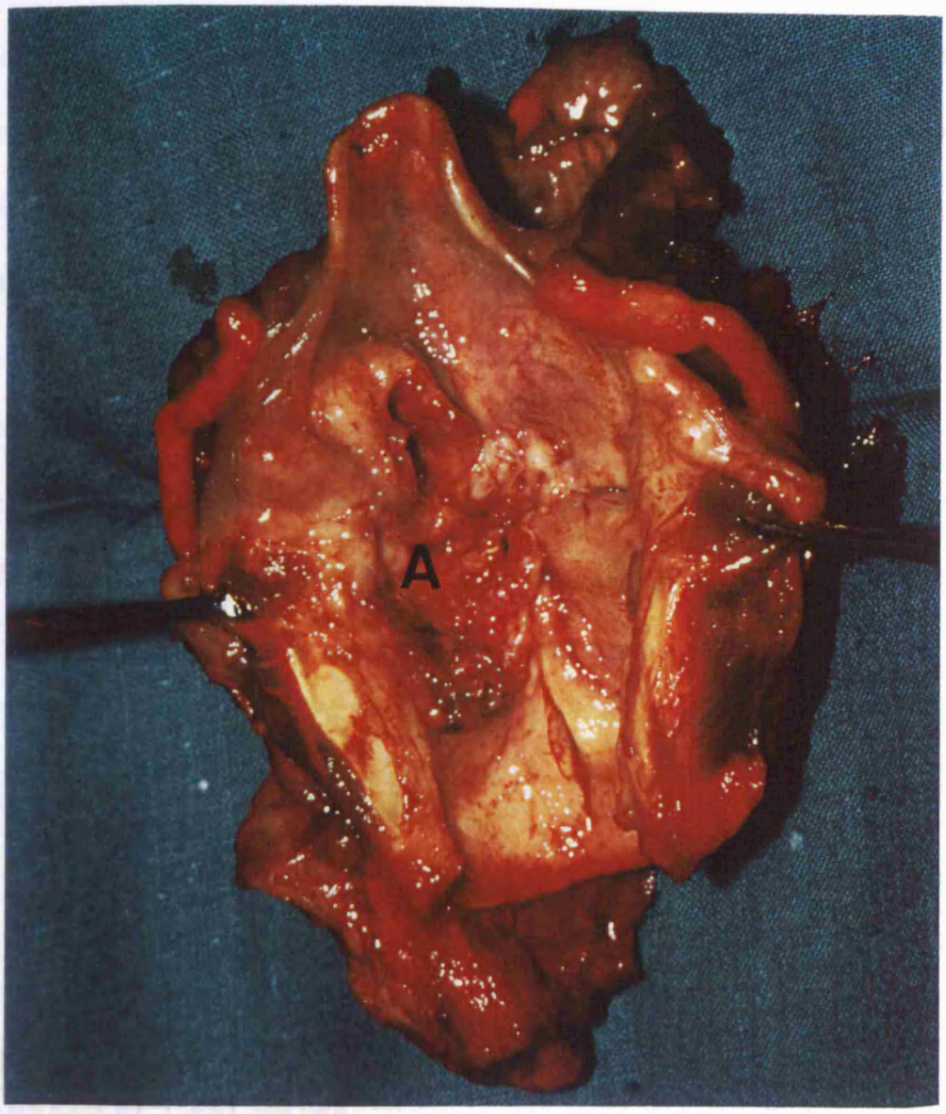


Illustration 1.2

A LARYNGECTOMY SPECIMEN SHOWING A CANCER

A vertical incision through the posterior larynx allows the tumour margins to be illustrated.

A Tumour



cords which includes the membranous portion of the vocal cord, and the cartilaginous portion of the vocal cord contributed by the vocal process of the arytenoid cartilage. The subglottis begins from the free edge of the true cords and extends inferiorly to the level of the inferior border of the cricoid cartilage(Weir N, 1987). There is some controversy over the exact junctions between these regions.

V Size

In the adult it is 3.6-4.4 centimetres in length and varies in diameter from the wider funnelled supraglottic area, to the narrower subglottis. The transverse diameter is greater than the anteroposterior. In adults the anteroposterior diameter is 2.6 and 3.6 centimeters, in females and males respectively(Weir N, 1987).

.V1 Function

The larynx transmits air to and from the lower respiratory tract. It prevents the entry of food, and alimentary tract secretions into the airway. The voice is generated by vocal cord motion acting on expired air.

2.Laryngeal cancer

.1 Epidemiology

Laryngeal cancer presents usually in the 7th-9th decade of life, affecting males more commonly than females. The incidence varies worldwide(Maran,A Gaze,M Wilson,J 1992). In high incidence countries such as France, India, Brazil there is a higher male preponderance(IARC, 1990)than in countries of low incidence such as the United Kingdom where the male preponderance is 5:1(Cancer statistics HMSO, 1988). Carcinoma of the larynx is an uncommon disease in the United Kingdom accounting for 7% of head and neck cancers(Powell J & Robin PE, 1983).

.11 Gross Pathology

All regions of the larynx are not equally affected. In the United Kingdom 60% of laryngeal cancers affect the glottis, 30% the supraglottis and 10% the subglottis. The lesions are often exophytic (illustration 1.2) but can be ulcerative, or infiltrative deep to normal appearing mucosa. The regions of the larynx most commonly involved differ in different countries. A proportion of cases on presentation will be transglottic, that is, affecting all areas of the larynx. It is not uncommon for tumours to spread to the adjacent hypopharynx and it can be difficult to determine the site of origin of tumours involving the laryngopharyngeal structures. Histology is not helpful in making a distinction.

Histology

90% of carcinomas at this site are of squamous cell origin (Illustration 1.3). These differ in the degree of cellular differentiation, though this has not traditionally been shown to be of great prognostic significance(Robin PE, Olofsson J, 1987). Quantitative morphometric methods(Fernandez-Nougueras Jimenez FJ & Esquivias Lopez-Cuervo JJ, 1992), immunohistochemical studies of proliferating cell nuclear antigen expression and densitometric image cytometry DNA analysis suggest that structural differences which closely reflect genetic instability and proliferating potential of the tumour are correlated with outcome(MunckWikland E et al, 1993). There are a number of rarer laryngeal histological types but this study is restricted to squamous cell carcinomas.

.111 Clinical features

Laryngeal tumours present with signs and symptoms of alteration of laryngeal function. Dysphonia is the earliest presenting symptom of glottic lesions because of difficulty in maintaining an adequate travelling wave through the glottic epithelium for normal phonation. Tumours in the supraglottis and subglottis tend to present late, because they only interrupt vocal cord movement by spread to the glottis or invasion of the recurrent laryngeal nerve.

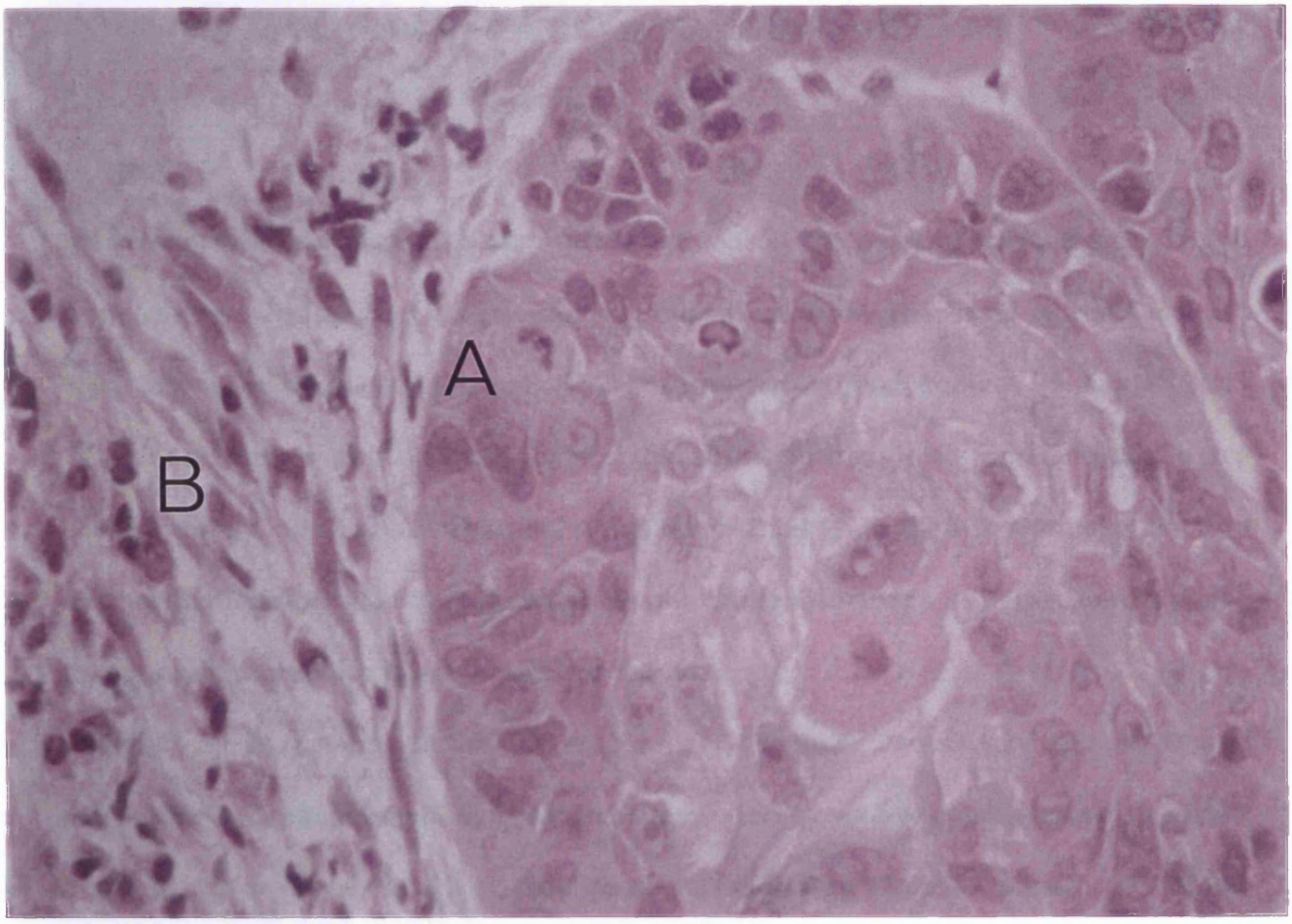
All tumours can eventually cause stridor, though this is invariably a late sign.

Illustration 1.3

HISTOLOGICAL APPEARANCE OF SQUAMOUS CELL CARCINOMA OF THE LARYNX

A Tumour edge

B Adjacent tissue displaying normal architecture



Extension into the hypopharynx can lead to dysphagia. Laryngeal incompetence with aspiration and episodes of pneumonia can occur due to loss of the cough reflex secondary to afferent denervation, or failure of glottic closure. The glottis fails to close either because tumour bulk precludes closure or the vocal cord is fixed by tumour infiltrating the cricoarytenoid joint. Paralysis of the adductor muscles of the larynx secondary to tumour invasion of the recurrent laryngeal nerve can also lead to failure of glottic closure.

Staging

Tumours of the larynx and pharynx are staged on the basis of tumour site, lymph node disease and distant metastases. This system forms the basis of the Tumour, Node, Metastases staging system of the International Union Against Cancer(UICC). The three main regions of the larynx the supraglottis,glottis and subglottis are divided into a number of subsites. A similar method is applied to the nasopharynx and hypopharynx. The T staging of oropharyngeal tumours is determined by size as opposed to subsite involvement by tumour.

In brief, early tumours T1 and T2 are localised to one or two sites within the region of origin. Laryngeal or pharyngeal tumours which extend to a contiguous region or beyond and/or cause vocal cord fixation are classified as T3 or T4 tumours. Evidence of such extension is based on physical examination, endoscopic findings and imaging

studies.

Nodal staging is based on the size of the nodes and their site. N0 being the absence of nodal involvement by tumour. N1 a single node less than 3 centimeters in diameter. N2 the presence of nodes greater than 3 centimeters but less than 6 centimeters in diameter. N3 the presence of any node greater than 6 centimetres in diameter(UICC, 1987).

The tumours in this study have been described as early and advanced based on their T stage as there was little evidence of distant metastases at presentation in the cases studied and the nodal metastases were not subjected to PCR testing.

3.Aetiology

.1 Tobacco and alcohol

Tobacco and alcohol are the main aetiological factors established by epidemiological research (Wynder EL et al, 1976 ;DeStefani E et al, 1987). The effects of which are believed to be multiplicative in the individual patient(Austoker J, 1994). The causal importance of tobacco is supported by animal work confirming the ability of cigarette smoke to induce pre-neoplastic change in tracheal mucosa(Thomassen DG et al, 1989). The demonstration of tobacco specific DNA chemical addition products(adducts) have highlighted the role of tobacco in carcinogenesis(Randerath E et al, 1986).

Alcohol may operate through secondary effects such as hypovitaminosis(Mak KM et al, 1984) and immune compromise or through a direct effect on exposed tissue. There is increasing evidence that vitamin A and C act as inhibitors of epithelial carcinogenesis in the head and neck. In favour of a direct effect of alcohol on tissue is the site predilection of the effect of alcohol which is more marked for supraglottic cancers (epilarynx) than glottic cancers, the type of alcohol being unimportant(Spitz MR, 1994). Alcohol free extracts of beer,whisky, brandy and wine have been shown to be mutagenic (Nagao M et al, 1981 ;Stoltz DR et al, 1982) supporting a direct effect of alcoholic beverages independent of ethanol or its' derived metabolites such as acetaldehyde.

Contaminant byproducts of alcohol production ubiquitously present in alcoholic beverages such as the N-nitroso compounds, (NOCs) may be the important cocarcinogenic agents. The importance of these agents is highlighted by work on N,N-Dimethylamine(NDMA) a volatile N-nitrosamine, though the nonvolatile N-nitroso compounds probably occur in higher amounts and greater frequencies(Hotchkiss JH, 1989). The most important food sources of NDMA in the United Kingdom are beer, fish and processed meat(MAFF, 1987).

NOCs undergo oxidation by cytochrome P450 to unstable alkylating agents which bind to nucleophilic DNA sites(Margison GP & O'Connor PJ, 1979) to form DNA adducts. The cytochrome P-450 system consist of a number of isozymes which differ

in their distribution and specificity for heterocyclic food derived amine mutagens(McManus M et al, 1988). Cigarette smoke induces cytochrome P-450 monoxygenase enzyme activity in the human liver and placenta(Sesardic D et al, 1990). High levels of laryngeal P450 and polycyclic aromatic hydrocarbon derived DNA adducts have been found in smokers consistent with metabolic activation of the hydrocarbons by laryngeal P450(Degawa M et al, 1994). The cytochrome P450 system may represent an end common pathway through which alcohol and tobacco exert their mutagenic effects.

.11 Other Environmental Carcinogens

The possible importance of environmental factors other than tobacco in DNA adduct formation is supported by the finding of similar measurable levels of 0^6-methyldeoxyguanosine(a DNA addition product) in women smokers and nonsmokers(Foiles PG et al, 1988).

A role for asbestos and atmospheric pollution in laryngeal carcinogenesis has been suggested(Wake M, 1993). The higher levels of aromatic hydrocarbon-DNA adducts found in industrial workers and urban dwellers compared to rural inhabitants may be the result of environmental pollution with polycyclic aromatic hydrocarbons(Motykiewicz G et al, 1995).

.111 Radiation and immunosuppression

Laryngeal tumours have not usually been attributed to previous radiation exposure. Laryngeal papillomas when treated by radiotherapy have undergone malignant squamous cell degeneration(Lie ES et al, 1994). In the absence of previous laryngeal pathology the few tumours induced by radiotherapy have been sarcomas rather than squamous cell carcinomas(Nageris B et al, 1994).

A correlation between immunosuppression and tumour development has been recognised though the relationship is one of both cause and effect. Patients with head and neck tumours illustrate poor cellular immunity(Wanebo HJ et al, 1975; Wolf GT et al, 1987). It is however difficult to determine in this patient group how much of the immunosuppression is secondary to the effect of the high alcohol intake(IARC, 1988) common amongst these patients as opposed to a direct tumour effect.

Host immunity to tumours is largely T cell mediated and T lymphocytic infiltration of tumours appears to affect the long term prognosis(Oliver RTD & Nouri AME, 1991). Individuals with defects in cellular immunity are thus at risk of developing neoplastic disease. These defects maybe genetic or acquired. In both groups the common tumours to develop are lymphomas, leukaemia, sarcomas, skin and lip cancers (Filopovich AH et al, 1992; Kinlen LJ et al, 1979; Hoover R & Fraumeni JF, 1973). One possible explanation is the dietary restriction and smoking restriction of

individuals on dialysis and kidney transplant protocols reducing the risk of the more common solid tumours(Oliver RTD & Nouri AME, 1991).

Immunotherapy with alpha-interferon and interleukin-2 is increasingly being assessed as an adjunct treatment modality in head and neck cancer(Urba SG et al, 1992). The combination is especially promising in view of the documented reduced expression of HLA type I antigens in undifferentiated laryngeal tumours(Ruiz-Cabello F et al, 1995), and the ability of alpha-interferon to bring about up-regulation of HLA class I genes(Fellous M et al, 1982). Increased surface expression of HLA type I antigens would be an expected consequence of up regulation of class I genes. Tumour cells would then be more likely to bear antigens recognisable by active T cells. This beneficial effect should be further augmented by interleukin-2 stimulation of T cell activity(Urba SG et al, 1993).

.IV p53

There is increasing evidence of the role of tumour suppressor genes in laryngeal carcinogenesis(Clark LJ, 1993). Mutated P53 has a longer half-life and is more easily detected than wild type. A number of studies have illustrated increased levels of P53 in head and neck squamous cell carcinoma(Burns JE et al, 1993; Brachman DG et al, 1993) consistent with P53 mutation.

Mutated P53 may shift the cellular genetic balance towards proliferation thus

inducing cancer(Prindull G, 1995). High levels of tobacco and alcohol may predispose to laryngeal cancer through P53 mutation(Field et al, 1992 & 1994). This view has not received universal support and remains difficult to prove because of selection bias(Franceschi S et al, 1995).

.V HPV

There are a group of patients with no history of tobacco or alcohol use who develop laryngeal squamous cell carcinoma. A number of factors have been suggested to be responsible for laryngeal carcinogenesis in these cases including oncogenic viruses(Simon M et al, 1994). The development of the highly sensitive polymerase chain reaction for viral detection has promoted this work. The technique allows the identification of viral DNA by progressive amplification of a target sequence of nucleic acids(Saiki RF et al, 1985; Markham AF et al, 1993).

The Human papilloma virus(HPV) is the aetiological agent in recurrent laryngeal papillomatosis(Terry RM et al, 1987 & 1989; Rimmel F et al,1992). Laryngeal papillomas have progressed to invasive squamous cell carcinoma, and the associated viral DNA postulated as responsible for cellular transformation(Zarod AP et al, 1988; Simon M et al, 1994; Lie ES et al, 1994). Radiation(Zur Hausen H, 1977; Lindeberg H & Elbrond O, 1991), immunosuppresion(Bradford C, 1990) and Bleomycin(Lie ES et al, 1994) have been reported to act as cofactors in malignant progression, in the

absence of tobacco exposure.

The cellular mechanisms by which HPV infection may lead to neoplastic change has been studied. HPV16 and 18 DNA is found integrated into the host genome, integration precedes cell immortalization and transformation. Integration of HPV DNA results in nonrandom breaks in the viral DNA at the E1-2 region(see chapter 2.1). It has been shown that such breaks reduce the function of the upstream regulatory region, leading to proliferation of the E6/E7 gene products(Scurry J & Wells M, 1992).

These products bind with human tumour suppressor genes wild type p53 and the retinoblastoma gene (pRB)(Munger K et al, 1989; Werness BA et al, 1990) offering a plausible explanation for malignant transformation. It would seem reasonable to assume that the presence of HPV would alter the tissue expression of P53. This has not been confirmed and in both laryngeal cancer and pre-cancer HPV and P53 expression appear to be independent(Lee NK et al, 1993; Fouret P et al, 1995). Laryngeal cancer is multifactorial and different mechanisms are probably operating in different cases.

HPV6 and HPV11 DNA have not been found to be integrated into the malignant cell's DNA, but occur as episomal DNA. Episomal HPV6 and HPV11 have been shown to bring about cell transformation. Augmented transcription through nucleic acid alterations in the purine-thymidine rich area of the upstream regulatory region is

the postulated mechanism(Kasher MS & Roman A, 1988). Duplications and insertions have been found in the nucleic acid sequence in the upstream regulatory region of low risk papillomaviruses when they are present in high risk lesions(Boshart M & Zur Hausen H, 1986; Rubben A et al, 1992). These alterations could have led to deregulated growth. It is also possible that HPV6 and 11 DNA is integrated at less than 1 copy per cell and thus undetectable with present technology.

It is most likely that if HPV has a role to play in laryngeal carcinogenesis this will be as a cofactor. Interestingly it has been reported that cigarette smoking has a dose dependent effect on the occurrence of oncogenic HPV in cervical tissue(Burger MPM et al, 1993).

.V1 Genetic factors

A genetic predisposition to cancer is highlighted by the Li-Fraumeni syndrome and the basis of this susceptibility may be mediated through relative defects in tumour immunity controlled by the human leucocyte antigen system(Coffin CM et al, 1991). Laryngeal squamous cell carcinoma has not been reported to occur more frequently in patients with genetically determined immunocompromise(Buehler SK et al, 1975; Morrell D et al, 1987) though tumours of lymphoid origin and skin cancer occur more commonly in these patients(Roitt IM et al, 1985).

CHAPTER 2

HUMAN PAPILLOMA VIRUS

1.Structure-

.1 Morphology

.11 Classification

2.Laryngeal Squamous Cell Carcinoma

3.Transmission of HPV-

.1 Genital

.11 Laryngeal

.111 Conclusion

1.Structure

.1 Morphology

Human papilloma viruses are a group of small DNA viruses composed of capsids enclosing 8-kilobase circular segments of double stranded DNA(Arends MJ et al, 1990; Chang F, 1990). The viral genome consists of a regulatory region, and a series of protein coding regions known as open reading frames which are classified as early and late with respect to the viral replication cycle. There are usually 7 early

labelled E1-E7, and 2 late correspondingly L1-2 open reading frames(Scurry J & Wells M, 1992)(illustration 2.1).

.11 Classification

Papillomaviruses were typed by the degree of DNA cross hybridization. That is less than 50% cross hybridization by reassociation kinetics between different HPV types(Coggin JR & Zur Hausen H, 1979). Subtypes such as HPV6a, 6b and 6c do not vary significantly in DNA cross hybridization but have different restriction endonuclease digestion patterns(Arends MJ et al, 1990).

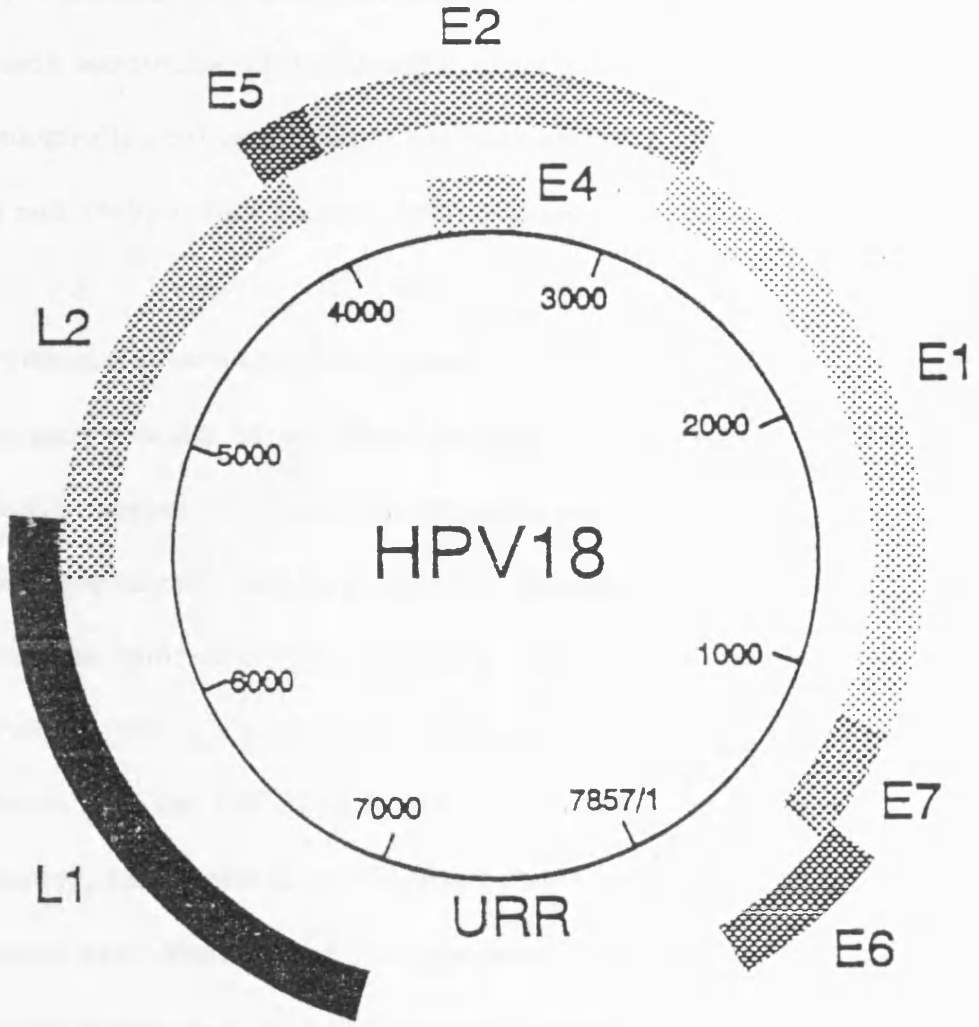
In 1991 the Papillomavirus Nomenclature Committee proposed a new taxonomic system. New papilloma virus types are now defined on the basis of a detailed knowledge of their genotype. A new type must show less than 90% nucleotide sequence homology in the E6, L1 and upstream regulatory regions with established HPV types. Mathematical algorithms are being employed in the construction of a phylogenetic tree which may lead to a better understanding of papillomavirus induced pathology(Van Ranst MA et al, 1993).

It is established that the different types of HPV show a high degree of site and disease specificity. HPV types 6,11,16,18 and 33 have been described in the human larynx (Terry RM et al, 1987; Morgan D et al, 1991; Hoshikawa T et al, 1990; Kiyabu M et al, 1989; Ogura H et al,1991) in association with benign and malignant disease.

Illustration 2.1

HPV 18 GENOME

Early and late open reading frames labelled E and L respectively



The site specificity and oncogenic potential of these viruses vary. HPV16 and 18 are primarily described as oncogenic types, and HPV6 and 11 as benign(Scurry J & Wells M, 1992; Kasher MS & Roman A, 1988).

The majority of evidence is in favour of the above, however some reports are at variance. Sutton GP et al, (1987) reported an association between HPV6 and invasive squamous cell carcinoma(SCC) of the vulva. HPV6 has also been isolated in verrucous carcinoma of the genital tract(Okagaki T et al, 1984). In addition morphologically nontumour tissue has been shown to harbour the oncogenic subtypes HPV16 and 18(Syrjanen S et al, 1990; Maitland N et al, 1989).

2. Laryngeal Squamous Cell Carcinoma

A wide range 5-88% of squamous cell carcinoma cases have demonstrated HPV infection, subtypes 6,11,16,18 and 33 being found(Dekmezian RH et al, 1987; Bryan RL et al, 1990; Brandasma JL et al, 1990; Kashima J et al, 1986). The wide variation is partly accounted for by the use of different techniques with different sensitivities. The Southern blot technique(Southern EM et al, 1975) showed a 5% prevalence rate for HPV 6,11 & 16 in control and tumour larynges(Brandsma J & Abramson A, 1989). Similar low levels of 5% for type 16, and 8% for type 11 was found with insitu hybridisation and PCR(Syrjanen S et al, 1990).

Polymerase chain reaction techniques have found higher levels of HPV, 18-40% in

laryngeal squamous cell carcinoma. Atypical laryngeal carcinoma's namely those in females and younger patients showed a greater prevalence of HPV16 as opposed to other laryngeal carcinomas(Hoshikawa T et al, 1990; Kiyabu M et al, 1989; Morgan D et al, 1991).

Case mix in the studies may also account for the differences. There maybe different rates of HPV infection in different populations despite similarities in age,sex and site of tumour cases(Hoshikawa T et al, 1990; Ogura H et al, 1991; Nunez DA et al, 1994).

Control tissue is seldom studied, but whenever assessed suggest that the isolation rate in tumour cases is not different to that in the control tissue. The control tissue is usually adjacent histologically normal sites, or cases with benign laryngeal pathology(Bryan RL et al, 1990; Morgan D et al, 1991). These groups are not satisfactorily representative of the general population.

The ability to isolate HPV by PCR on formalin fixed paraffin embedded autopsy larynges has been established(Nunez DA et al, 1993). A group of control laryngeal tissue can thus be obtained from autopsy cases without laryngeal disease for comparison with the tumour group.

In the present study tumour cases were compared with autopsy controls. The PCR reaction was used because of its high sensitivity, which is useful when studying relatively low DNA yielding formalin fixed tissue(Warford A et al, 1988).

3.HPV Transmission

.1 Genital

HPV is thought to be a venereally transmitted agent(Barrett JJ, 1954; Oriel JD, 1983; Fairley CK et al, 1994). In a study of female partners of men with penile condylomata acuminata an increased risk of cervical neoplasia compared to controls was found(Campion MJ et al, 1985). Transmission of HPV is clinical lesion and HPV type specific(Barrasso R et al, 1987; Koutsky L et al, 1988; Manos M et al, 1990). High HPV isolation rates of up to 90% have been reported in cervical neoplasia by DNA hybridisation(Lancaster WD et al, 1986) and polymerase chain reaction methodology(Manos M et al, 1988; Pao CC et al, 1990). A geographical variation in prevalence rates seems likely and would explain some of the variation in several studies as well as a variation in the sensitivity of different techniques of virus isolation(Reeves D et al, 1989; Nunez et al, 1994). Sampling error is an alternative explanation, since only 33% of penile squamous carcinoma tissues were positive for HPV types 16 and 18 when sampled at more than one site(McCance DJ et al, 1986). Inadequate test reliability may also contribute to the variation noted(Schiffman MH & Schatzkin A, 1994).

Squamous cell cervical carcinoma is epidemiologically related to measures of sexual activity. The earlier the age of first intercourse, frequency and number of sexual partners(Buckley JD et al, 1981; Rotkin ID et al, 1973). The theory of a venereally

transmissible agent seems probable, and HPV is a candidate virus. There are conflicting reports but these are in the minority and are often flawed.

A population based survey carried out in Greenland and Denmark which found no association between HPV and cervical carcinoma after comparing the prevalence of cervical HPV in both countries which have a 5-6 fold variation in cervical carcinoma incidence rates, had a sampling bias. Fifteen percent of the subjects selected were not studied for logistical reasons or patient refusal. The cohorts finally studied varied significantly in the proportion having previous cervical smears. It is also interesting considering the expected incidence rate of cervical carcinoma in the populations studied that none of a sample of 1247 had more significant cervical disease than dysplasia(Kajer DK et al, 1988).

Twenty-one percent of anogenital samples from commercial sex users and providers attending a STD clinic in Amsterdam were found to be HPV positive. The HPV negative and positive groups sampled did not vary sufficiently in their sexual lifestyles to demonstrate that differences in sexual behaviour were predictive of HPV isolation(Van Doornum GJJ et al, 1992).

Adjusting for age does not abolish the relationship between HPV and cervical squamous cell carcinogenesis. Ten percent of the tumour cases in Meanwell et al's study were not squamous cell carcinomas, furthermore the control group was younger than the tumour group and interestingly 25% of HPV positive cases when

sampled at different sites were negative.(Meanwell CA et al, 1987)

Several large multi-centred studies have shown that 35-62% of women with intraepithelial or invasive cervical carcinoma were HPV non-type specific or HPV16/18 positive as opposed to 9% of women with normal cervical cytology(DeVillers EM et al, 1987) and 32% of age-matched randomly selected controls(Reeves WC et al, 1989). HPV appears to have an aetilogical role in areas with a high incidence of cervical carcinoma.

Some studies while acknowledging that sexual behaviour is strongly associated with cervical cancer found it was not associated with HPV infection(Reeves WC et al, 1989; Kiviat NB et al, 1989). Studies which found evidence of HPV infection in sexually active adolescents and women versus its absence in virgins suggest that sexual activity does determine genital HPV detection(Andersson-Ellstrom A et al, 1994; Rylander E et al, 1994). Nonsexual transmission(Cason J et al, 1995) probably confounds attempts to correlate genital HPV infection with sexual behaviour and may account for the disagreement in the literature(Jenison SA et al, 1990). In addition an age-specific effect cannot be ruled out as studies which were restricted to women in the second and third decades found high prevalence rates dependant on the number of sex partners(Bauer HM et al, 1991).

A novel suggestion is that trauma associated with sexual intercourse is acting as a stimulus for proliferation of otherwise latent HPV infection(Jenison SA et al, 1990).

Hence the predisposition for HPV isolation at sites of trauma the posterior introitus and cervix in females(Nagington J & Rook A, 1979; Oriel JD, 1971).

This introduces the concept of latent viral infection. Latency as applied to the Herpes virus family implies the presence of either integrated or episomal viral DNA in otherwise normal host cells. The viral DNA while being capable of replicating infectious viral particles at some latter date in response to trigger factors expresses only some genes e.g EBNA(Corey L & Spear PG, 1986; Liebowitz D, 1994).

HPV DNA exist in otherwise normal host cells at various anatomical sites(Maitland NJ et al, 1989; Nunez DA et al, 1994). What is, however, uncertain is the ability of the cell localised viral DNA to generate infectious viral particles in response to a trigger factor. The increase in shedding of HPV DNA from the genital tract with pregnancy(Rando RF et al, 1989) suggest increased viral replication. The infectivity of virus generated at this time can be assumed from papers suggesting vertical transmission of HPV(Sedlacek TV et al, 1989). Further work is however needed to confirm this, and for the time being latency as applied to HPV relates to occult infections detected by molecular biological techniques.

The recent development of serological tests will allow this hypothesis and alternative modes of transmission to be tested more widely. Children without a history or clinical evidence of HPV infection were as likely to be seropositive for HPV6,16 and 18 as patients attending a STD clinic with HPV DNA positive lesions(Jenison SA et

al,1990). This was further confirmed by finding similar rates of PCR positive HPV6 and 16 DNA in buccal mucosa from pre-school children and adults.

A study of normal children and women with HPV 16 DNA-positive Cervical intra-epithelial neoplasia(CIN) found a higher HPV seroprevalence among the women(Cason J et al, 1992). L1 serodeterminant proteins(Jenison SA et al, 1988; 1989) which are highly conserved across HPV species(Baker C, 1987) were utilised in the study, but the method of subject selection and age distribution of the CIN patients were not reported. Age may have a bearing on the rate of HPV detection(Bauer HM, 1991). All the CIN samples were checked for HPV16 but not for HPV6 or 11 and multiple infections may have occurred(Young LS et al, 1989). Seropositivity to the L1 protein reported by this study may therefore not always be consistent with HPV16 exposure, but it does suggest a difference in HPV seroprevalence between the study groups.

The most common immunoglobulin in serum is IgG(Roitt et al, 1985). The Jenison et al(1990) study does not state the type of immunoglobulin isolated. The findings of Jenison et al(1990) and Cason et al(1992) may be complementary in that past exposure to HPV may lead to IgG clonal specificity which does not allow for a detectable difference in the STD and child population. Forty percent of seropositive children were under the age of 6 months and the role of maternal transmitted IgM was not fully excluded(Cason J et al, 1992).

Pao CC et al(1991) illustrated that peripheral monocytes harbour HPV, offering a nonsexual transmission route plus a site for latent infection to maintain high levels of immunoglobulin expression.

Clinical expression of HPV either in condylomata acuminata or in cervical and other cancers maybe a by-product of host stimulation by chemicals or hormones. Oestrogen stimulation or altered immunity have been postulated to explain the increased incidence of HPV lesions seen in pregnant women in the third trimester, with a return to low levels post-partum(Rando RF et al, 1989; Schneider A, 1987). There are no reports suggesting that these women become symptomatic because of intercourse with new sexual partners. The stimulatory effects of steroid hormones on viral transcription up stream regulatory genes has been shown(Schneider A et al, 1987; Gloss B et al, 1987; Pater MM et al, 1988).

The effect of co-infection with other viruses is also being studied, and so far herpes simplex which was the previous candidate aetiological virus for cervical cancer has been shown to effect up-regulation of HPV18 transcription(Gius D & Laimins LA, 1989; McCusker CT & Bacchetti S, 1988). Human Herpes virus 6(HHV6) a ubiquitous and usually latent herpes virus(Chen MC et al, 1994) is active and widespread in AIDS (Corbellino M et al, 1993). HHV6 enhances the expression of HPV oncoproteins(Chen MC et al, 1994b). A cooperation between HPV and HHV6 in facilitating oncogenesis is however so far unproved(Chen M et al, 1994).

.11 Laryngeal

A number of reports have shown that HPV is present in the absence of clinically apparent disease of the larynx(Lie ES et al, 1994; Mounts P et al, 1984). The traditional view is that young mothers with condylomas delivering vaginally predispose their infants to juvenile onset Recurrent Respiratory Papillomatosis(RRP)(illustration 2.2). This opinion is based on retrospective studies. This is refuted by the finding that caesarean section does not abolish the occurrence of juvenile onset laryngeal disease(Shah K et al, 1986; Abramson AL et al, 1987; Kashima HK et al, 1992).

Prospective studies have been less successful than retrospective ones in proving vertical transmission. Commercially available hybridisation detection techniques(ViraPap/ViraType) found 18% of mothers and 2.8% of neonates at 24-72 hours after birth to have evidence of HPV infection(Smith EM et al, 1991). However the types of HPV identified from the oropharynx in the two positive neonates were HPV16 and 31/33/35 and not 6 and 11 the RRP associated virus types. Sedlacek TV et al(1989) in a similar study did not type the HPV found in the neonates and none of the mothers typed positive for HPV6/11 casting doubt on the birth canal as a route of transmission of HPV6/11. St.Louis ME et al(1993) in their study of vertical transmission of HPV did not find evidence of HPV6/11 subtypes in the oral cavity of the HPV positive children.

The confounding finding of neonates with HPV born to mothers with no evidence of the infection in the genital tract(Sedlacek TV et al, 1989) may represent sampling error or be indicative of non-genital transmission of HPV to the neonate(St. Louis ME et al, 1993). The role of alternative routes of transmission including autoinoculation and close non- sexual contact is supported by the 17-23% prevalence of HPV6 and HPV16 in preschool children(Padel AF et al, 1990; Jenison SA et al, 1990).

111.Conclusion

The overwhelming body of evidence is in favour of venereal transmission of anogenital HPV. Vertical transmission of HPV to the larynx is not entirely supported and this maybe due to alternative routes of transmission not as yet clearly elucidated.

4.Aim of Study

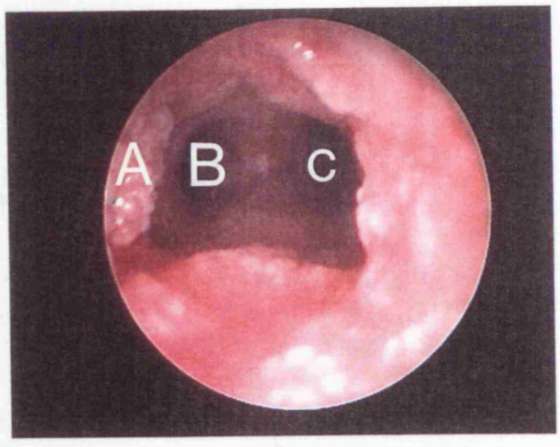
It is anticipated that if the oncogenic HPV16 type plays a major role in the aetiology of squamous cell carcinoma of the larynx it will be found in a high proportion of laryngeal tumours but uncommonly in the nondiseased larynx.

The present study determines the prevalence of Human papilloma virus types 6, 11 and 16 in a series of formalin fixed paraffin embedded laryngopharyngeal squamous

Illustration 2.2

ENDOSCOPIC VIEW OF CHILD'S MAIN BRONCHUS WITH LARYNGEAL PAPILLOMA

A- Site of papilloma
B- left main bronchus
C- right main bronchus



cell carcinoma specimens, utilising the polymerase chain reaction. The findings are compared with the results in a series of nondiseased autopsy larynges.

CHAPTER 3

HPV PERSISTENCE

1.Introduction

2.Age

3.Major Histo-compatibility Complex

4.Immunosuppression-

.1 Genetic

.11 HIV

.111 Iatrogenic

5.Immunotherapy in head and neck cancer

1.Introduction

Human papilloma virus induced lesions of the larynx show a predilection for the squamo-columnar junctional glottis(Kashima H et al, 1993). In this respect HPV is behaving as it does elsewhere in the body, most commonly proliferating at junctions of different epithelial types(Toon PG et al, 1987; Singer, 1976; Zur-Hausen H & De Villers E-M, 1994), the transformation zone in the cervix and the squamo-columnar junction in the anal canal(Frazer IH et al, 1986). Tissue tropism is also illustrated

by the Epstein-Barr virus another candidate oncogenic human DNA virus, which has a predilection for lymphoid tissue(Wright DH, 1985; Geddes JF et al, 1992). Cytomegalovirus on the other hand which is not an oncogenic candidate virus, does not illustrate significant tissue tropism(Robbins SL, 1979; Gehrz RC, 1991).

Do all individuals exposed to HPV become infected, and what proportion become immunocompetent? Serological evidence of HPV infection had been lacking until the work on synthetic peptides(Dillner J et al, 1989) and fusion proteins(Li CC et al, 1987; Jenison SA et al, 1988) was reported. Host factors predisposing to susceptibility and persistence of HPV infections can now be studied further. Immunity to viruses beyond the nonspecific actions of lysosome and natural killer cytotoxic cells is largely cellularly mediated(Roitt IM et al, 1985).

Other viral infections offer models of factors which may predispose to HPV persistence. Herpes simplex virus is a suitable virus for comparison. Serological evidence of Herpes infection varies in different populations. In the western world by the fourth decade 41% of individuals are seropositive to the herpes virus common antigen though a much smaller proportion are symptomatic with recurrent Herpes labialis(Corey L & Spear PG, 1986). Previous serological studies in the 1940's and 50's found a 90% seroprevalence in all populations studied (Nahmias AJ & Rozman B, 1973). There is no evidence however of a pandemic of herpes labialis at that time suggesting a mismatch between serological conversion and disease expression. A

number of reasons may account for this including subclinical infection and altered host responsiveness determining severity of infection and viral latency. Herpes labialis latency is maintained by viral infection of neurons(Fraser NW et al, 1981; Galloway D et al, 1979).

Comparison of recurrent herpes labialis(RHL) cases and controls shows an association with resistance to RHL clinical expression and HLA-B35 genotype(Gallina G et al,1987). Class I and II major histocompatibility complex restricted T-cells play a major role in protection from HSV infection(Corey L & Spear PG, 1986; Yasukawa M & Zarling JM, 1984; Rouse BT & Lawman MJ, 1980).

It would not be surprising if host immunocompetence had a role to play in the persistence and pathological expression of HPV infections. Latent HPV epithelial infection has been proposed(Raab-Traub N, 1989); latent being used to describe the demonstration of HPV nuclear material in the absence of cytological manifestation of viral infection. The lack of persistence or rather detection of HPV nuclear material on repeat sampling maybe secondary to alterations in host immunity determining HPV replication(Caussy D et al, 1990).The immunosuppressive effect of pregnancy may account for the increased detection of HPV associated with pregnancy(Caussy D et al, 1990) rather than up-regulation by hormonal stimulation of endocrine responsive regulatory viral genes (Mitrani-Rosenbaum S et al, 1989; Chan WK et al, 1989).

2.AGE

In the immunocompetent host HPV persistence may be an age related effect(Hildesheim A et al, 1993; Meanwell CA et al, 1987). Twenty percent of cytologically normal women of all ages harboured HPV types but persistence for more than 3-6 months was uncommon. HPV16 infections in a 30 year and over age group proved persistent(Hildesheim A et al, 1994). The proportion of the intrasubject variation in HPV isolation at different times due to sampling error is uncertain but probably lies between 25%-66%(McCance DJ et al, 1986; Meanwell CA et al, 1987). An age related variation in the prevalence of genital warts in male hetero and homosexuals has been shown(Judson FN et al, 1980).

3.The Major Histocompatibility Complex(MHC)

The MHC is a collection of genes located on the short arm of chromosome 6 which determine self recognition. These genes are arranged in two main groups in humans, MHC class I and class II and encode for cell bound protein complexes which are antigenic(Rayfield LS & Challacombe SJ, 1987). The main function is to allow presentation of endogenous antigenic peptides to cytotoxic T lymphocytes. The cytosol derived antigenic peptides are thought to be transported by proteins encoded for by genes located in the class II region(Kaklamanis L et al, 1994).

Illustration 3.1

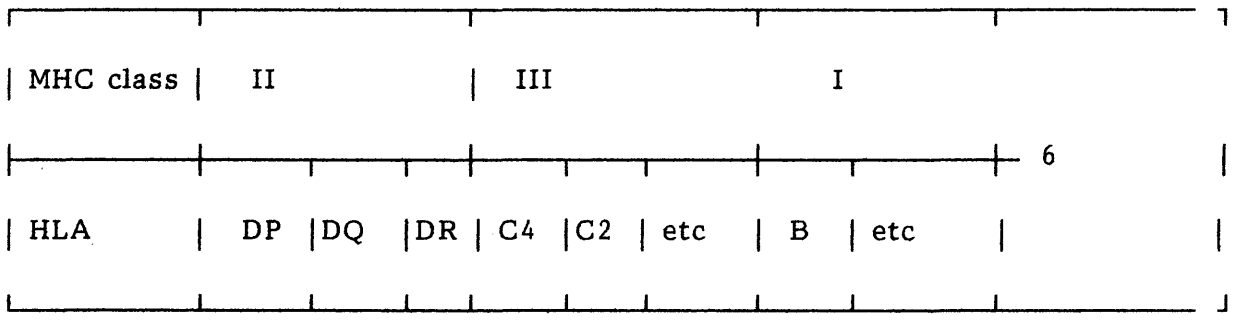
THE MAJOR HISTOCOMPATIBILITY LOCUS

DR DP and DQ loci code for HLA class II molecules

A B and C loci code for HLA class I molecules

C2 Bf and C4 loci (HLA class III)do not play a role in cell recognition but are important in the complement pathway

6=chromosome 6



Antibody dependent cell mediated cytotoxicity which represents a point of cooperation between humoral and cellular immune systems is dependent on the presentation of foreign antigens in association with MHC class II molecules by antigen presenting cells to T helper lymphocytes(Roitt IM et al, 1985; Cromme FV et al, 1993). Not all cells bear MHC I and II molecules. MHC I is expressed by almost all nucleated human cells but not by erythrocytes. MHC II is expressed by B lymphocytes, macrophages, antigen presenting cells and activated T cells. The MHC(also know as the Human leucocyte Antigen) two major regions are coded for by genes at loci A B and C for MHC I, and DP DQ and DR for MHC II(Rayfield LS & Challacombe SJ, 1987).

There is a wide heterogeneity at these loci in human populations(Benacerraf B, 1981) though linkage disequilibriums have been shown for several of the genes. Thus specific allelic associations have been found to occur more frequently than chance the so called haplotypes. Disease associations have been established for specific HLA haplotypes which maybe secondary to their effect on immune mediated disease. Thus HLA-B27 and ankylosing spondylitis, HLA D/DR4 and Rheumatoid arthritis(Rayfield LS & Chalcombe SJ, 1987).

HLA B35 is associated with resistance to clinical expression of RHL(Corey L & Spear PG, 1986; Gallina G et al, 1987). More importantly an elegant study illustrated that HLA a1B8DR3 is associated with an increased risk of seroconversion on exposure to,

and progression of HIV infection once established(Steel CM et al, 1988). A number of investigators have found HLA associations with HIV related disease including Kaposi's sarcoma(Enlow RW et al, 1983; Scorza Smeraldi R et al, 1986; Fabio G et al, 1990).

The Shope virus model infection of domesticated rabbits, shows an association between viral persistence and tumour development with major Histocompatibility class II antigen determined alleles. Clearance was associated with a linkage disequilibrium for DQaE and DRaB(Han R et al, 1992). A similar pattern is demonstrated in humans by the predisposition to cervical cancer in bearers of particular HLA DR and DQ haplotypes(Wank R & Thomassen C, 1991; Apple RAJ et al, 1994).

T-cell immune responses are depressed either in response to immunosuppression or denovo in many tumour patients(MCDougall CJ et al, 1990). This has been shown to be associated with a loss of HLA class I and II antigen expression in the spontaneous group as opposed to its maintenance in the immunosuppressed group(Oliver RTD & Nouri AME, 1991). It is postulated that increased turnover and shedding of HLA antigen is responsible for this under-expression(Swan F et al, 1989). Structural genetic change, transcriptional change modulated by regulatory genes(Ruiz-Cabello F et al, 1988) or post-transcriptional alteration(Cromme FV et al, 1993) may also be responsible for the reduction in the MHC products.

It is reasonable to propose that some individuals will be at greater risk of HPV

infection and persistence, thus demonstrating more severe forms of HPV related disease based on their HLA haplotype(Duggan-Keen, personal communication). At present such a relationship between dysplasia, the presence of HPV, and MHC expression has not been shown(Cromme FV et al, 1993; Jochmus I et al, 1993). Longitudinal studies would be necessary to illustrate the role of alterations in MHC expression on progression of CIN or prognosis for HPV related carcinoma.

4.Immunosuppression

.1 Genetic Susceptibility

Poor host cell mediated immunity in the genetically determined epidermodysplasia verruciformis or neoplasia-papilloma syndrome is associated with the development of squamous cell cancer from preexisting HPV types 3 and 5 associated skin warts. Ultra-violet radiation from sunlight is the cofactor in these cases(Carson LF, 1986; Boyle J et al, 1984). Ultra-violet(UV) light both induces dysplastic cellular DNA damage(Boyle J et al, 1984), importantly mutation of P53(Dei Tos AP, 1994) and suppresses the immune response to anaplastic cells(Parrish JA, 1983). In the murine model UVB induces suppressor T cells which reduce the ability to reject UVB induced carcinogenesis(Fisher MS & Kripe ML, 1982; Kripke ML et al, 1981). Defects of cell mediated immunity are not always associated with a high level of HPV infection, example severe combined immunodeficiency(Stephan et al, 1993) or

idiopathic CD4 lymphocyte deficiency(Goodrich et al, 1993).

.11 HIV

Immunodeficiency secondary to Human immunodeficiency virus(HIV) infection increases the shedding of herpesvirus 7(Di Luca D et al, 1995); it is unknown if the shedding of HPV from the genital tract of infected individuals is similarly increased. HIV infected patients have high levels of anogenital HPV infection which is assumed to be a reflection of poor immunity(Judson FN et al, 1980). Bradbeer's study(1987) of HIV patients found a high level of HPV infection but importantly the patients studied had a high level of infection with a range of sexually transmitted diseases. The high level of HPV infection in HIV patients may be a reflection of increased exposure to HPV infection rather than a reduced immunity to infection. This is supported by the lack of correlation between symptoms and signs of immunodepression and the presence of cervical HPV or dysplasia in a series of 33 HIV positive women(Vernon SA et al, 1994) .

Cervical dysplasia is associated with sexual promiscuity. High levels of cervical intraepithelial neoplasia are inconsistently reported in studies of HIV positive patients(Bradbeer C, 1987; Crocchiolo P et al, 1988). The discrepancy may be a reflection of differences in the sexual lifestyles of patients who have acquired HIV sexually and those who have acquired HIV through infected blood

transfusions(Spurret MA et al, 1988).

Epidemiological correlates of homosexuality are associated with an increased incidence of anorectal dysplasia in men(Daling JR et al, 1982). It is suggested that anorectal dysplasia may as cervical dysplasia be aetiologically linked with HPV infection hence a higher incidence of HPV is to be expected in homosexual males. Studies reporting a higher level of anogenital warts in homosexual males often subject homosexual subjects to more extensive investigation example proctoscopy compared to heterosexual subjects(Short SL et al, 1984). Judson FN et al(1980) in a study restricted to genital HPV and with equal sampling strategies in heterosexual and homosexual males found the incidence of genital HPV to be lower in the homosexuals. The difference in reported prevalence rates of HPV infection in homosexual males may be a reflection of differences in the rate of HIV infection in the subjects studied. The prevalence of anogenital HPV infection in HIV positive males and females does not correlate with independent measures of sexual activity but correlates inversely with total CD4+ counts(Melbye M et al, 1990; Caussy D et al, 1990; Williams AB et al, 1994).

HIV infection is known to predispose infected individuals to a range of malignancies the most common being Kaposi's sarcoma. This effect was initially thought to be due to a general reduction in cytotoxic T cell activity. The increasing presentation of Herpes Zoster in HIV infected haemophiliacs along with falling CD4+ levels(Aronstam

A et al, 1993) supports a failure of immunity as a factor in the increased risk HIV patients have of developing some cancers.

A specific HIV induced failure to clear oncogenic viruses such as EBV and HPV is an alternative or additional explanation for Acquired Immunodeficiency syndrome (AIDS) related oncogenesis(Birx DL et al, 1986). A direct aetiologic role for EBV in AIDS related lymphomas is at present unproved but is gaining support(Carbone A et al, 1993; Cremer KJ et al, 1990; Hamilton-Dutoit SJ et al, 1991; Neri A et al, 1991; DeAngelis LM et al, 1992; Geddes FJ et al, 1992).

The role of specific viral effects rather than a general suppression of T cell oncologic surveillance as the important factor in AIDs related oncogenesis is highlighted by the work on Kaposi's sarcoma. A virus was isolated from both AIDS and non-Aids Kaposi's cases(Huang YQ et al, 1992; Chang Y et al, 1994; Su I et al, 1995) and postulated as the cause. HHV-8 has been proposed as the causative virus for both sporadic KS and KS in HIV patients(Lennette ET et al, 1996). Immunocompromised solid organ transplant recipients suffering from KS have also been shown to harbour HHV-8 DNA (Tasaka T et al,1997; Alkan S et al,1997). A possible sexual transmission route for HHV-8 independent of HIV is suggested by the presence of HHV-8 DNA in spermatozoa and mononuclear cells(Huang YQ et al, 1997).

This means that great caution needs to be exercised in accepting explanations of a generalized reduction of T cell function to account for the tendency of CIN to occur

in HIV infected women(Maiman M et al, 1993; Maiman M et al, 1993b; Johnstone FD et al, 1994). The same caution has to be exercised in interpreting the relationship between anal dysplasia, HPV and HIV which is difficult to unravel(Frazer IH et al, 1986; Douglas JM et al, 1986). It is quite likely that oncogenic HPV viruses are leading to SCC independent of HIV just as HHV-8 leads to Kaposi's sarcoma.

.111 Iatrogenic

Renal transplant recipients have a higher rate of HPV infection and skin malignancies than the general population(Welsh KI et al, 1989; Boyle J et al, 1984). HPV,EBV and other viruses may have an oncogenic role in renal transplant recipients who develop cerebral sarcomas, lymphomas(Schenk SA & Penn I, 1971; Hoover R & Fraumeni JF, 1973), premalignant keratoses, and squamous cell carcinoma(Euvrard S et al, 1993). Renal transplantation leads to an alteration in the humoral immunity to HPV(Lewensohn-Fuchs et al, 1993). HPV IgG levels diminished after transplantation with some patients being rendered seronegative. IgA levels increased. This contrasted with the lack of change in titres to adenoviruses and measles, and an increasing IgG titre to CMV.

The increase in IgA was directed against an E2 peptide, suggesting infection or re-infection of mucosal surfaces. The presence of HPV DNA in circulating monocytes has been demonstrated(Pao CC et al, 1991). This combined with an immunoglobulin

A induced shift in EBV tropism(Sixby JW & Yao Q, 1992), offers a route for mucosal reinfection from HPV loaded monocytes.

Why this phenomenon should become more apparent after renal transplantation is uncertain? The types of tumours which occur in the immunosuppressed all have evidence of an oncogenic viral aetiology, and display mechanisms by which they escape immunesurveillance(Euvrard S et al, 1993) . This may occur through a reduction in cell mediated immunity related to the use of immunosuppressant drugs in transplant recipients. Inversion of the helper-inducer/cytotoxic T cell ratio occurs in renal transplant recipients. Langerhan cell levels(the antigen presenting cells) in epithelial tissue are also reduced. Chronic graft antigenic stimulation depletes the effector cells of the immune response(Euvrard S et al, 1993). HPV bearing cells lose their antigenicity by a reduction in viral antigen expression(Khanna R et al, 1993). Down regulation of antigen presentation and cell adhesion molecule production are additional points in the immune response which are muted(Keller R et al, 1995). The transplant recipient illustrates a multifactorial reduction in immune surveillance which obviously favours graft acceptance but which predisposes to the development of tumours with an infectious aetiology.

5. Immunotherapy in Head and Neck cancer.

Tumours develop partly by eluding the host defense mechanism or selectively

occurring in host with poor immune function. Immunotherapy in head and neck cancer has been tried in the past mostly with BCG. The theory being that vaccination would bring about a general stimulation of white cell function, but the results were not sufficiently encouraging for this technique to be adopted routinely. Immunotherapies directed at the sites in the immune response which appear to be diminished in the tumour host are being developed and subjected to evaluation in clinical trials. Lattime et al(1996) have shown that cytokine encoded vaccinia virus productively infects tumour cells after tumour injection. This offers a method of selectively increasing interlukin-2 activity which should enhance tumour cell cytotoxic activity. Tumour infiltrating lymphocytes which recognize unique melanoma tumour cell antigens are being used to clone the genes associated with tumour regression. Adoptive transfer of lymphocytes and active immunization are being used in cancer vaccine trials(Rosenberg SA, 1996).

Directed chemotherapy has been show to be effective in vitro and offers the chance of reducing associated undesirable side effects(Nakaki M et al, 1997). The use of vaccines directed against the oncogenic viruses responsible for some tumours offers another avenue of treatment for evaluation. It is most likely that hepatitis B which causes a large proportion of tumours in the developing world will be the first for which results will be available as a vaccine already exists. It will understandably take 10 to 15 years before the results will be known but the potential impact for such

an immune- preventative approach in human oncology is immense(Oliver RTD & Nouri AME, 1991). The induction of antibodies in the female genital tract in response to parenteral vaccines suggest that vaccines against HPV will work effectively in reducing the incidence of HPV associated cervical cancer.

The field of gene therapy may offer further solutions. Individuals with defects in tumour suppressor genes such as P53 could undergo genetic manipulation with insertion of appropriate deleted segments or regulatory genes thus rendering them capable of mounting an effective immune response to cancer cells.

CHAPTER 4

METHODS

1.Plan of investigations and sampling

1. Sequence of investigations

.11 Sample collection for comparison of HPV6 and 11 isolation

.111 Sample collection for Comparison of HPV16 isolation

2.DNA extraction

3.Confirmation of DNA extraction

.1 PCR with bcl complementary primers

.11 PCR with beta-globin complementary primers

4.Evidence of HPV infection

.1 PCR with L1 complementary general primers

.11 PCR with L1 specific complementary primers

.111 PCR with E6 complementary primers

5.Electrophoresis

1.1.Sequence of Investigations

Laryngeal specimens from autopsy cases were used as the non-tumour cases for the experiments carried out. The specimens were obtained prospectively without randomisation from teaching hospitals in Leeds and Leicester. All postmortems were performed with fresh instruments, and when multiple cases were done in the same session these were carried out on separate tables. The larynx specimens were gathered by the pathologist carrying out the postmortems, and handed over to the investigator who placed them in a formalin filled container for transport. Each specimen was transported in an individual container, which was not reused to reduce the risk of cross contamination. Specific site sampling was carried out by the investigator in the laboratory utilising fresh blades for each specimen. The tissue samples were processed separately. The supraglottis,glottis and subglottis of each larynx was sampled. These sites were analysed separately in each case.

In the first series of experiments tumour cases were collected from archival files of laryngeal squamous cell carcinoma in Leeds. DNA was extracted by proteinase K digestion, followed by alcohol precipitation. Adequate extraction of DNA was confirmed by a polymerase chain reaction with beta-globin primers.The tumour cases were compared with the nontumour cases for evidence of HPV 6 and 11 infection, using PCR primers complementary to the E6 region of HPV. Positive results were classified as such only if repeatable.Table 4.1

TABLE 4.1

OUTLINE OF INVESTIGATION SEQUENCE

Experiment 1

1. Harvesting tumour cases and non-tumour control larynges
2. DNA extraction with beta-globin primer mediated confirmation
3. Testing for prevalence of HPV6 and HPV11

Experiment 2

1. Harvesting tumour cases and non-tumour control larynges
2. DNA extraction with bcl primer mediated confirmation
3. Controls vs tumours for HPV16 with E6 and L1 primers

(bcl primers were used for the second experiment because this work was carried out in a Laboratory with experience in the use of bcl primers for DNA extraction confirmation. Beta-globin primers were not routinely stocked. The size of the expected PCR products are similar and thus it was appropriate to interchange the primers without having a detrimental effect on the interpretation of the study results. In addition as this was a second phase of the study, the results are interpreted separately without prejudicing the first experiments.)

In the second series of experiments the presence of HPV16 was sought in archival squamous cell carcinomas of the larynx and laryngopharynx. The tumour cases were numbered in the order of presentation of histological tissue to the pathology department. A random number series was generated by a Casio FX-82C calculator, and archival cases for study were retrieved on the basis of these random numbers. The tumour DNA prepared as before was compared with DNA from nontumour autopsy larynges. Primers complementary to the L1 and E6 regions of the viral genome were used. (See Outline table 4.1).Agarose gel electrophoresis was carried out to identify the PCR product in all cases.

.11 Sample Collection for investigation of HPV6 and 11 in the larynx.

Twelve normal appearing post mortem larynges were collected sequentially from teaching hospitals in Leeds whenever there was a hospital autopsy and the pathologist did not require the same for further investigation. The larynges were harvested by the pathologist performing the autopsy and each placed in an individual formalin filled container. The specimens were collected by the investigator no more than 2 hours after harvesting and processed the same day in all cases. The aryepiglottic folds were chosen for the supraglottic sample, an area between 1 and 2 centimetres below the edge of the vocal cord was selected for the subglottic sample and the vocal cords themselves for the glottic sample (illustrations 4.2-4.3). The

tissue was fixed in formalin and embedded in paraffin prior to the preparation of haemotoxylin and eosin slides. The slides were reviewed to exclude microscopic evidence of dysplasia. Cases with evidence of hyperkeratosis were not excluded. Details of the autopsy cases cause of death, history of exposure to alcohol, tobacco, steroid use, recent major surgery, transfusion or trauma were recorded along with age and sex details.

The prevalence of HPV6 and 11 in this series was compared with a non-random series of archival laryngeal squamous cell carcinoma cases from the departmental files of the University department of pathology at Leeds.

.111 Sample collection for investigation of HPV16 in laryngeal squamous cell carcinoma.

Cases

The histopathology files of the pathology department at Leicester Royal Infirmary were searched using key words larynx and squamous cell carcinoma from 1990 to 1992. Patients were numbered in the order of presentation. The initial presentation only was numbered when patients presented more than once in the study period. Cases for histological review were selected on the basis of a generated random number table. Cases of squamous cell carcinoma confirmed by histology review had representative blocks with areas of invasive squamous carcinoma sampled for further

study.

Controls

The initial autopsy series of tissue was increased using a similar strategy, i.e. larynx tissue without evidence of laryngeal disease was collected at the time of autopsy by pathologist in a non-random fashion over the years 1990-1993 from postmortems carried out in Leicester. These specimens were similarly processed on the day of harvesting.

2.DNA extraction

Two to four, 10 µm sections were cut from the designated paraffin blocks and placed in eppendorf tubes. The tissue was de-waxed by washing in xylene. Two washes each of 1 ml in volume. The tubes were centrifuged at 13000 revolutions per minute(rpm) for two minutes prior to removal of the xylene each time. Caution was exercised at this stage to prevent the tissue section which is poorly visible in xylene being removed with the xylene wash fluid.

The resulting specimen was further washed in 1 ml volumes of Industrial Methylated Spirit(IMS). Two washes in 99% concentration IMS followed by washing in 95% IMS, the sample being centrifuged as before prior to removal of the wash fluid in each case, and caution exercised to prevent the study tissue being inadvertently removed

Illustration 4.2

AUTOPSY LARYNX PRIOR TO SAMPLE HARVEST

Larynx divided vertically through posterior lamina of cricoid
A- Aryepiglottic fold
B- vocal cord
C- subglottis

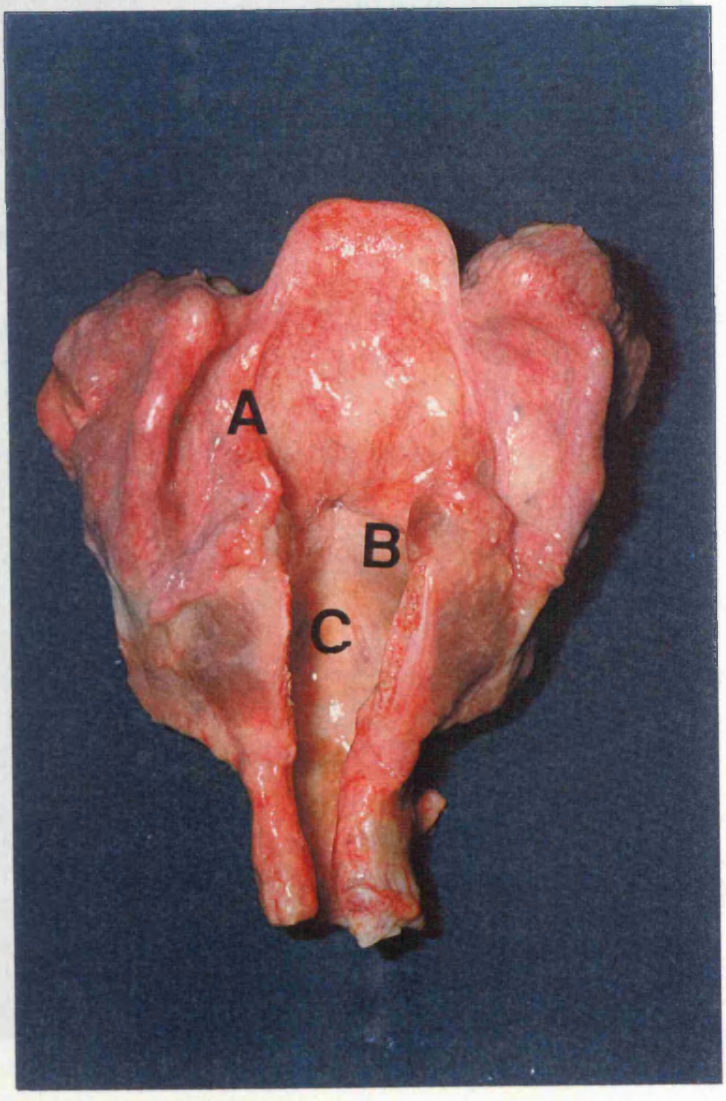
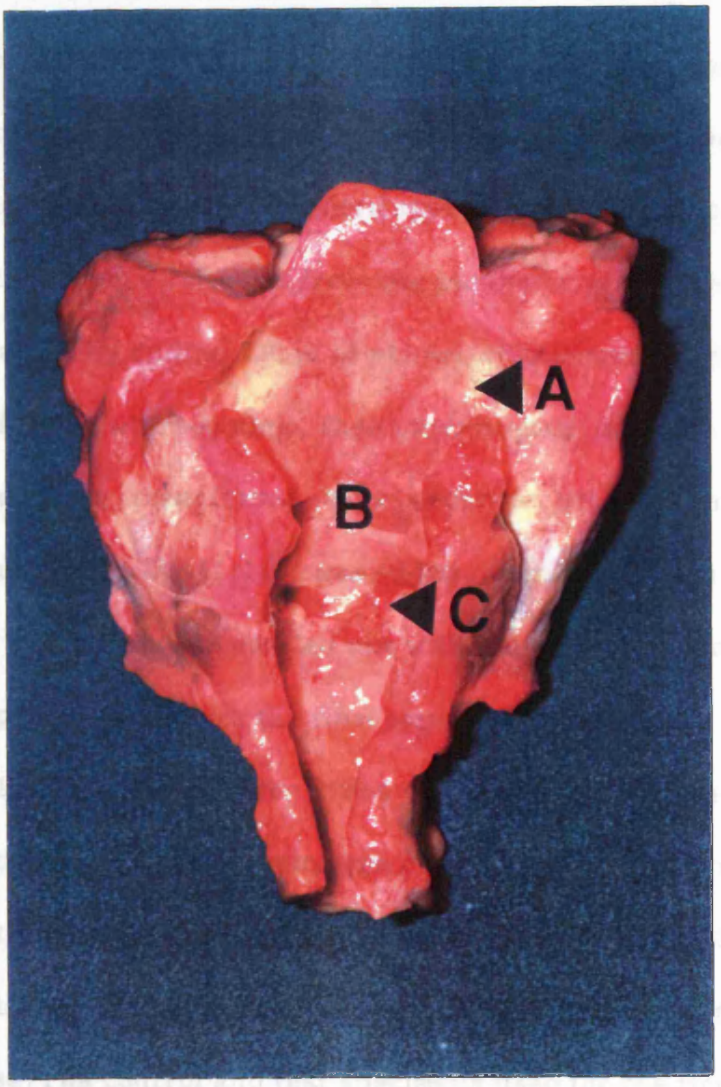


Illustration 4.3

AUTOPSY LARYNX AFTER SAMPLES HARVESTED

A- Aryepiglottic fold site harvested for supraglottic samples
B- Vocal cords excised for glottic samples
C- Site 1-2 centimeters below vocal cord at which subglottis was sampled



by the micropipette. All pipette ends and tubes were sterilised prior to use. Each extraction pipette end was used only once. The tubes were blot dried after the last 95% IMS was removed, and then vacuum dried for 1 hour.

A 1mg/1ml digestion buffer of Proteinase K(Boehringer Manheim GmBH, Manheim, Germany) in a solution of 5 mM tris-hydroxymethylaminomethane hydrochloride(TRIS-HCL) pH 8.0/1% sodium dodecyl sulphate was made up. 250 µl of the proteinase K digestion buffer was added to each specimen tube, which was whirl-mixed and momentarily centrifuged at 13000 rpm prior to incubation at 37^0C. The specimens were removed from the incubator after five to seven days and the DNA extracted by adding 250 µl of phenol/chloroform/Isoamyl alcohol(IAA) to each eppendorf tube, whirl-mixing, then centrifuging at 13000 rpm for 3 minutes. The supernatant was transferred to a separate tube by micropipette. 250 µl of phenol/chloroform/IAA(1:1:1) was added, and the process of extraction repeated. A further extraction was carried out with chloroform/Isoamyl alcohol(24:1).

DNA was precipitated from the final supernatant by adding one tenth of the volume of 1M sodium chloride, three times the volume of filtered absolute alcohol and refrigerating at -70^0C for a minimum of 1 hour. The tubes were centrifuged for 10 minutes at 13000 rpm at 4^0C, before removing the fluid by blot and vacuum drying. The resulting pellet was resuspended in 1 ml of ultrapure water and stored at 4^0C for further use. The control and test specimens used for the first series of experiments

to test for the presence of HPV6 and 11 had the precipitation step carried out at -20^0C overnight. The precipitate was collected by centrifuging at 15000 g for 10 minutes before removing the supernatant and drying. The DNA was resuspended in 50 µl of ultrapure water.

Strict laboratory protocols were always observed to prevent specimen contamination. These involved the use of separate rooms for DNA preparation from those used for the polymerase chain reaction. Dedicated pipettes were used to manipulate the reagents for amplification in the PCR room and no other procedures were routinely performed in the areas designated for DNA amplification.

Larynges could have been contaminated prior to arrival in the laboratory. In the case of tumour larynges harvested in the operating room environment contamination from the oral cavity and oropharynx at intubation is a possibility. In addition flora from the nasal cavity and nasopharynx could be introduced if a nasogastric tube is passed before the larynx is removed. In the autopsy specimens clean instruments and separate dissection tables are routinely used in all autopsies in the departments that the specimens were harvested from. The larynges were potted into individual formalin filled containers at the autopsy table. These containers were sealed and transported by the investigator to the laboratory for processing. The larynges were harvested over a period of 2 years from 3 different hospitals making consistent contamination unlikely.

3. Confirmation of DNA extraction

.1 PCR bcl

5 µl aliquots of 1:1 dilutions of the samples were amplified, using primers complementary to the minor coding region for the bcl gene on chromosome 18 (illustration 4.4). This generates a 118 base-pair product. The reaction mixture contained 0.4nM of each primer (volume 5µl),30 µl of reaction buffer(see illustration 4.5), sample DNA and ultrapure water made up to a total reaction volume of 50µl. A negative control in which ultrapure water replaced sample DNA and a positive control utilising laboratory control laryngeal DNA(from paraffin embedded material) known to amplify with the bcl primers was included in each run of samples tested(Control DNA details, illustration 4.6).

0.5 units of Thermophilus aquaticus(Taq) polymerase (Promega Taq, Madison WI,USA) was added to each tube containing the aliquoted reaction constituents. The tubes were microcentrifuged for 10 seconds and a layer of mineral oil placed over the contents before transfer to the thermocycler (Hybaid, Omnigene Ayelsbury, UK). An initial denaturation cycle at 94^0C for 2 minutes was followed by 39 cycles of 94^0C, 58^0C and 72^0C for 1,1 and 2 minutes respectively and a final extension cycle of 72^0C for 5 minutes. The reaction products were either kept at room temperature overnight or stored at 4^0C prior to electrophoresis.

In the event that 5 µl of 1:1 test sample DNA failed to amplify, or only a weak band

Illustration 4.4

BASE SEQUENCE OF COMPLEMENTARY PRIMER PAIRS USED TO CONFIRM DNA EXTRACTION

Primers (bcl)

bcl 2B(24mer)

AATTCCCTTCTGAAAGAAACGAAA

PCR product 118 basepairs

bcl 2D(23mer)

GCCCTGGAGGAATTTTTAATGTG

Primers (beta-globin)

beta-globin 1(20mer)

ACACAACTGTGTTCACTAGC

PCR product 110 basepairs

beta-globin 2(20mer)

CCACTTGCACCTACTTCAAC

Illustration 4.5

MISCELLANEOUS REAGENTS

Reaction Buffer

1 M TRIS pH 8.8

1 M (NH4)2SO4

1 M MgCl2

10mM DNTP mix containing 100 mM each dATP,dTTP,dGTP and dCTP

20mg/ml BSA

was obtained at the 118 bp position the reaction was repeated with 1 and 9 µl volumes of the sample at the same concentration followed by 1,5 and 9 µl volumes at 1:100 concentration.

In cases where no result was obtained at any of these dilutions the extraction process was repeated once. In the event that no positive results were obtained on repeat extraction the sample was removed from the study. The volume and concentration giving an easily identifiable band of the expected molecular weight was used for the HPV reactions. Ultra-pure water was used instead of sample DNA in one of the PCR reaction tubes, as a negative reaction control. Non laryngeal tumour DNA which was established to undergo amplification was similarly used as a positive reaction control(details illustration, 4.6).

.11 PCR beta-globin

Samples prepared similarly to that for the bcl reaction were analysed with primers complementary to sequences of the human beta-globin gene shown in illustration 4.4. 1.0 unit of TAQ was used in each reaction tube. The reaction was carried out in a programmable cycler(MJ Research, Waterdown, MA USA) with an initial denaturation cycle of 94^0C for 5 minutes. Annealing and amplification with extension was carried out by 39 further cycles of 94^0C, 60^0C and 72^0C for 1,1 and 1 minutes respectively and a final extension cycle of 72^0C for 5 minutes. An ultra-pure water negative

control was included in each PCR batch of samples analysed.

4. Evidence of HPV isolation

.1 PCR HPV L1 general primer

A general primer mediated PCR was carried out utilising consensus primers complementary to homologous sequences within the L1 open reading frame of HPV (illustration, 4.7), (Manos M et al, 1989). The primers ability to detect 20 ng of recombinant HPV16 and HPV6b in a 50µl reaction volume was confirmed(illustration, 4.8). The weakness of the bands obtained with this large quantity of DNA suggest that the primers were not highly sensitive. The priming conditions were changed several times to obtain the conditions which resulted in the best quality bands on electrophoresis.

A recombinant positive HPV16, 11 or 6 control and a negative control was included in each reaction set. The reaction mixture contained 0.05 nM(0.6 µl) of each primer, 30 µl of reaction buffer, test DNA and ultrapure water to a reaction volume of 50µl. The samples were otherwise prepared as before. The thermocycler was programmed to carry out 40cycles of 96^0C for 80 seconds, 58^0C for 90 seconds and 70^0C for 2 minutes.

Gel preparation, electrophoresis and product visualisation was as before. Cases illustrating banding at the expected molecular weights 448bp for HPV6 and 11, 451bp

for HPV16 and 454bp HPV18(Innis MA, 1991) were classified as positive.

This strategy failed to obtain any positive results despite clear responses with the control plasmid DNA. The sequence of investigation was thus modified with two sets of HPV16 specific primers being used instead of 1 general primer and 1 specific primer. An E6 specific primer pair was used (illustration 4.9, HPV16 A & B) in addition to a L1 specific primer pair CD(illustration 4.10) as described below.

.11 PCR HPV16 with L1 specific primers

The optimal temperatures for primers C and D were arrived at by pilot studies comparing the PCR product bands obtained under different conditions see illustration 5.7. The clearest reaction product positive and negative were obtained with the thermocycler set at 96^0C, 58^0C and 72^0C for 1.3,1.5 and 2 minutes respectively for 40 cycles.

.111 PCR HPV6,11 & 16 with E6 specific primers

In a series of 12 control larynges the rate of isolation of HPV6 and HPV11 was determined by PCR using primers complementary to the E6 open reading frame (see illustration 4.9). Recombinant DNA was used as a positive control along with water as a negative control. A 50 µl reaction volume was used.

HPV16 E6 primers were likewise used to compare the presence of HPV16 in a series

of control and laryngeal SCC cases. The reactions were carried out on a thermocycler programmed for an initial denaturation cycle of 94^0C for 5 minutes, 55^0C for 1.5 minutes, 72^0C for 2 minutes followed by 39 cycles of 94^0C, 55^0C and 72^0C for 0.5,1.5 and 2 minutes respectively and a final extension cycle of 72^0C for 5 minutes in the case of HPV6 and 11.

The programme for HPV16 primers A and B was determined by pilot studies first with two sets of primer pairs at varying conditions. Illustration 4.11 shows the product from plasmid positive and water negative reactions with 2 sets of HPV16 E6 specific primer pairs AB and EF. The latter primer pair plasmid positive lanes 1 and 8, were not used because the product generated was not of the predicted size 316bp.

The clearest reaction product positive and negative with primer pair AB, were obtained with the thermocycler set for initial denaturation at 90^0C for 5 minutes followed by 39 cycles of 90^0C, 54^0C, and 72^0C for 0.5, 1.5 and 2.0 minutes with final extension at 72^0C for 5 minutes prior to cooling(plasmid positive and negative lanes 2 and 3 respectively, illustration 4.11). Initial melt at 94^0C for 5 minutes followed by 39 cycles of 94^0C, 50^0 and 72^0C for 0.5, 1.5 and 2.0 minutes respectively plasmid positive lane 9 negative lane 10(illustration 4.11) show some banding with the negative control at the higher denaturation temperature resulting in a loss of specificity. The choice of the lower denaturation temperature may have reduced the ability to obtain positive results with the paraffin embedded specimens used in these

experiments.

5.Electrophoresis

45µl of the PCR reaction product heated to 65^0C for 10 minutes immediately prior to electrophoresis, was transferred to a 500µl eppendorf tube to which 5µl of 10X loading buffer had been added. The tube was flick mixed and centrifuged briefly at 13000 rpm. 40 ul of this solution was added to the well of an agarose gel. A mixed 3% Nusieve and 1% Seakem agarose gel was prepared by micro-waving at high temperature the appropriate weights of agarose in a conical flask containing Tris-acetate-ethylenediamminetetraacetic acid(TAE). Approximately 250mls of the solution was added to each gel plate/comb assembly and left to solidify at 4^0C. Electrophoresis was carried out in a tank containing 1xTAE at 100volts for 45 minutes.

1 well in each gel was filled with 2 µl of a molecular weight marker PHIX174(Promega, Madison WI, USA) or an alternative added to 16 µl of Tris-ethylenediamminetetracetic acid and 2µl of 10X loading buffer to determine the molecular weights of the reaction products. The gel was stained in 250 µl of 1XTAE containing 70 µl of 1% ethidium bromide for 1 hour followed by two washes in ultra-pure water.The gel bands were visualised under UV light and a photographic record made with polaroid 667 black and white film.

Illustration 4.6

BCL PRIMERS B AND D CONFIRM DNA EXTRACTION WITH CONTROL SPECIMEN

118 bp bands obtained with 1 in 1000 and 1 in 500 dilution of extracted DNA lanes 2 and 3. Negative 1 in 10 dilution (PCR amplification inhibited by high concentration of starting DNA, lane 1) and ultrapure water lane4. DNA marker PHIX174(Digest HaeIII) lane 5.

Details-positive control paraffin-embedded laryngeal tissue from male 66 years old who died with aortic valve disease.

Rows: A=expected PCR product 118bp, B=primer dimers.

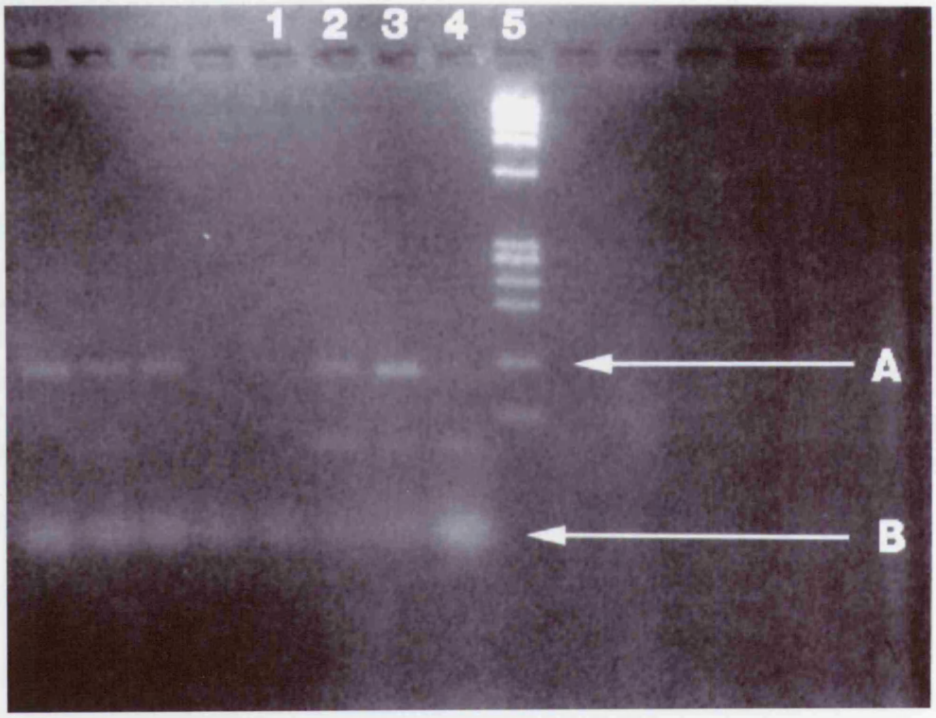


Illustration Table 4.7

BASEPAIR SEQUENCE OF HPV L1 COMPLEMENTARY PRIMERS

MY11(20mer)

GCMCAGGGWCATAAYAATGG

MY09(20mer)

CGTCCMARRGGAWACTGATC

where M=A+C

R=A+G

W=A+T

Y=C+T

Predicted Size of L1 PCR product

HPV06 448bp

HPV11 448bp

HPV16 451bp

HPV18 454bp

Illustration 4.8

AGAROSE GEL SHOWING HPV16 AND 6B PLASMID PCR PRODUCTS 451 AND 448 BASEPAIRS RESPECTIVELY WITH L1 COMPLEMENTARY PRIMERS

lanes1-5 negative specimens with primer dimers only

lane 6 recombinant HPV16 451 basepairs

lane 7 recombinant HPV6b 448 basepairs

lane 8 ultrapure water

lane 9 DNA marker PHIX174(Digest HaeIII)

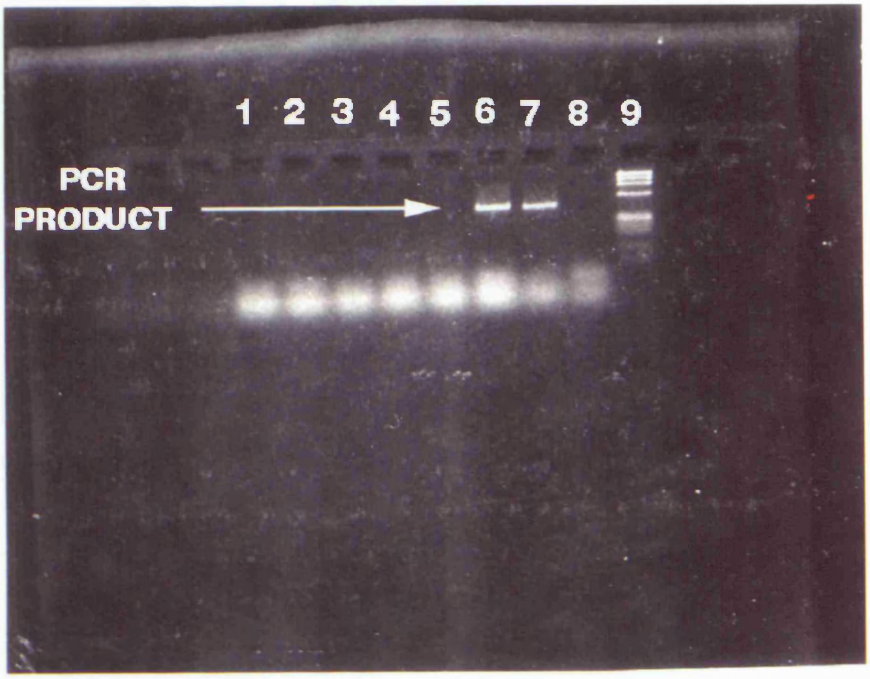


Illustration 4.9

HPV TYPE SPECIFIC PRIMER PAIRS COMPLEMENTARY TO THE E6 REGION

HPV6 A(21mer) HPV6 B(20mer)

GCTAATTCGGTTGCTACCTGT CTGGACAACATGCATGGAAG

PCR product size 237bp

HPV11 A(20mer) HPV11 B(20mer)

CGCAGAGATATATGCATATG AGTTCTAAGCAACAGGCAACA

PCR product size 90bp

HPV16 A(20mer) HPV16 B(20mer)

TCAAAAGCCACTGTGTCCTG CGTGTTCTTGATGATCTGCA

PCR product size 110bp

Illustration 4.10

HPV16 TYPE SPECIFIC PRIMER PAIR COMPLEMENTARY TO THE L1 REGION

HPV16 C(20mer;EMBL6582)	HPV16 D(22mer;EMBL6675)
GCWCAGGGWCATAAYAATGG	TAGTTTCTGAAGTAGATATGGC

where W=A+T

Y=C+T

PCR product size 93bp

Illustration 4.11 HPV16 with E6 specific primer pairs AB and EF

lane 1 plasmid positive HPV16 with primer pair EF conditions 1

lane 2 plasmid positive HPV16 with primer pair AB conditions 1

lane 3 negative with primer pair AB conditions 1

lane 4 negative with primer pair EF conditions 1

lane 5 blank

lane 6 PhiX with Hae digest basepair ladder

lane 7 blank

lane 8 plasmid positive HPV16 with primer pair EF conditions 2

lane 9 plasmid positive HPV16 with primer pair AB conditions 2

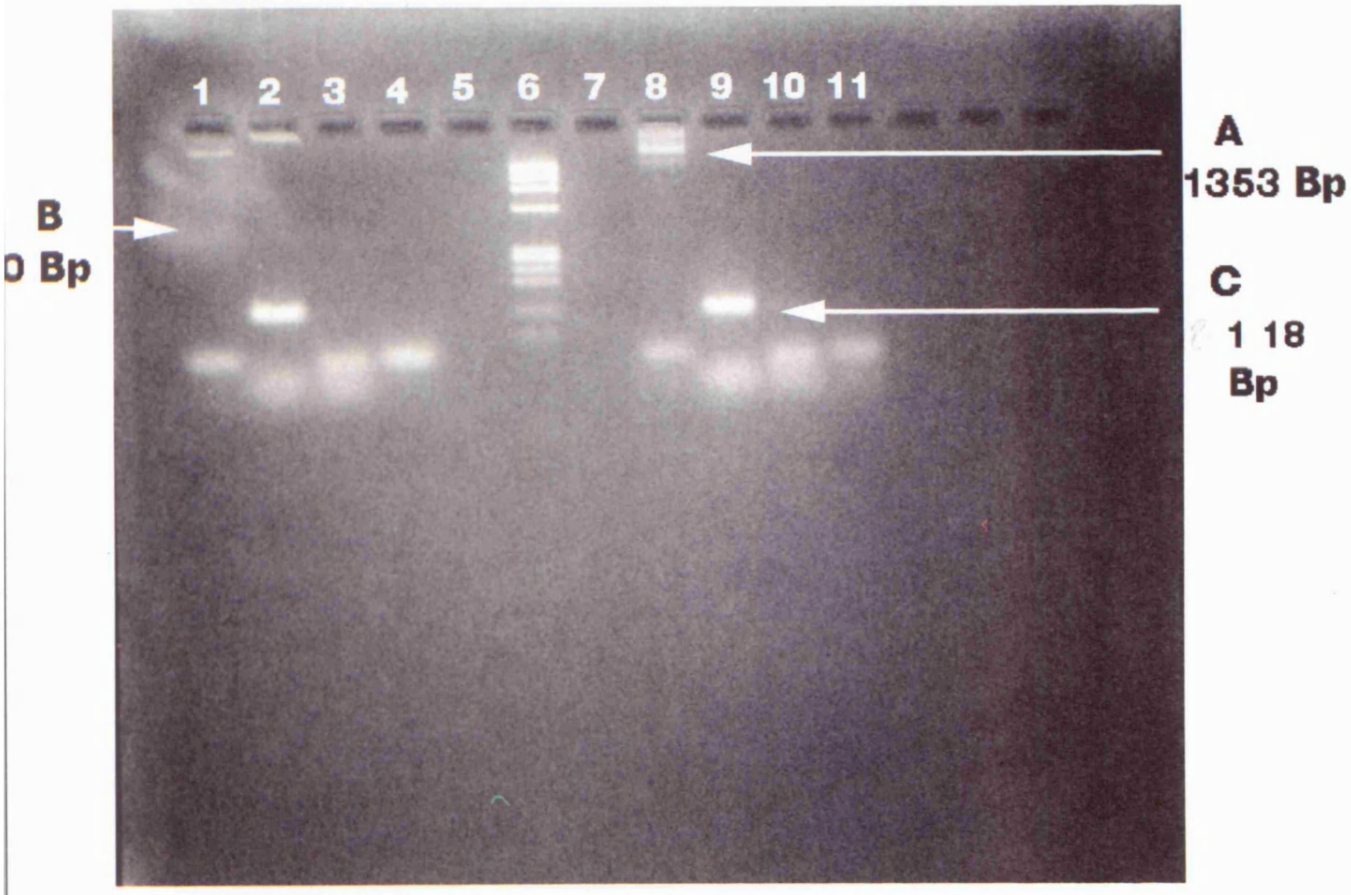
lane 10 negative with primer pair AB conditions 2

lane 11 negative with primer pair EF conditons 2

Conditions 1 - initial denaturation at 90^0C for 5 minutes followed by 39 cycles of 90^0C, 54^0C, and 72^0C for 0.5, 1.5 and 2.0 minutes with final extension at 72^0C for 5 minutes prior to cooling.

Conditions 2 - Initial melt at 94^0C for 5 minutes followed by 39 cycles of 94^0C, 50^0 and 72^0C for 0.5, 1.5 and 2.0 minutes.

A- observed PCR product with EF primer pair 1353bp(marker), B- expected position of PCR product with EF primer pair 316bp(marker310bp). C observed and expected position of PCR product with AB primer pair coincide at 110bp(marker118).


1 2 3 4 5 6 7 8 9 10 11
A
1353 Bp
B
C
1 18
Bp

CHAPTER 5

RESULTS

1.DNA extraction

2.Comparison of HPV6 and 11 isolation in tumour and nontumour larynges

3.Comparison of HPV16 isolation in tumour and nontumour larynges

1.DNA extraction

A PCR reaction either with complementary beta-globin or bcl primers was carried out. DNA from 12 autopsy larynges was confirmed by beta-globin PCR to be satisfactory for further study. Formalin fixed paraffin embedded DNA from 7 archival laryngeal SCC cases was likewise satisfactorily extracted(tables 5.1, 5.2). In the case of the HPV16 comparison all the autopsy larynges yielded satisfactory DNA after extraction with proteinase K either on the first or second attempt. A total of 3 tumour cases were excluded because of inadequate DNA extraction(table 5.11). This despite repeating the extraction process. It maybe that leaving the precipitation step for longer would have increased the yield, but the initial study protocol required that these cases be withdrawn.

41% of control larynges were positive for HPV6 and or HPV11 on two or more occasions(table 5.1). A case was not classified as positive unless the same result was obtainable on repeat testing. There was no statistical difference in the isolation rate of HPV6 and 11 in a series of 7 laryngeal cancer cases, 71% when compared with the control series(table 5.2). The sample size used has only a 15% chance of showing a difference of the order of 30% between these two groups. 40 specimens would have to be studied in each group to achieve a power of 80% and robustly exclude the chance of a type 2 error.

The male:female ratio was 1.4:1 in the controls and 2.5:1 in the tumour cases. The sex ratios are not within the range expected for a typical laryngeal carcinoma series. The mean ages of the samples were similar.The gel shown in illustration 6.2 shows the positive HPV11 results for different sites from control specimen 1 and the positive results for tumour case 3.

It is interesting to note that of the 5 controls without a history suggestive of immunosuppresion only 1 case was positive for HPV while the other 3 HPV positive cases occurred in transplant recipients or patients with malignancy. A history in keeping with immunosuppresion was not a statistically significant determinant of HPV infection.

There however was a significant difference in the isolation of HPV6 and HPV11 in the nontumour larynx in this small series when results are analysed with respect to

nontumour larynx in this small series when results are analysed with respect to smoking (Fishers exact p=0.003), illustration 5.4. This suggest that tobacco use predisposes to laryngeal expression of HPV. It may also be that the patients who smoked had microscopic laryngeal pathology.

Histology slides were available for 6 patients. This was rendered possible because of a parallel study being undertaken on some of the harvested tissue. Slides were available for nontumour larynges 1-6 shown in table 5.1. These were reviewed blind to the results of the PCR and case 3 showed some areas of dysplasia. The others were all normal. No histology slides were available for autopsy larynges 7-12.

In essence therefore all active smokers in this study had evidence of HPV6/11 infection. In 1 case the histology is unknown, in another there was evidence of dysplasia and in three cases the larynx was microscopically normal. All nonsmokers failed to show evidence of laryngeal infection with HPV6/11, in 2 laryngeal histology was normal and in 4 unknown. The further patient with uncertain smoking history also failed to illustrate laryngeal infection with HPV.

In view of these findings it was especially pertinent that the experiments on HPV16 documented the histology in all the controls. HPV positivity may be a surrogate marker for laryngeal epithelial dysplasia. It was therefore decided that all nontumour cases for the second arm of the investigation, that is the investigation of HPV16 prevalence had to be normal on microscopic histological evaluation of the sites elected

Illustration 5.1

EXPERIMENT 1 HPV6/11 IN THE NONTUMOUR LARYNX

Case no.	sex	age	Associated Disease	Result
1	m	43	ischaemic heart disease	11+
2	f	68	renal failure/transplant	6+
3	m	27	renal transplant	6&11+
4	m	54	craniotomy cerebral tumour	11+
5	m	80	diverticular disease	-
6	m	82	GI haemorrhage	-
7	f	81	pancreatic carcinoma	-
8	f	68	intestinal obstruction	-
9	m	66	aortic valve disease	-
10	m	74	myeloproliferative disorder	-
11	m	61	diabetic nephropathy	-
12	f	71	bronchus carcinoma	11+

Illustration 5.2

EXPERIMENT 1 HPV6/11 IN LARYNGEAL CARCINOMA CASES

Case	sex	age	Site & Stage	Result
1	m	66	glottic T1	-
2	m	61	uncertain	6+
3	m	55	glottic T1	11+
4	f	46	supraglottic T4	-
5	f	72	uncertain	6+
6	m	59	uncertain	6+
7	m	69	supraglottic T4	6+

Illustration 5.3

RELATIONSHIP OF HISTORY OF SMOKING TO HPV6/11 RESULTS IN THE CONTROL LARYNX

	Active Smoker	
	positive	negative
HPV6/11 positive	5	0
HPV6/11 negative	0	7

for study. Cases excluded because of gross abnormality, were not recruited by the pathologist performing the autopsy. Thus no details were made available to the investigator.

3.HPV 16 in tumour and nontumour larynges

The change in sample harvesting strategy outlined above resulted in the exclusion of seven cases from the first experiment because of abnormal or unavailable histology. The five cases with normal histology cases 2-6(illustration 5.5), were submitted to this arm of the investigation.

14 further autopsy larynges were harvested in Leicester but 1 was excluded because of dysplastic change(case 9, illustration 5.5). The only other histological abnormality found in the series was evidence of hyperkeratosis in one case. This case was not excluded from the investigation.

Initial runs of Recombinant HPV16 DNA with HPV16 L1 complementary primer pair CD to determine the optimal annealing temperature revealed that a temperature of 58^0C was best(illustration, 5.7). The other optimal temperature requirements are as described in chapter 4 for this primer pair.

Initially 30 tumour cases were accessed from archival pathology files anticipating a HPV16 positive yield of 30%. Three cases could not be accessed because the paraffin blocks were missing. Review of the histology from the blocks accessed failed to

reveal evidence of squamous cell carcinoma on 1 block which was thus excluded.

A further 14 archival laryngeal tumour cases were accessed, 2 of which did not amplify on bcl PCR. The site and tumour stage of the 40 SCC cases of the larynx including the 3 with inadequate DNA extraction are shown in table 5.10. These details were obtained blind to the results of the PCR.

The initial strategy required the amplification of the tumour and control DNA with HPV16 L1 directed general primers(Manos et al,89). The absence of any positive results in the 79 DNA samples assessed warranted a change of strategy in view of the results anticipated from the literature.

An additional HPV16 E6 complementary primer pair AB was obtained (gift from F.Lewis, Leeds University)(table 4.3) and used with a modified strategy for assessing HPV16 positivity (illustration 5.4). HPV16 E6 primers A and B amplified DNA in 5 of the 18 controls and 9 of the 37 amplifiable tumour cases(illustration, 5.9). The presence of HPV16 was however not confirmed by amplification with L1 specific complementary primers C and D.

The male to female ratio was 2:1 in the controls and 5.6:1 in the tumour cases. The mean ages were 67years(s.d., 9 years) in the tumour cases and 66(s.d.,18) years in the controls. The tumour cases excluded did not vary from those studied male:female ratio 6:1, mean age 68years(s.d., 9 years).

The tumour cases were evenly matched between early and advanced stage disease.

T1/T2 cases accounting for 43% and T3/T4 disease for 38% of the 37 cases studied(table 5.12). Twenty-two cases were intralaryngeal in origin, 8 arose from the pharyngolarynx. The case notes either could not be traced or were lacking in sufficient detail to judge site of origin in 10 cases.

Illustration Chart 5.4

SEQUENCE OF INVESTIGATIONS TO CONFIRM HPV16 POSITIVE

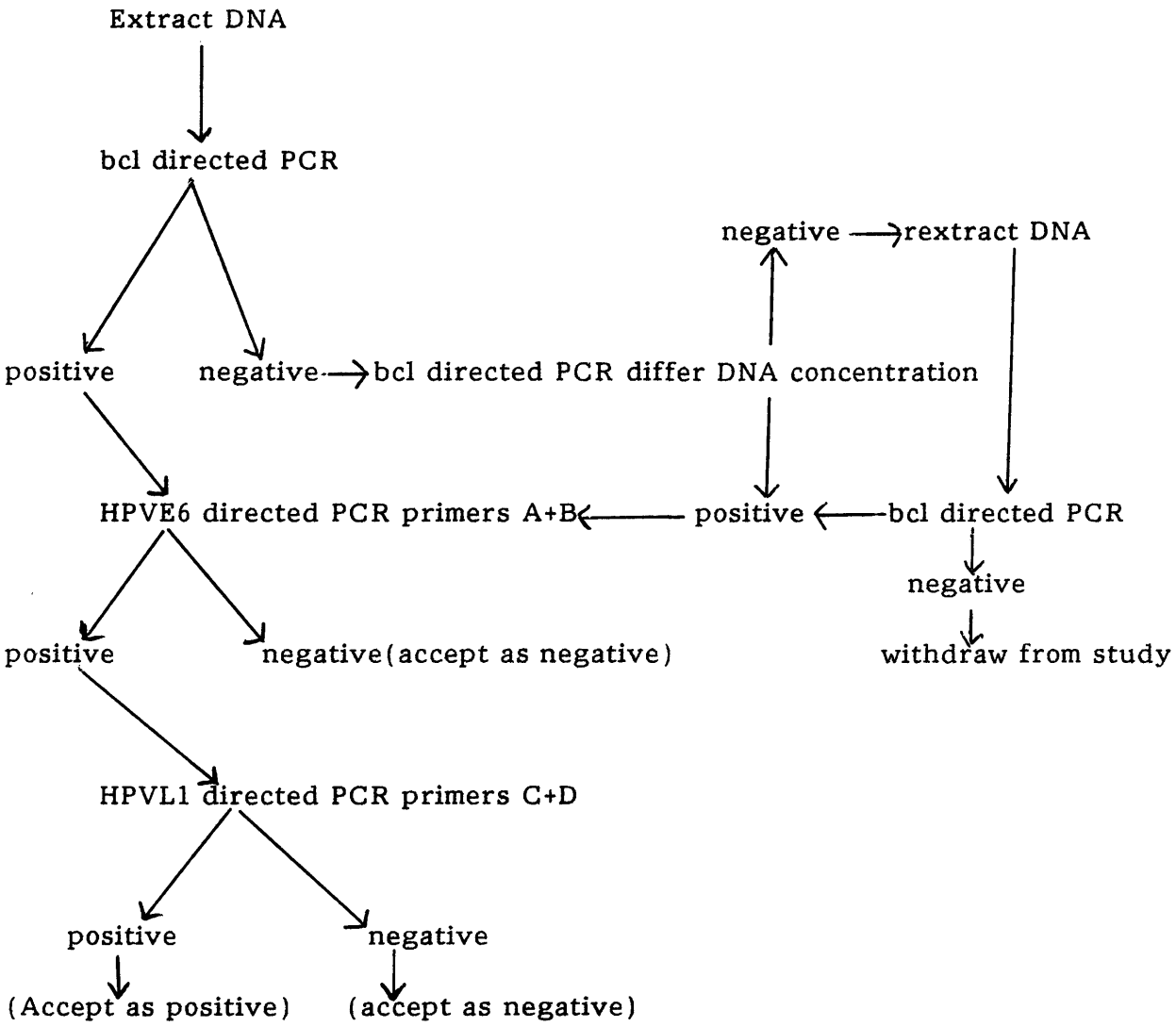


Illustration 5.5

HPV16 IN THE NONTUMOUR LARYNX

Number	HPV16 on AB	sex	age	Cause of death & associated disease
1		f	81	Exclusion dysplastic larynx
2	+	m	77	heart failure, pulmonary fibrosis
3		m	81	aortic aneurysm
4	+	m	53	heart failure, gastric carcinoma
5		f	75	breast cancer
6	+	f	86	myocardial infarct
7	+	m	44	gastric cancer,hepatic failure/mets.
8	+	m	41	Disseminated Hodgkins disease/Chemo.
9		m	80	Exclusion dysplastic larynx
10		m	87	gastric cancer, emphysema
11		f	86	heart failure, heart valve disease
12		f	68	GI haemorrhage, liver cirrhosis alch.
13		m	68	pulmonary embolus, MI, diabetic
14		m	67	obstructive airway disease
15		m	43	ischaemic heart disease
16		f	68	renal failure/transplant
17		m	27	renal transplant
18		m	54	craniotomy cerebral tumour
19		m	82	GI haemorrhage
20		f	81	pancreatic carcinoma

Illustration 5.6

HPV16 IN THE NONTUMOUR LARYNX RELATIONSHIP TO SMOKING

Number	HPV16 on AB	sex	age	Smoker
1		f	81	Exclusion dysplastic larynx
2	+	m	77	no
3		m	81	yes
4	+	m	53	unknown
5		f	75	no
6	+	f	86	no
7	+	m	44	ex
8	+	m	41	unknown
9		m	80	Exclusion dysplastic larynx
10		m	87	ex
11		f	86	ex
12		f	68	unknown
13		m	68	unknown
14		m	67	yes
15		m	43	yes
16		f	68	yes
17		m	27	yes
18		m	54	yes
19		m	82	ex
20		f	81	no

Illustration 5.7

PCR PRODUCT ILLUSTRATING 58^0C AS THE OPTIMAL ANNEALING TEMPERATURE FOR HPV16 L1 COMPLEMENTARY PRIMERS CD

PCR PRODUCT 93bp

lanes 1,4,6,9-12 blank

lane 2 HPV16 DNA anneal temperature 58^0C

lane 3 Ultrapure water anneal temperature 58^0C

lane 5 (PHIX174 HaeIII digest) molecular weight ladder

lane 7 HPV16 DNA anneal temperature 64^0C

lane 8 Ultrapure water anneal temperature 64^0C

Note: Bright band A obtained with positive HPV16 when utilising an annealing temperature of 58^0C, compared to the faint band B when the annealing temperature is raised to 64^0C.

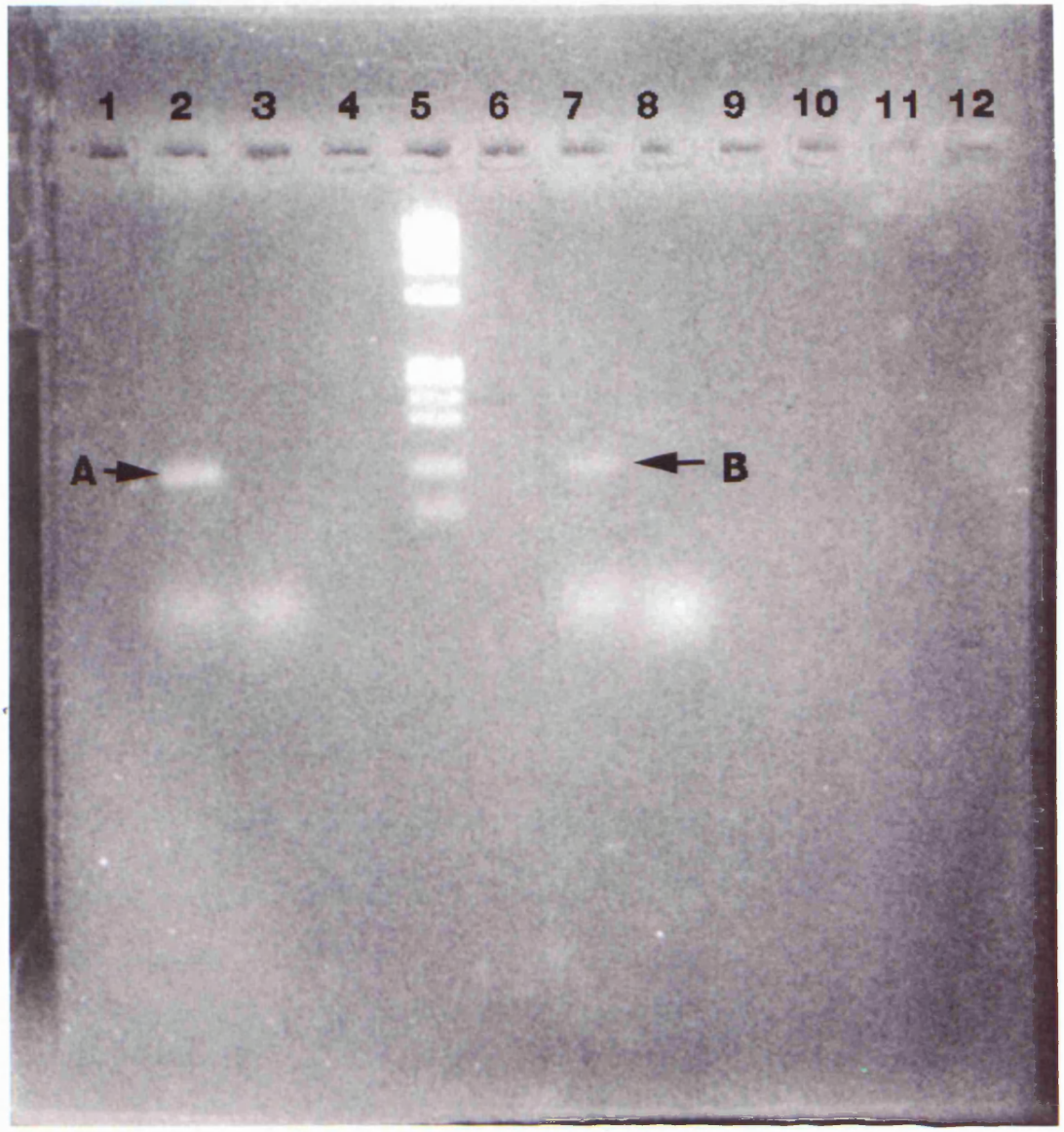

1 2 3 4 5 6 7 8 9 10 11 12
A
B

Illustration 5.8

A NUMBER OF CASES AFTER AMPLIFICATION WITH bcl PRIMERS

PCR PRODUCT 118bp

lanes 1-3 nontumour larynx 13 3 sites 1:100 dilution

lanes 4-6 nontumour larynx 13 3 sites 1:1 dilution

lanes 7-9 nontumour larynx 12 3 sites 1:100 dilution

lanes 10-12 nontumour larynx 12 3 sites 1:1 dilution

lane 13 1:100 dilution positive control

lane 14 1:1 dilution positive control

lane 15 negative control ultrapure water

lane 16 tumour larynx 1:100 dilution

lane 17 (PHIX174 HaeIII digest) basepair ladder

A- position of PCR product 118 bp

B- position of primer dimers

PCR positive control(nontumour larynx 6 established as positive with bcl primers in previous PCR experiment)

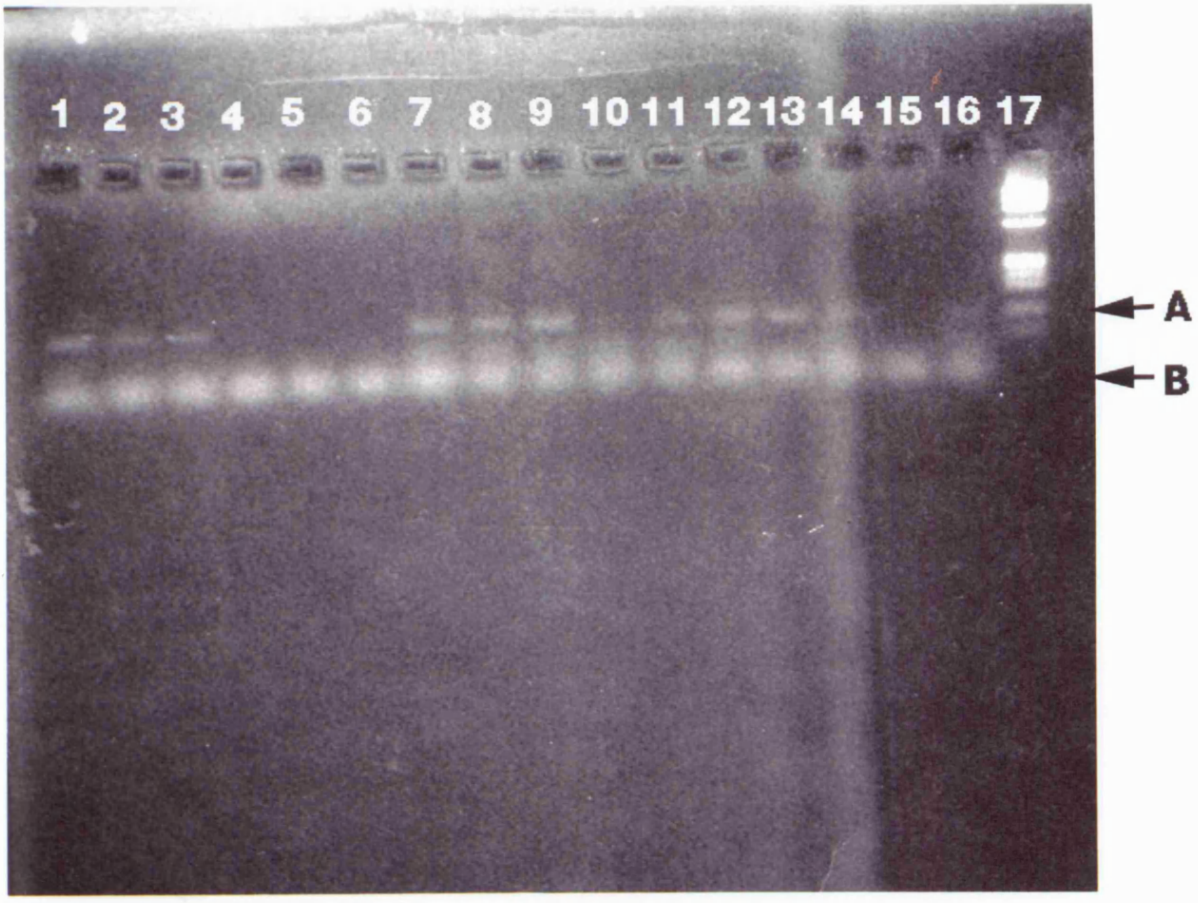

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
A
B

Illustration 5.9

CASES SHOWING POSITIVE AMPLIFICATION WITH HPV16 PRIMERS AB

PCR PRODUCT 110bp
lanes 1-3 negative 3 sites from nontumour case test
lanes 4-10 positive from sites in nontumour cases 2 and 4
lane 11 control 11
lane 12 control negative
lane 13 blank
lane 14 (PHIX174 HaeIII digest) basepair ladder

See discussion chapter 6 for extra bands.

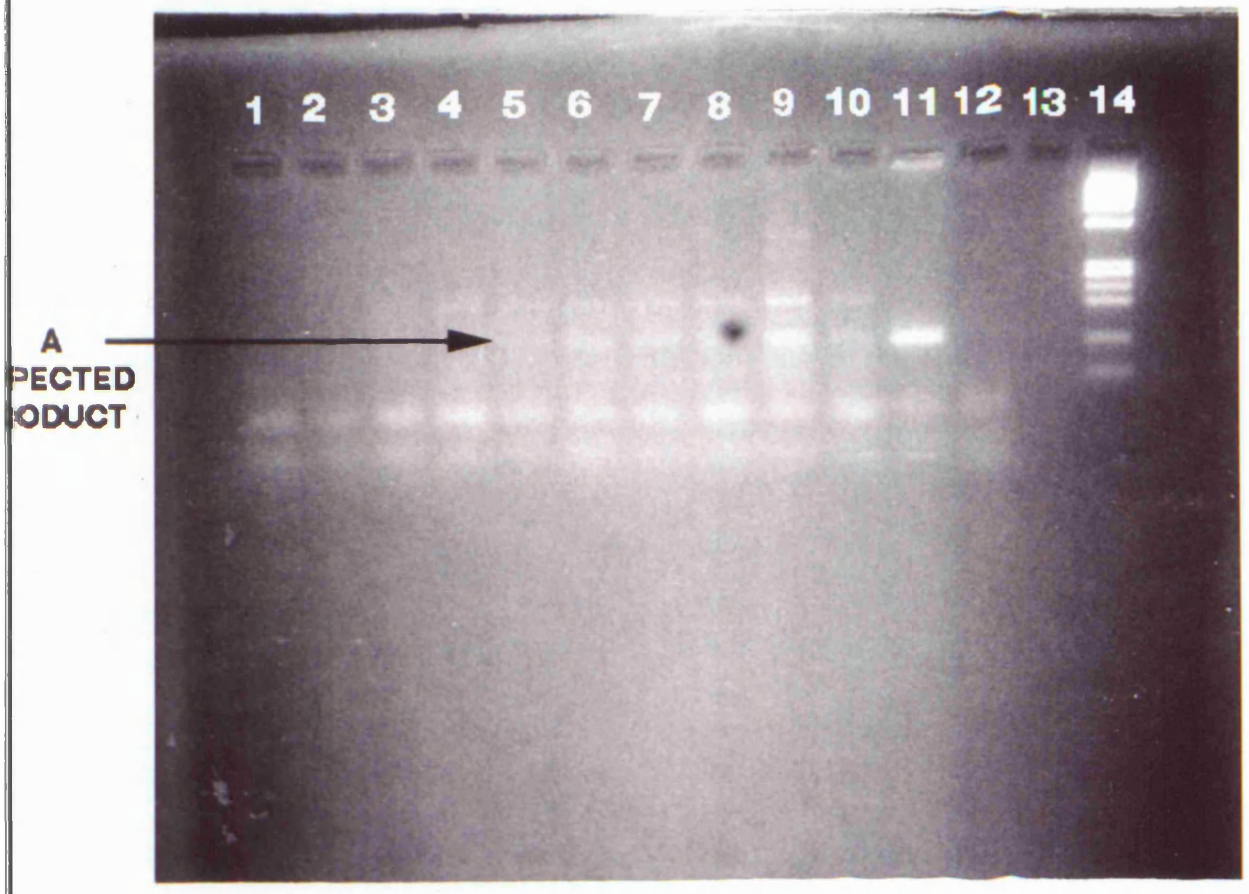


Illustration 5.10

HPV 16 IN LARYNGEAL TUMOUR CASES

Case	site	sex	age	stage
1	glottic	m	73	BCL - on 2 extractions
2	glottic	m	72	T2 N0
3	glottic	m	65	T2 N0
4	glottic	f	63	T2 N1 + A/B E6
5	unspecified	m	68	Tx Nx
41	glottic	m	72	T1 N0
42	transglottic	f	72	T4 N0 + A/B E6
43	supraglottic	m	73	T2 N0
9	laryngopharynx	m	47	T4 N0 + A/B E6
10	glottic	m	45	T2 N0
11	supraglottic	m	67	T2 N1
12	glottic	m	51	T3 N0 + A/B E6
13	unspecified	m	76	Tx Nx
14	unspecified	m	75	Tx Nx
15	unspecified	m	60	Tx Nx + A/B E6
16	unspecified	m	70	Tx Nx
17	supraglottic	f	76	T2 N0
18	laryngopharynx	m	65	T4 Nx
19	supraglottic	m	71	T2 N0 + A/B E6
20	transglottic	m	60	T3 N0 + A/B E6
21	laryngopharynx	m	67	T4 N0
22	glottic	m	52	T3 N0
44	glottic	m	65	T1 N0
24	unspecified	m	82	Tx Nx
25	glottic	f	74	T1 N0
26	laryngopharynx	m	60	T3 N0
27	glottic	m	77	T1 N0
28	supraglottic	m	56	T2 N0 + A/B E6
29	transglottic	m	56	T4 N0 BCL -
30	laryngopharynx	m	66	T4 N1 + A/B E6

Illustration 5.10 Continued

HPV 16 IN LARYNGEAL TUMOUR CASES

Case	site	sex	age	stage	
31	supraglottic	m	71	T2 N1	
32	supraglottic	m	79	T4 N0	
33	glottic	m	64	T2 N0	
34	laryngopharynx	m	60	T2 N0	
35	laryngopharynx	f	77	T4 N3	
36	laryngopharynx	m	69	T4 N0	
37	supraglottic	m	86	T3 N0	
38	glottic	f	74	T1 N0	
39	supraglottic	m	57	T4 N2	BCL -
40	unspecified	m	60	Tx Nx	

Illustration 5.11

TUMOUR CASE EXCLUSIONS

Case	sex	age	Reason for exclusion
6	m	77	block misfiled
7	f	64	archival block missing
8	m	76	archival block missing
23	m	76	no evidence of SCC on block
1	m	73	bcl - on 2 extractions
29	m	56	bcl -
39	m	57	bcl -

Table 5.12

bcl POSITIVE TUMOUR CASES EXPERIMENT 2 SITE AND T STAGE

	T1	T2	T3	T4	Tx	n
site						
supraglottic	0	6	1	1	0	8
glottic	4	5	2	0	0	11
subglottic	0	0	0	0	0	0
transglottic	0	0	1	2	0	3
laryngopharynx	0	1	1	6	0	8
unspecified	0	0	0	0	7	7
n	4	12	5	9	7	37

CHAPTER 6

RATIONALE OF EXPERIMENTAL PROTOCOL

AND CONCLUSIONS

1. Archival tissue versus fresh

2. Autopsy controls

3. HPV detection

.1 Available techniques

.11 PCR

.111 L1 primers

.1V False positive

4. Confirmation of DNA extraction

5. Positive Control strategies

6. Conclusions

.1 HPV6/11 findings

.11 HPV16 findings

.111 Future direction

1.Archival tissue versus fresh

The incidence of laryngeal cancer in the UK population and the use of a random number of cases at presentation would have necessitated over 3 years of sample collection in Leicestershire to approach the sample size of the second arm of this study. Formalin fixed archival material offered a ready source of study material and reduced the sample harvesting time considerably, thus allowing the study to be completed.

This strategy may have reduced the number of positive cases of HPV16 isolated since DNA extraction is more efficient on fresh as opposed to formalin fixed tissue(Guerin-Reverchon I et al, 1990; Jackson DP et al, 1991). Three cases were discarded from the archival series because of inability to detect bcl. The cases excluded from study did not vary in age and sex from those studied.

2.Autopsy controls

Reports of an association between HPV and laryngeal carcinogenesis have invariably been marred by a lack of study of control tissue . This is partly due to ethical dilemmas. The best control tissue would be nondiseased individuals from the same population but the likelihood of obtaining ethical approval or obtaining volunteers to submit to laryngeal biopsy with its attendant morbidity is unlikely. A proxy for

control tissue has been adjacent tissue to the tumour site(Morgan D et al, 1991). This has obvious limitations because the normal appearing larynx in patients in remission from laryngeal papillomatosis when subjected to molecular biological assessment has been shown to contain HPV(Rihkanen H et al, 1994). This is quite likely to be the pattern of laryngeal involvement in carcinoma of the larynx. Hence the finding of similar isolation rates in control and tumour tissue in studies using such a strategy.(Morgan D et al, 1991).

The use of laryngeal biopsy tissue from non-tumour cases is another strategy adopted by researchers. This is more representative of reality but since occult HPV infection has been postulated (Scurry J & Wells M, 1992) and is illustrated in the larynx(Rihkanen H et al, 1994) it is possible that patients with laryngeal symptoms who prove to have benign histological disease will have a higher yield of HPV than the general population.

An autopsy series of larynges was chosen to overcome these criticisms but also raises problems. Not all individuals in the community come to autopsy and this is therefore a biased group. At first glance the bias should have no effect on the findings of this study but closer inspection reveals potential contributing factors. Host immuno-compromise favours laryngeal involvement by HPV(Bradford C et al, 1990). In the control tissues studied 30% of cases were potentialy immunocompromised because of tumour, organ transplantation or steroid use. This certainly had a

bearing on the expression of HPV6/11 as determined in the initial series of 12 control larynges(Nunez DA et al, 1994a). It however had no discernible effect on the expression of HPV 16 positivity and the latter finding is probably reflective of the true control population.

3. HPV detection

.1 Available techniques

Histological appearance was the first method of detecting HPV infection. This depended on evidence of hyperplasia of the basal cell layer(acanthosis), thickening of the superficial layers or hyperkeratosis and the presence of koilocytes. The latter are cells in which the cytoplasm has become vacuolated(Jenson AB et al, 1987; Koutsky LA et al, 1988) and the nucleus hyperchromatic and crenated. The Koilocyte is considered a marker of HPV infection(Arends MJ et al, 1990). Viral detection by cell culture is not routinely used for HPV(Trofatter KF, 1997), and electron microscopic methods have been inconsistent (Derkay CS & Darrow DH, 1994). Immunohistochemical methods aimed at identifying core and late antigens with peroxidase-antiperoxidase staining and antibodies respectively were developed(Tsuchiya H et al, 1991). These are not as sensitive as techniques based on the detection of viral DNA. Southern blotting, in situ hybridisation and PCR are the techniques used for detecting viral DNA. The PCR is the most sensitive of these

techniques(Chuen Lee P & Hallsworth P, 1990) while Southern blotting is the most specific(Zur Hausen H & de Villers EM, 1994).

.11 PCR

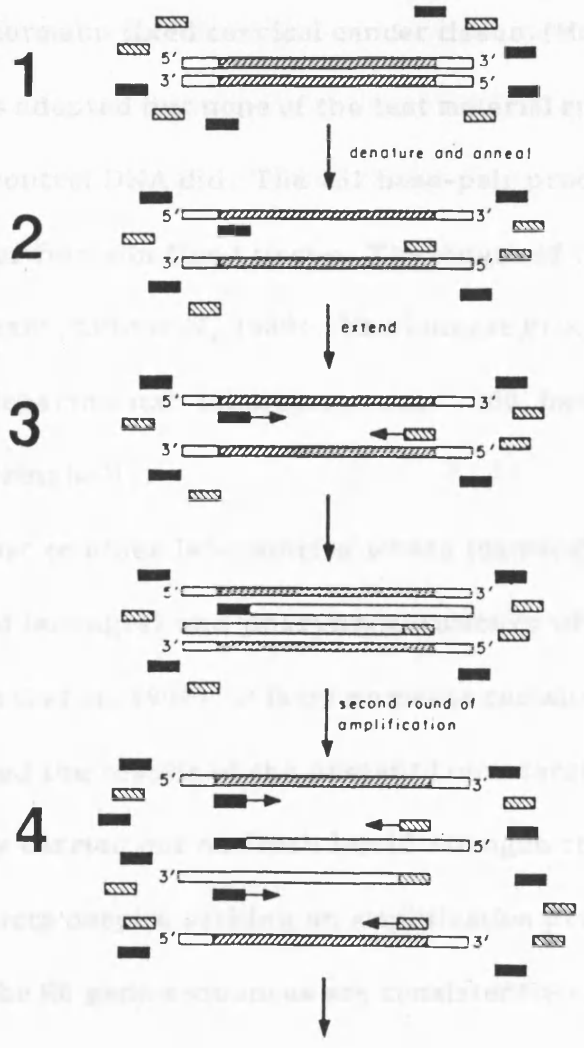
The principle of the PCR reaction was first described by Khorana and colleagues(Taylor GR, 1991). It has gained widespread application in the field of viral detection. The ability to detect low copy numbers of the test genome with PCR is established(Saiki RK et al, 1985; Nunez DA et al, 1994a). Review of the literature confirmed that laryngeal tissue yielded higher positive rates for HPV isolation when PCR was used as opposed to older techniques(Dekmezian RH et al, 1987; Hoshikawa T et al, 1990; Morgan D et al, 1991). PCR methodology was thus adopted for this study.

The basis of the technique is outlined in illustration 6.1. The test DNA is first denatured by raising the temperature thus unravelling the DNA double stranded helix. Pre-determined oligonucleotide primer pairs complementary to the nucleic acid sequences bridging the area of DNA of interest are added. These primers which are in excess, anneal with the single strands of test DNA as the temperature of the reaction mixture falls. Once this has occurred the excess of deoxynucleotides present in the reaction mixture along with the action of DNA polymerase leads to synthesis of DNA strands which mirror the area of DNA between the primers. As the

Illustration 6.1

STEPS IN THE POLYMERASE CHAIN REACTION

1. Denaturation of target DNA
2. Annealing of primer pair to single stranded DNA
3. Primer initiated DNA replication(extension)
4. Target and synthesized DNA acting as templates for further synthesis



reaction continues DNA strands that mirror the area of interest come to be produced in excess.(Taylor GR, 1991; Markham AF, 1993).

.111 L1 primers

General primer PCR for HPV with primers directed against the late coding region of the viral genome have been developed and used extensively by other workers in the investigation of formalin fixed cervical cancer tissue.(Manos M et al, 1989). Initially this strategy was adopted but none of the test material amplified with these primers, though plasmid control DNA did. The 451 base-pair product expected was probably over optimistic for formalin fixed tissue. The length of the sequence to be amplified by PCR is important(Akhtar N, 1989). The longest PCR product identified on such tissue in the departmental laboratory was 300 basepairs in length(personal communication,Pringle H).

This is in contrast to other laboratories where identical L1 primers identified HPV in 46% of archival laryngeal and pharyngeal tumours when fresh frozen tissue was studied(Clayman G et al, 1994). It is by no means certain that the use of fresh tissue would have altered the results of the present Leicestershire series. A study of SCC of the oral cavity carried out on fresh liquid nitrogen frozen tissue failed to detect L1/L2 PCR products despite seeking an amplification product of only 173 bp. It was concluded that the E6 gene sequences are consistently retained while the late genes

are often deleted when the HPV16 genome is integrated into the cell genome(Maitland N et al, 1989). Further evidence of L1 coding genes in laryngeal cancer cases is provided by the findings of HPV amplification with consensus primers to the L1 region with rates of 22% in a series of laryngeal cases in the West Midlands(Salam MA et al, 95).

The failure of the general L1 primers to work as expected prompted the use of an E6 directed HPV16 specific primer pair in addition to a HPV L1 complementary type 16 specific primer pair(see methods, chapter 4). The expected PCR product for the L1 specific HPV16 primer pair was 93 bp and for the E6 specific HPV16 complementary primer pair 110bp.

.1V False positives

There is a potential for false positive reporting with an overly sensitive technique. A number of studies have sought to correct for this by mounting dot blot or southern blot reactions with radioactive probes against the PCR products either separately or after gel electrophoresis. Confirmation is almost invariable and would only be negative in situations where the product genomic sequence was different to that expected.

A different strategy was adopted in this study. It was thought that contamination during the handling of the test material could be a potential source of spurious

results. In order to guard against this in the HPV6/11 part of the experiments all findings were double checked prior to reporting as positive. It is unlikely unless all the specimens were rendered positive by contamination that the identical isolated specimens would be positive secondary to contamination generated during the course of the PCR on two separate occasions. Test negative material was also always included to preclude the latter possibility.

Contamination at the point of collection of the specimens was guarded against in the case of both tumours and controls by always using individualized containers at the point of harvest. In an operating theatre the strict protocols observed in handling human tissue make it an unlikely area for cross contamination to occur. The time span over which the specimens were collected also means that the specimens were not handled by the same individuals. The control specimens were harvested from 2 cities and 4 hospitals by different pathologists again reducing the potential for systematic contamination. All the DNA extractions were carried out by the investigator but again the time span over which the study took place in addition to the adherence to laboratory protocols for the handling of tissue should have reduced the chance of systematic contamination. It is however true that no series of precautions could absolutely exclude the possibility of contamination at some point in processing the study material.

In the case of the HPV16 experiments different primer pairs were used on separate

occasions to confirm a specimen as positive for HPV. Initially it was planned to use a L1 complementary general primer pair,and an E6 specific primer pair. The failure of the general primer pair to amplify any target sequences in the material tested despite the DNA in some specimens undergoing amplification with E6 specific primers, suggested the general primer pair utilised was defective. The experimental strategy was changed to overcome this possibility . Two sets of specific primers were used instead complementary to the L1 and E6 regions of the HPV16 genome respectively. This change of strategy did not alter the result however, as none of the test material meet the criteria to be classified as HPV16 positive.

Reviewing the positive gel for HPV16 with the AB primer pair(illustration 5.3) shows that multiple banding occurred in all the positive specimens. This is probably due to non-specific priming(Taylor GR, 1991). Contrast this result with that for the HPV6 positive gel (illustration 6.2). The lanes with positive PCR product in this gel shows more clearly defined single bands at the anticipated product size of 237 base-pairs. Mis-priming does not exclude the presence of HPV16 specific product, but the failure to generate product with L1 specific primers suggest the presence of HPV16 L1 variants or the deletion of L1 sequences while retaining the E6 gene. Maitland et al(1989) similarly obtained multiple banding on PCR analysis of a series of upper aero-digestive tract(oral cavity) squamous cell carcinomas. Direct nucleotide sequencing employing an E6 primer confirmed the presence of E6 gene

Illustration 6.2

CASES SHOWING POSITIVE AMPLIFICATION WITH HPV6 PRIMERS

lanes 3-5 negative 3 sites non-tumour larynx 4

lane 6 negative control ultra-pure water

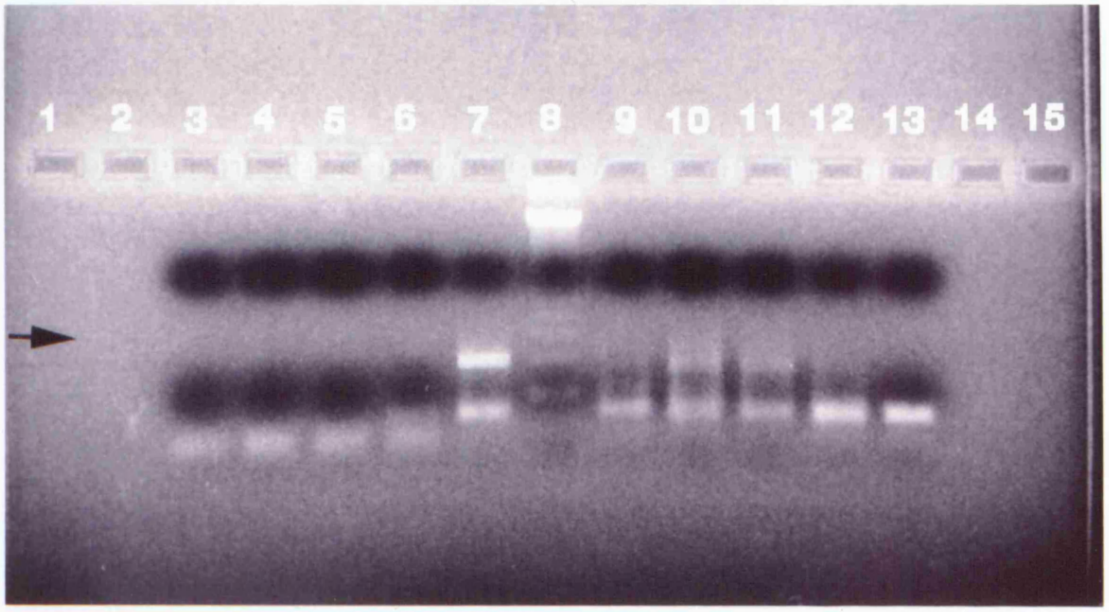
lane 7 plasmid HPV6 positive control

100 base pair DNA ladder(commercial)

lanes 9-11, 3 sites non-tumour larynx 3(positive result lane 10&11)

lanes 12-13 tumour cases with negative results

Position of PCR product shown corresponding to 237 base-pairs


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
PCR
ODUCT

sequences but they were unable to confirm if the frequently negative results with late region primers was due to a late gene variant or deletion of late genes. Experiments involving cloning of the entire HPV16 homologous sequences from laryngeal cultures will be required to distinguish between these possibilities.

In the case of the HPV6 and 11 results there remains the possibility that the specimens could have become contaminated at the DNA extraction phase. This is however unlikely because DNA extraction was never carried out in areas where PCR product was manipulated and stringent laboratory protocols to prevent contamination were observed. Contamination at specimen collection can not be excluded but the control specimens were harvested by varying pathologist from 4 units which would reduce systematic contamination bias.

All specimens once placed in formalin were then sampled by the investigator and while this provides an opportunity for systematic contamination bias all the specimens were not HPV positive. The investigator did not have the completed proforma containing details of patient smoking status prior to sample processing, and would therefore not have been able to selectively introduce contamination to achieve the results showing an association between smoking and the presence of HPV6/11. The close correlation between the known smoking status of the patients from whom the control tissue was harvested and the findings of HPV6 and 11 DNA in their laryngeal tissue(page 78) could not easily be explained by random or systematic

contamination.

4. Confirmation of adequate DNA extraction

Primers complementary to the beta-globin and bcl genes were chosen as checks on the adequacy of DNA extraction. Testing the extracted genomic DNA for amplification by the PCR reaction, namely the same method to be applied in the experiments but with primers complementary to ubiquitous human genes is a good strategy. It confirms that adequate amounts of viable DNA for amplification had been extracted.

The beta-globin and bcl genes were specifically chosen, because of local laboratory experience with these and the easy availability of tested primers and established PCR protocols(Griffin NR, 1990 ;Rogers TA, 1987; Akhtar N, 1989). The PCR product with the betaglobin primers was 110 basepairs in length and with the bcl primers 118 basepairs in length. The PCR products generated by the test primers used for identifying HPV were all of similar or shorter length namely 93bp L1 HPV16, 110bp E6 HPV16, 90bp E6 HPV11 with the exception of HPV6 237bp and the general primer products which varied from 448-454 bp in length. This means that by including only specimens generating PCR products with the bcl and betaglobin primers there is no reason why the HPV11 and 16 specific primers should not have worked. The HPV6 specific and HPV general primer products being longer would require longer lengths

of test strand DNA than was confirmed to be present.

The expected PCR products with the HPV6 and 11 primers used were 237 base-pairs and 90 base-pairs respectively. HPV16 AB primers yielded a 110 base-pair product and HPV16 CD primers a 93 base-pair product. The L1 general primer products were 448-451 base-pairs. The long length of the L1 general primer products compared to the verified adequacy of the harvested DNA to yield amplified products of 110-118 base-pairs with beta-globin and bcl primers probably accounts for the negative results. That is, the harvested DNA while of sufficient quality to generate shorter PCR products was not of sufficient quality to allow 448-451 base-pair products to be generated.

In the case of HPV16 AB and CD primers the same could not hold and thus the failure to obtain product consistently with both primer pairs suggest the absence of HPV16 or the presence of some new variant not before established. The purpose of the study was to determine the presence of known HPV16 infection rather than identify novel HPV16 types or subtypes, thus no further attempt was made to pursue this finding in the present study. The HPV6 product was larger than the beta-globin and bcl product, thus there maybe more positive HPV6 specimens which were not identified because of low quality DNA. The HPV11 findings should stand.

Two approaches were used to determine the optimum concentration of test DNA to be used. Optical density as determined by UV transillumination, and serial

concentration amplification against control primers. In the former case an optimum of 50pg of DNA was sought. The limitation of this approach being the lack of certainty that all the DNA available could be amplified. However this was checked by beta-globin amplification,and found to be sufficient.

The alternative approach adopted involved finding the test DNA concentration yielding the most discernible band on gel electrophoresis, after amplification with bcl complementary primers in a series of amplification experiments. This method has the advantage of confirming that the DNA is amplifiable, rather than merely being present. It however is no more certain than the former technique in determining the ease of illustrating HPV positivity on the test samples.

The ideal method would have been a general primer mediated PCR run at varying test DNA sample concentrations, to determine which test sample concentration gave the most easily identifiable post-electrophoretic band. The test sample concentration giving this result could then be used in a type specific PCR. The L1 primers were intended for this purpose but as no positive results were obtained, the concentration of test sample giving the most clearly defined bands on bcl electrophoresis were used for the HPV16 experiments.

5. Positive Controls

Plasmid DNA controls of the HPV types under test were used. This is not a limitation

when some of the test DNA amplifies with the test primers as in the HPV6/11 arm of the investigation. In the case of the HPV16 work however positive amplification of test DNA occurred with one pair of primers but not the other. This may reflect a relative lack of sensitivity of the second primer pair or technical failure, rather than an absence of HPV16. It is generally easier to amplify chromosomal or plasmid DNA than human genomic DNA(Akhtar N, 1989).

The study could thus have been improved by using HPV16 positive human genomic DNA. It is doubtful however that this would have made any difference to the results obtained.

6. Conclusion

.I The HPV6/11 findings

The finding of moderate levels of HPV6 and 11 in control and test tissue suggest that it would be difficult to attribute a great deal of significance to the finding of these types in laryngeal carcinoma cases(Nunez DA et al, 1994a). Brandasma JL & Abramson AL(1989) using Southern blot hybridization techniques on a sample of 60 cases while finding a lower prevalence for HPV6/11(in keeping with the lower sensitivity of Southern blot techniques) were unable to show a difference against a series of control laryngeal tissue. It does question the validity of reports asserting a direct causal association between the finding of these HPV subtypes in cases of

laryngeal cancer(Zarod AP et al, 1988). It is more likely that these viral subtypes are important as carcinogenetic cofactors in cases of carcinoma ex papilloma along with tobacco, radiotherapy and chemotherapy(Kashima H et al, 1986; Lie ES et al, 1994).

The level of HPV6 and 11 illustrated in the control larynx(Nunez DA et al, 1994) is in agreement with that shown for the human nasopharynx(Bryan RL et al, 1990), and hypopharynx(Nunez DA et al, 1994c). The finding of HPV6 and 11 in the control larynges was clearly associated with the smoking status of the subjects($p<0.01$). This association has not been shown before.

Tobacco is the commonest predisposer to laryngeal carcinogenesis and thus it would be difficult when studying laryngeal cancers to collect a large series of cancers in patients without tobacco exposure. Bradford C et al(1990) postulated that it was more common to find HPV in laryngeal cancer patients who were none smokers and suggested that viral oncogenesis was a separate route to cancer in these patients. Fouret P et al's(1977) findings of HPV oncoproteins in the presence of P53 mutations in individual patients, suggest that HPV oncogenesis is not a seperate route exclusive to tobacco and other oncogenic promotors in head and neck cancer. The present work suggest that HPV6 and 11 may become established in the larynges of smokers at a subclinical level. Longitudinal studies would be required to determine if this association was commonplace, and what impact it has on the long-term

development of laryngeal cancer.

The association of HPV with cervical SCC is strong(chapter 2). Smoking is a predisposing factor in the development of cervical dysplasia when its effect is studied independent of other factors(Becker TM et al, 1994; Brisson J et al, 1994). Smoking has been shown to predispose to cytologically evident HPV infection of the cervix(Kataja V et al, 1993) but this association is not confirmed when Southern blot evidence of HPV infection is used(Sikstrom B et al, 1995). PCR studies of exfoliated cervical cells have also failed to show a correlation between the level of smoking related DNA damage and the presence of HPV infection in the cytologically normal cervix(Simons AM et al, 1995). In conclusion, in the cervix smoking does not appear to predispose to HPV infection. Studies of head and neck SCC which conclude likewise that there is no positive association between smoking and the presence of HPV infection have been statistically weak(Snijders PJ et al, 1996; Suzuki T et al, 1994).

In the present study tobacco smoke was found to predispose to infection with HPV6 and 11 in the control larynx. This is interesting because it suggest that smoking may be a risk factor for adult onset recurrent respiratory papillomatosis. Recurrent respiratory papillomatosis shows a bimodal age specific presentation pattern. Children presenting with the disease are more commonly the first born children of teenage mothers delivered vaginally. Adults presenting with respiratory

papillomatosis do not have a birth history different from the general population, and the mode of infection remains uncertain though orogenital contact is a commonly proposed route(Kashima H et al,1992).

Rihkanen et al(1994) found a 19% prevalence of HPV6 and 11 infection in patients with benign laryngeal lesions excluding papillomatosis but found no association with smoking. The role of smoking in predisposing patients to develop adult onset laryngeal papillomas is unknown, but the current findings suggest that this area requires further study. This is especially important because patients with laryngeal papillomas are at increased risk of developing laryngeal carcinoma in later life especially in the presence of continuing tobacco exposure(Lie et al, 1994).

.II The HPV16 findings

HPV16 has an established carcinogenic effect on host uterine cervical tissue(ZurHausen H, 1977; Scurry J & Wells M, 1992), and HPV18 and 33 have been shown to be associated with laryngeal carcinogenesis in a paediatric patient in the absence of other cofactors(Simon M et al, 1994). HPV18 associated laryngeal carcinogenesis has only consistently been reported by Japanese workers(Ogura H et al, 1991; Suzuki T et al, 1994) and this may represent a geographical variation, thus it was decided to concentrate on the isolation of HPV16 in the present study. The absence of HPV16 in the control tissue was different to that reported by

Maitland, N et al.(1989) for normal oral cavity tissue and suggest that latent HPV16 laryngeal infection does not occur. There are reports of HPV16 in control laryngeal tissue, but these reports are limited to cases of laryngeal carcinogenesis in which adjacent tissue has been sampled(Morgan D et al, 1991; Snijders PJ et al, 1996). It was also impossible to illustrate HPV16 in the human hypopharynx(Nunez et al, 1994c), and thus it appears that in the absence of morphological disease this HPV type is absent despite using the highly sensitive PCR reaction for detection. The failure to detect HPV16 in a series of 22 squamous cell carcinomas of intra-laryngeal origin is at variance with previous reports of a 40% isolation rate in the literature (Morgan D et al, 1991; Kiyabu M et al, 1989)(Fishers exact test,$P<0.05$).

There are a number of possible explanations. The primers used were sensitive enough to return positive results in the case of HPV6 and HPV11 in the larynx. There is however a small possibility that the HPV16 primers which failed to confirm the presence of HPV16 in human genomic DNA were not sufficiently sensitive or failed for technical reasons. That is at least the CD primers failed because the AB primers did find evidence of HPV16 infection. An HPV16 variant as discussed on page 94 would offer another explanation. In the event that the discordance is interpreted as absence of clear evidence of HPV16 infection how could this be interpreted? Two possibilities may account for these negative findings.

A geographical variation with no evident HPV16 being found in the Leicestershire

Illustration 6.3

Laryngeal HPV16 identified by PCR

Year	Author	Specimens	n	T1-2 stage	HPV16 + findings		
					Untreated	Treated	Unknown
1997	Nunez D	paraffin	37	16	0/37	-	-
1997	Fouret P et al	paraffin	187*	unknown	6	-	-
1996	Snijders P et al	frozen	15	5	0/4	4/11	-
1995	Salam MA et al	paraffin	36	unknown	-	-	2/36
1994	Clayman G et al	paraffin	59	28	24/59	-	-
1991	Morgan DW et al	frozen	10	0	1/3	3/7	-
1990	Perez-Ayala et al	frozen	48	unknown	26/48	-	-
1989	Kiyabu M et al	paraffin	10	unknown	-	-	4/10

*a series of head and neck SCC cases, the number of laryngeal carcinomas is unstated.

laryngeal SCC population, as opposed to the high level of 40% reported in a London population. In uterine cervical SCC which has a high association with HPV16 most studies reporting HPV DNA in about 90% of cases(Zur Hausen H & de Villers E-M, 1994), the prevalence of HPV16 in a Leicestershire population using similar methodology to that in this study, was 33%(Rogers TA, 1987). Furthermore only a 5% prevalence of HPV16 was found in a contemporaneous West Midlands series of laryngeal SCC cases(Salam M et al, 1995). A previous unpublished study of a large archival series of laryngeal SCC cases in Edinburgh also failed to find any evidence of HPV16 infection though this was based on less sensitive in-situ techniques (Arends MJ et al, 1990).

An alternative or additional explanation on comparing this laryngeal tumour series with others is the disease stage of the cases represented. The majority of cases from Morgan's study(1991) were undergoing laryngectomy for advanced or recurrent disease, whereas the patients in this study were almost 50% T1/T2 disease(that is early disease). It is possible that with more advanced disease HPV16 becomes more apparent, because of increased viral genome proliferation due to up-regulation by the upstream regulatory region(Hoppe-Seyler F & Butz K, 1994). A stage dependent difference in the expression of HPV types has been shown by Clayman G et al.(1994), with only 1 of 12 T1 larynges showing any HPV's as opposed to 40% of more advanced cases.

The role of previous treatment on the isolation of laryngeal HPV is ill defined. It is important to remain aware of the impact of immunosuppressive treatment such as radiotherapy on a predisposition to HPV infection(Bradford C et al,1990). PCR based studies of series of previously untreated laryngeal carcinomas are rare(table) and often biased towards patients with advanced disease(Snijders PJ et al,1996; Clayman GL et al,1994; Morgan DW et al,1991). Perez-Ayala M et al(1990) or do not report the stage of disease in their previously untreated cases. No series randomized case selection for study.

In conclusion in a series of previously untreated laryngeal cancers, selected at random to reflect the distribution of disease seen at presentation in a Leicestershire population, there is no evidence of HPV16 persistence. This is the first study to randomize the archival cases studied.

.III Future Developments

The further study that should be carried out would be a comparison of isolation rates of HPV16 in stage 3/4 laryngeal SCC, with the rates seen in this study. This would determine if the absence of HPV16 is a factor of disease stage as opposed to a true low prevalence in the local population.

The question concerning the role of immunocompromise and prevalence of HPV6 and 11 infection is difficult to solve unless a study could be carried out on a

cross-section of the normal population, say on laryngeal brushings obtained at intubation from patients undergoing routine anaesthesia. This is probably the best way of determining the true level of HPV infection in the population, but will require ethical committee approval, and a pilot study to determine the applicability of laryngeal brushings/smears for HPV DNA isolation.

The importance of smoking both active and passive in predisposing to clinical laryngeal papillomatosis requires further study. The evidence of a link will offer a method of reducing the incidence of respiratory papillomatosis, and also the late developement of laryngeal carcinoma.

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Human papilloma viruses: a study of their prevalence in the normal larynx

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Abstract

The association of human papilloma viruses (HPV) with laryngopharyngeal squamous cell carcinoma is under investigation. The suitability of control tissue in the reported series, invariably obtained from histologically normal tissue adjacent to a squamous cell carcinoma or from patients with benign laryngopharyngeal disease, is questionable. The present study determined the prevalence of HPV in a series of normal larynges.

Twelve autopsy larynges were collected. DNA was obtained by SDS proteinase K digestion. Evidence of HPV infection was documented by the polymerase chain reaction using oligonulceotide primers complementary to sequences in the E6 region of HPV types 11, 16 and 18.

Four female and eight male larynges, mean age 65 years (SD=16 years) were collected 72 hours postmortem (median value). HPV type 11 was isolated from three specimens. A 25 per cent prevalence rate for HPV 11 was found. No other HPV types were isolated.

Key words: DNA; Larynx; Polymerase chain reaction; Viruses

Introduction

Human papilloma viruses (HPV) types 6 and 11 have an established aetiological role in laryngeal papillomatosis (Terry *et al.*, 1987). The oncogenic potential of HPV in the human larynx is being investigated (Brandasma and Abramson, 1989; Morgan *et al.*, 1991).

The prevalence of HPV in laryngeal verrucous carcinoma, carcinoma *in situ* and invasive squamous cell carcinoma varies from five to 88 per cent; 6, 11, 16 and 33 being the subtypes isolated (Kashima, *et al.*, 1986; Dekmezian *et al.*, 1987; Brandasma and Abramson, 1989; Bryan *et al.*, 1990; Morgan *et al.*, 1991). Recent work suggests that the isolation of HPV is no greater in the carcinomatous larynx than in the control larynx, thereby questioning its aetological significance (Brandasma and Abramson, 1989; Bryan *et al.*, 1990).

Reported control tissue consists of biopsy specimens from adjacent nondiseased sites, or biopsy specimens taken from patients undergoing direct laryngoscopy in whom there is no evidence of respiratory papillomatosis or malignancy. The controls therefore invariably consist of tissue obtained from a group of patients with laryngeal pathology, albeit sometimes benign, and are unlikely to be representative of the general population.

A number of techniques have been used for the identification of HPV in the larynx which vary in their sensitivity and specificity. The polymerase chain reaction (PCR), the most sensitive available technique (Bryan *et al.*, 1990; Morgan *et al.*, 1991) was used to determine the prevalence of HPV in this series of normal autopsy larynges.

Methods

Larynges appearing normal were collected at autopsy with local ethical committee approval. The supraglottis, glottis and subglottis of each larynx was sampled. HPV sequences were detected by PCR using oligonucleotide primers complementary to the sequences of the E6 region of HPV types 11, 16 and 18 as described previously (Griffin *et al.*, 1990) In brief 4 × 10 μm paraffin sections were cut using a microtome, slide mounted and left on a hot plate for five minutes. The tissue was dewaxed in xylene, rehydrated through graded alcohols to water, and then scraped off into a 1.5 ml microcentrifuge tube to be incubated in 100–500 μl of digestion buffer containing 10 mM tris-hydroxymethylaminomethane hydrochloride (TRIS-HCL(pH 8.8, 100 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.5 per cent sodium dodecyl sulphate (SDS) and 2 mg/ml proteinase K at 37°C for five days. The aqueous digest was then extracted once with phenol:chloroform (1:1), once with chloroform:isoamyl alcohol (24:1) and the DNA was precipitated with two volumes of ethanol and 1/10 volume of 3 M sodium acetate at –20°C overnight. The DNA was collected after centrifugation at 15000 g for 10 minutes, dried and redissolved in 50 μl of ultrapure water: 10 μl aliquots were

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amplified to detect the presence of beta-globin before proceeding to HPV detection.

HPV detection was carried out using a reaction mixture containing 1 nM of each primer pair, 200 μM of each dNTP, 10 × reaction buffer (10 mM TRIS-HCL, pH 8.3; 50 mM KCl; 7.5 mM $MgCl_2$; 0.01 per cent gelatin: 0.1 per cent TRITON × 100; 1.0 unit of Taq polymerase (Super Taq: HT Biotechnology Ltd) in a total reaction volume of 50 μl. A positive control containing a HPV recombinant plasmid and a negative control in which ultrapure water was substituted for the aliquoted DNA were also amplified for each series of DNA specimens tested.

Temperature cycling was performed using a programmable cycler (M.J. Research, Waterdown, MA) programmed to perform one cycle at 94°C for five minutes, 55°C for 90 s, 72°C for two minutes and 39 cycles at 94°C for 30 s, 55°C for 90 s and 72°C for two minutes. The amplified products were visualized under ultraviolet light after electrophoresis for one hour on a two per cent agarose gel.

Stringent laboratory conditions were routinely used to reduce the risk of cross-contamination and false positivity. Furthermore the consistency of the findings were confirmed by repeating the PCR test protocols for all specimens on a minimum of two occasions, one week apart.

Results

Four female and eight male larynges were studied. The patients had a mean age of 65 years (SD=16 years). The larynges were harvested at a median time of 72 hours postmortem (range 24–144 hours). Three larynges each were harvested from nonsmokers (cases 7, 8 and 11) and ex-smokers (Table I). Five of the other cases were active smokers and one case had an indeterminate history of tobacco use.

All of the specimens underwent amplification with human betaglobin primers. HPV 11 was isolated from three cases, no other viral subtypes were found.

Discussion

The 33 per cent prevalence rate of HPV 11 found in the present study is not significantly different from that shown for sites of invasive squamous cell carcinoma or adjacent histologically normal sites in the larynges studied by Morgan *et al.* (1991) (Fisher's exact test: $p>0.05$). The results are also within the 95 per cent confidence range of 17/69 per cent (calculated on the standard error of a proportion using the binomial approximation) illustrated by Bryan *et al.* (1990) for the histologically normal nasopharynx.

TABLE I
AUTOPSY DETAILS AND PCR RESULTS OF THE CASES STUDIED

Patient no.	Age (years)	Sex	Postmortem time (hours)	Smoker	HPV type
1	43	male	144	yes	
2	68	female	48	yes	
3	27	male	72	yes	11
4	54	male	48	yes	11
5	80	male	144	ex	
6	82	male	48	ex	
7	81	female	24	none	
8	68	female	48	none	
9	66	male	96	ex	
10	74	male	48	?	
11	61	male	72	none	
12	71	female	96	yes	11

HPV 18 was not demonstrated. There is however only one report of a laryngopharyngeal cancer positive for HPV 18 in the literature (Ogura *et al.*, 1991). The absence of HPV 16 in this study is different from the prevalence rate of 40 per cent found in other studies utilizing the polymerase chain reaction on invasive squamous cell carcinomas of the larynx Kiyabu *et al.*, 1989; Hoshikawa *et al.*, 1990; Morgan *et al.*, 1991) ($p<0.5$: Fisher's exact test one-sided). The failure to isolate these types in the present study supports an aetiological role for HPV 16 and possibly 18 in laryngeal cancer. The prevalence of HPV 11 found suggests that this viral type is not tumour-specific and can occur in the absence of laryngeal pathology.

The use of autopsy material precludes the sample from being representative of the general population, though it avoids the bias inherent in studies which obtain material from patients undergoing laryngeal procedures. A large scale, population-based, cross-sectional survey is the only assured method of determining the true prevalence of laryngeal HPV carriage.

Conclusion

This is the first study to confirm the viability of isolating HPV subtypes in autopsy larynges and provides evidence of HPV 11 infection in a larynx which appears normal.

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Human papilloma viruses in the human hypopharynx

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Human papilloma viruses in the human hypopharynx

The possible association of human papilloma viruses (HPV) with laryngopharyngeal squamous cell carcinoma is under investigation. Recent work suggests regional differences in the prevalence of HPV infection in the hypopharynx. The present study investigates the prevalence of HPV in tissue obtained from a series of piriform fossae.

Piriform fossa epithelium was harvested from 12 autopsy cases free of local disease. DNA was obtained by SDS/Proteinase K digestion. Evidence of HPV infection was documented by the polymerase chain reaction using oligonucleotide primers complementary to sequences in the E6 region of HPV types 11, 16 and 18.

All the specimens were positive for beta-globin. HPV11 was isolated from two patients. None were positive for HPV16 or HPV18. An 18% prevalence rate for HPV11 in the normal human hypopharynx was found.

Keywords *Human papilloma virus hypopharynx polymerase chain reaction*

Human papillomaviruses have been shown to be associated with benign disease of the upper aerodigestive tract.[1,2] The association between these viruses and malignant disease of the anogenital region, especially the cervix, is well documented.[3] HPV infection in carcinomas of the upper aerodigestive tract has been shown, but not consistently at all sites.[6,7] The causal link between the presence of HPV infection and malignant change in the upper aerodigestive tract is therefore still under debate.[8,9] Ogura *et al.*[10] suggested that regional differences in the type and prevalence of HPV in the laryngopharynx may occur, which emphasizes the need for studies to determine the prevalence in these sites, in the normal population. Studies have assessed the presence of HPV at pharyngeal sites adjacent to benign disease and the results for the oro- and hypopharynx have invariably reported the absence of HPV infection.[4,9] This contrasts with isolation rates of 36% and 43% for HPV6 and 11, respectively in the histologically normal nasopharynx.[11] The prevalence of HPV infection in the normal hypopharynx in the British population is unknown. The present study determined the prevalence of HPV11, 16 and 18, in the normal hypopharynx.

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Methods

A triangular wedge of the epithelial lining of the piriform fossa was harvested at autopsy. Samples were obtained from both piriform fossae in each case, fixed in formalin, and embedded in paraffin.

The DNA was precipitated with 2 volumes of ethanol and 1/10 volume of 3 M sodium acetate at −20°C overnight, centrifuged at 15 000 *g* for 10 min, dried and re-dissolved in 50 μl of ultra-pure water.

Ten microlitres aliquots were amplified in the presence of beta-globin to determine whether amplifiable DNA was present in the test samples before proceeding to HPV detection. HPV detection was carried out by the polymerase chain reaction (PCR) utilizing a 50 μl reaction mixture containing 1 nM of each primer pair, 200 μm of each dNTP, 10× reaction buffer (10 mM TRIS HCL pH 8.3, 50 mM kCL, 7.5 mM $MgCl_2$, 0.01% gelatin, 0.1% TRITON x-100, 1.0 unit of Taq polymerase (Super Taq, HT Biotechology Ltd, Promega, Madison, Wisconsin, USA). A positive control consisting of the test viral recombinant nucleic acid and a negative control in which ultra-pure water was substituted for the aliquoted DNA were amplified for each series of specimens tested.

Temperature cycling was carried out with a programmable

Table 1. Subjects studied, causes of death, associated diseases and treatments

Patient number	Cause of death	Associated disease/treatment
1	Cardiac arrest	Ischaemic heart disease/bypass
2	Cardiac arrest	Renal failure/transplant
3	Paracetamol overdose	Hepato-renal failure/renal transplant
4	Cerebral tumour	Craniotomy
5	Cardiac arrest	Diverticular disease/bowel resection
6	Gastric carcinoma	Gastro-intestinal haemorrhage
7	Pancreatic carcinoma	
8	Cardiac arrest	Intestinal obstruction
9	Cardiac failure	Aortic valve disease
10	Bronchopneumonia	Myelo-proliferative disorder
11	Cardiac arrest	Diabetic nephropathy
12	Bronchial carcinoma	Superior vena cava obstruction

Table 2. HPV status of hypopharyngeal tissue by subject

Patient number	Age (years)	Sex	Post-mortem time (h)	HPV type
1	43	M	144	
2	68	F	48	
3	27	M	72	11
4	54	M	48	
5	80	M	144	
6	82	M	48	
7	81	F	24	
8	68	F	48	
9	66	M	96	
10	74	M	48	
11	61	M	72	
12	71	F	96	11

M, Male; F, Female.

cycler. Electrophoresis on agarose gel containing ethidium bromide allowed visualization of the PCR product under ultraviolet light.

Results

Piriform fossa epithelium was harvested from eight men and four women (mean age 65 years), 72 h (median, range 24–144 h) post-mortem. All the specimens were positive for beta-globin. HPV11 was isolated from two (see Table 2). None were positive for HPV16 or HPV18.

Discussion

The PCR, capable of identifying low copy numbers of the HPV genome of the order of 100–200 copies per reaction mixture[12] has been applied to studying histologically benign tissue from the nasopharynx, histologically normal sites in the pharynx adjacent to sites of squamous cell carcinoma, and inflamed non-neoplastic oropharyngeal tissue.[5,9,11] PCR was used in the present study to determine the prevalence of HPV infection in the non-diseased hypopharynx.

The finding of a 18% prevalence rate for HPV11 in the normal hypopharynx is within the range for the normal nasopharynx and larynx.[11,13] This is, however, higher than the rate of clinical detection of hypopharyngeal papillomas.[2] It is not possible to extrapolate from the present study to the general population because 50% of the subjects may have been immuno-compromised because of malignancy or recent organ transplantation. It has been shown, though not conclusively, that laryngeal tumours associated with HPV infection occur in tissue transplant recipients such as patient 3.[14] Thirty-three per cent of the six potentially immuno-compromised patients were positive as compared with none of the other cases (Fisher's exact text $P > 0.05$). A more representative sample of the general population would need to be studied to determine the true prevalence.

HPV16 and 18 have been found in cases of hypopharyngeal malignancy.[5,10] The failure to identify HPV16 or 18 in this study supports a potential oncogenic role for these viral types in the hypopharynx. The role of HPV11 infection in the hypopharynx is uncertain.

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