P53 Presentation and Other Prognostic Indicators in Oral Squamous Cell Carcinoma.

Frances Rita Maloof

BDS(Hons),FRACDS

A thesis submitted in partial fulfilment of the requirements

for the degree of

Master of Dental Science

(Oral and Maxillofacial Surgery)

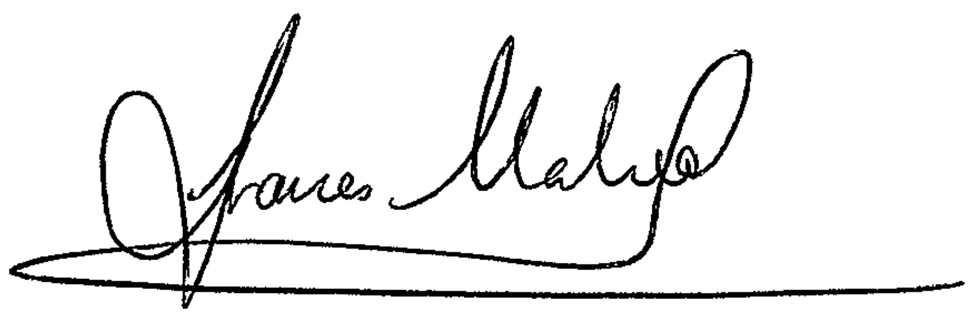
Department of Oral and Maxillofacial Surgery

The University of Sydney

December 1996

Statement of Authorship

The contents of this thesis consists of original work carried out by the author unless otherwise stated and duly acknowledged.No part of this thesis has been submitted in whole or in part for any other degree.



Frances R Maloof
December 1996

Abstract

P53 presentation and other prognostic indicators in oral squamous cell carcinoma.

Mutations in the p53 gene are the commonest genetic errors so far identified in human malignancies, including oral squamous cell carcinomas (OSCC).Using an immunohistochemical technique employing two monoclonal p53 antibodies D0-7 and 1801, a peroxidase-labelled secondary antibody and appropriate controls, to detect abnormal p53 protein expression in OSCC, the present investigation of surgical excision specimens of 30 primary OSCC from 30 patients reached the following conclusions:-

1. p53 was expressed at the resection margin in 10 / 30 cases (33%) and within the primary tumours in 13/ 30 cases (43%).

2. There was a strong association between the immunohistochemical detection of p53 at the resection margin and the tumour ($p=0.004$) supporting the potential of p53 mutant protein detection to be used as a marker of malignancy and allow diagnosis of disease at an earlier stage.

3. The immunohistological detection of p53 at the margin was mainly found in dysplastic epithelium however no statistically significant association was found ($p=0.16$).

4. p53 expression was not related to the site of the primary tumour or tumour grade ($p=0.4$).

5. It appeared that patients with p53 negative tumours tended to survive longer,though no statistically significant association was found ($p=0.10$).

6. No correlation was found between the site of the primary tumour and survival though 7 of 30 patients died within 2yrs of diagnosis.

7. The detection of mutant p53 protein in oral smears by immunostaining is likely to be of value in the early stages of carcinogenesis since p53 positive cells were observed at the periphery of the tumour and deep invasive margins.

8.The staining patterns using D0-7 were comparative to those of 1801 and the application of the microwave technique using D0-7 in 10 OSCC samples showed more intense staining which was statistically significant (P=0.04) compared with the background signal .

9. p53 protein was occasionally expressed in the 10 samples of clinically normal oral mucosa from subjects who smoked.

10. Reproducibility of the assessment of p53 positivity was shown to be good with low subjective error in interpretation .

11. The histological malignancy (Bryne) grading had a predictive value in assessing survival and was strongly correlated with primary tumour depth (p=0.02) and lymph node metastases (p=0.04).The tumour depth had a definite correlation with lymph node status (p=0.03) but no direct association with survival.

There is still no single histological or clinical parameter that is able to precisely forecast the outcome of a patient with primary OSCC. Although assessment of the data collectively increases the ability to predict the prognosis of primary OSCC , it is still however approximative. The immunohistochemical detection of p53 although singly shown to be a weak prognostic factor , has potential to be used as a tumour marker in oral cancer, especially in diagnosis.It may prove to have application for the monitoring of disease progress and treatment effectiveness.

Acknowledgements

I would like to express my thanks to my supervisor Professor Murray Walker, for his guidance during my candidature.I am most grateful for his encouragement and inspiration throughout this study.

I am indebt to Miss Janice Matthews for her pointing me in the right direction during take-off , and for her invaluable assistance and willingness to help.I am also grateful to Mrs Mara Cvejic for her life saving efforts, ready encouragement and warm support.

Thanks to Dr Michael Bilous and staff of the Anatomical Pathology Department ICPMR, Westmead Hospital, for the use of resources and teaching of the basic histopathological technique.Thanks also to Dr Ian Kalnins for the use of patient material.Gratitude is also extended to Dr Roger Reddel and Elsa Moy of the Children's Medical Research Institute for the donation of cell lines and readiness to help.

Thanks to the faculty of Dentistry for financially supporting this research. I extend my thanks to Dr Karen Bythe for her invaluable assistance in the statistical analysis of data.Thanks also to Dr Alexandra Jones for ready discussion of data and use of material.

Heart felt gratitude is extended to Dr Elpida Vlach for her encouragement and support.To my parents, who have encouraged perserverence, hard work and whos support I truly appreciate.Thankyou to my fiancé Simon Grace for his patience and encouragement during this study.

Most importantly I would like to thank God , while ever watchful with His gentle love, mercy and guidance has made all things possible.

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List of Abbreviations

A	Adenine
a.a	Amino acid
AHH	Aryl hydrocarbon hydroxylases
C	Cytosine
CEA	Carcinoembryonic antigen
c-myc	Cellular myelocytoma oncogene
DNA	Deoxyribonucleic acid
EBV	Epstein-barr virus
ELISAs	Enzyme-linked immunosorbent assays
G	Guanine
GSH	Gamma-glutamyl-cysteinyl-glycine
GST	Glutathione-s-transferase
HPV	Human papilloma virus
hsc 70	Heat shock protein 70
HSV	Herpes simplex virus
IAP	Immunosuppressive acidic protein
IgG1	Immunoglobulin G1 class
IgG2b	Immunoglobulin G2b class
IHC	Immunohistochemistry
IQ	Interquartile
LFS	Li-Fraumeni syndrome
mm	Millimeters
*m*RNA	Messenger ribonucleic acid
NEM	Normal epithelial margin
OSCC	Oral squamous cell carcinoma
Pab1801	Primary antibody 1801
Pab240	Primary antibody 240
Pab246	Primary antibody 246

PabD0-7	Primary antibody DO-7
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
ras	Rat sarcomas oncogene
SCC	Squamous cell carcinoma
SCCA	Squamous cell carcinoma antigen
SM	Surgical margin
STNMP	Site tumour node metastasis pathology
T	Thymine
TD	Tumour depth
TM	Tumour margin
TNM	Tumour node metastasis
UICC	Union Internationale Centre Le Cancer
UV	Ultraviolet
WHO	World health organisation
wt-p53	Wild-type p53

Chapter One

REVIEW OF THE LITERATURE

Oral Squamous cell Carcinoma.

1.1 Introduction

1.1.1 Epidemiology

In 1985 an estimated 412,000 new cases of oral and pharyngeal cancer were diagnosed worldwide.(Macfarlane *et al.*1994).It is the fourth most common cancer in developing countries and is ranked eighth in developed countries .

Squamous cell carcinoma accounts for 90 per cent or more of all oral malignant neoplasms.In England and Wales 85 per cent of cases occur in patients over the age of fifty years.(Soames & Southam 1993)

Although the incidence and mortality rates of oral cancer showed a long decline earlier this century , in the recent years the incidence and mortality rates have increased in New South Wales ,Australia and other developed countries.(Macfarlane 1994 & Tables 1.1,1.2). In addition, the age of onset of oral cancer is declining, presumably reflecting changes in aetiological factors (Smith 1989).

In Australia and the United Kingdom, the lip and tongue are the most frequently affected

sites in both sexes ,followed by the floor of the mouth in males , the buccal mucosa in females,and then the alveolar ridge site.In India the most frequent site is the buccal mucosa, ascribed to tobacco smoking and the betel quid habit (Soames and Southam 1993).

Table 1.1. Actual number of new cases of cancer diagnosed per annum in New South Wales (total resident population 5.9 million in 1991) in males.

Site(ICD-9)	**1972**	**1973-1977**	**1978-1982**	**1983-1987**	**1988**	**1989**	**1990**	**1991**
lip	101	132	126	139	131	156	154	208
tongue	38	49	57	74	79	75	79	79
gum	11	7	7	8	14	12	10	12
floor of mouth	20	30	37	47	38	39	35	42
other parts of the mouth	20	24	29	40	40	65	50	48

Table 1.2.1.1. Actual number of new cases of cancer diagnosed per annum in New South Wales (total resident population 5.9 million in 1991) in females.

Site(ICD-9)	**1972**	**1973-1977**	**1978-1982**	**1983-1987**	**1988**	**1989**	**1990**	**1991**
lip	15	26	28	33	47	44	55	81
tongue	26	23	31	32	37	42	41	38
gum	7	6	6	7	7	8	11	10
floor of mouth	11	8	15	16	10	17	18	16
other parts of the mouth	10	14	17	21	20	25	31	32

The median age at which cancer of the lip was diagnosed was 62years of age in males and 70 years of age in females.

1.1.2 Aetiology

Many cancers appear to have a multifactorial aetiology ,with genetic and environmental factors forming two important groups.Epidemiological studies have examined the role of influences such as geographical distribution,racial prevalence,occupation,social class,diet,climate,hygiene,use of tobacco,and alcohol consumption,to elicit possible causative factors for the disease.

Although no common single factor has been identified, some habits plus host factors are

more traditionally supported and these include the influence of tobacco and alcohol.Infective agents, poor oral hygiene ,and nutritional deficiencies (Smith *et al* 1990) all potentially avoidable factors seem important in the aetiology of oral cancer , particularly tobacco smoking.

1.1.3 Tobacco

The association of tobacco use, related to dose and time,and combining with alcohol to give a multiplicative effect with oral cancer is based primarily on epidemiological studies(Wynder *et al* 1957 , Graham,1977).

Polycyclic aromatic hydrocarbons in tobacco ,are activated to ultimate carcinogens in cells by microsomal complex enzymes commonly referred to as aryl hydrocarbon hydroxylases (AHH).Trell and colleagues (1981) found a higher inducibility of AHH in cancer cases than in controls.Accumulation of mutations induced by such carcinogens, including proto-oncogenes and tumour suppressor genes or DNA repair genes are thought to play a key role in tumour development (Levine *et al* 1994).

Consequently, antioxidants or free radical scavengers such as vitamins A,C,and beta carotene E are widely used chemopreventive agents against oral cancer.

Suggestions have also been made linking interaction between tobacco smoking and the Herpes simplex type 1 virus in the carcinogenesis of oral cancer (Reviewed by Enwonwu and Meeks 1995).

1.1.4 Alcohol

Several reports have examined the possible mechanisms for alcohol related carcinogenesis.Alcohol intake ,apart from limiting food consumption, promotes increased urinary loss of ascorbic acid (Gaby and Singh,1991) and other essential nutrients (Gersons ,1990).

Alcohol induced immunosuppression, promotes impaired salivary gland function and oral mucosal immunity, as well as a reduction in the number of helper CD4 cells (Watson *et al* 1994).Alcohol is also said to increase the risk of oral cancer regardless of the form it takes.(Winn, 1995).Several studies suggest a synergistic effect of joint exposure to alcohol and smoking ,an observation supported by epidemiological evidence (Field *et al* 1992).

1.1.5 Nutrition

Dietary deficiencies ; especially of vitamins A and C , iron and certain trace elements are thought to predispose to oral cancer (Stitch *et al* 1988, La Vecchia *et al* 1993).

Malnutrition is characterised by a marked tissue depletion of anti-oxidant nutrients,including GSH (gamma-glutamyl-cysteinyl-glycine),a key cellular anti-oxidant (Enwonwu & Meeks 1995).Trickler *et al* in 1993 showed GSH to be protective against chemically induced oral cancer and leukoplakia in adult Syrian hampsters.Weizbicker *et al* in1989, suggest that dietary cysteine, found in meat sources and certain vegetables, may be even more important in the protection against oral cancer.

Epidemiological evidence in Scandinavia confirmed the importance of iron deficiency in the development of carcinoma of the mesopharynx and hypopharynx.Susceptibility also applied to the buccal mucosa, tongue and all levels of the oesophagus (Smith Pindborg, Binnie ,1990). Atrophy and reduced maturation of the oral epithelium has been suggessted to be associated with an increased susceptibility to the action of carcinogens (Binnie, 1991).Less attention however has been given to iron deficiency in recent epidemiological studies.

1.1.6 Infective agents

An infectious aetiology has been suspected in oral carcinoma and potentially malignant oral lesions. This has been the subject of much debate, though a failure to find viral particles in the neoplasms, or the absence of virions does not mean that viral nucleic acid might not be present (Cox *et al* 1991).

There has been little research evidence for involvement of retroviruses in oral carcinogenesis,and a role for human adenoviruses appears unlikely (Kumari *et al,*1982).Epstein-Barr virus products have not been found in oral carcinoma tissue (Talacko *et al,*1991).Serum antibodies to Herpes simplex virus type 1 (HSV) are increased in patients with head and neck carcinoma (Kumari *et al,*1982),and cigarette smoking is suggested to predispose mucosa to HSV infection by suppressing host defenses such as natural killer cell activity (Ferson *et al,*1979).

Human papillomavirus (HPV) antigens and genes have been detected in oral cancer and precancer (Loning *et al,*1985). Maitland *et al* 1987,found 46% of tongue and floor carcinomas to have a HPV -16 related virus.HPV has been identified in oral leukoplakias, head and neck metastases, and apparently normal oral mucosa.Cox *et al.* 1991 conclude that more proof is still required to show direct involvement of HPV in the aetiology of oral cancer.

1.1.7 Candida infection

Candida albicans was reportedly shown to cause squamous metaplasia in chick embryo ectoderm (Cawson &Binnie 1980),and some strains were shown to produce nitroso compunds (Krogh *et al.*,1987) which may act to promote experimental oral mucosal carcinogenesis (O'Grady *et al.*,1992).The presence of Candida albicans in an oral premalignant lesion or oral squamous cell carcinoma (OSCC) may however be the result

of supra-infection on pre-existing lesions or OSCC and therefore have no relevance to the carcinogenic process (Scully et al.,1991).

1.1.8 Syphilitic infection

White syphilitic lesions are normally present on the anterior two third of the dorsum of the tongue and are the result of secondary and tertiary infection of the oral cavity with *Treponema pallidum* (Binnie ,1976).These lesions were considered pre-malignant , but due to the introduction of the anti-microbial drug ,penicillin , the treatment of syphilis has not resulted in a decrease in oral cancer.The spirochaete is no longer considered to be of significance in the development of oral cancer as a direct causative agent, but rather, a possible association seems more favored (Daftary *et al.*, 1991).

1.2 Histological Grading of Oral Squamous cell carcinoma

This study concentrates on primary oral squamous cell carcinoma (OSCC),the commonest malignant tumour arising in the oral cavity (see figure 1.1).

Broders (1941) described a classification for the degree of malignancy of OSCC (see table 1.3.)

Figure 1.1 Oral squamous cell carcinoma sited on the lateral border of the tongue.

Table 1.3. Broders (1941) Classification for degree of malignancy.

Grade	Classification
1.	Well differentiated
2.	Moderately well differentiated
3.	Poorly differentiated
4.	Undifferentiated

The histological pattern of a **well differentiated** OSCC,consists of infiltrating islands or strands of prickle cells with a limited layer of peripheral basal cells often with central keratin whorls.Intercellular bridges are recognisable (figure 1. 2.). The **moderately differentiated** OSCC has a histological pattern where keratin whorls are sparse or absent.There may be cellular and nuclear pleomorphism. Mitotic figures may appear more abundant and some more atypical (figure 1.3 and 1.4). In the **poorly differentiated** OSCC, the epithelial cells are hardly recognisable as being squamous, with gross anaplasia , nuclear or cellular pleomorphism or nuclear hyperchromatism (figure 1.5). The cells may only be recognised by immunohistochemistry for cytokeratin intermediate filament proteins.

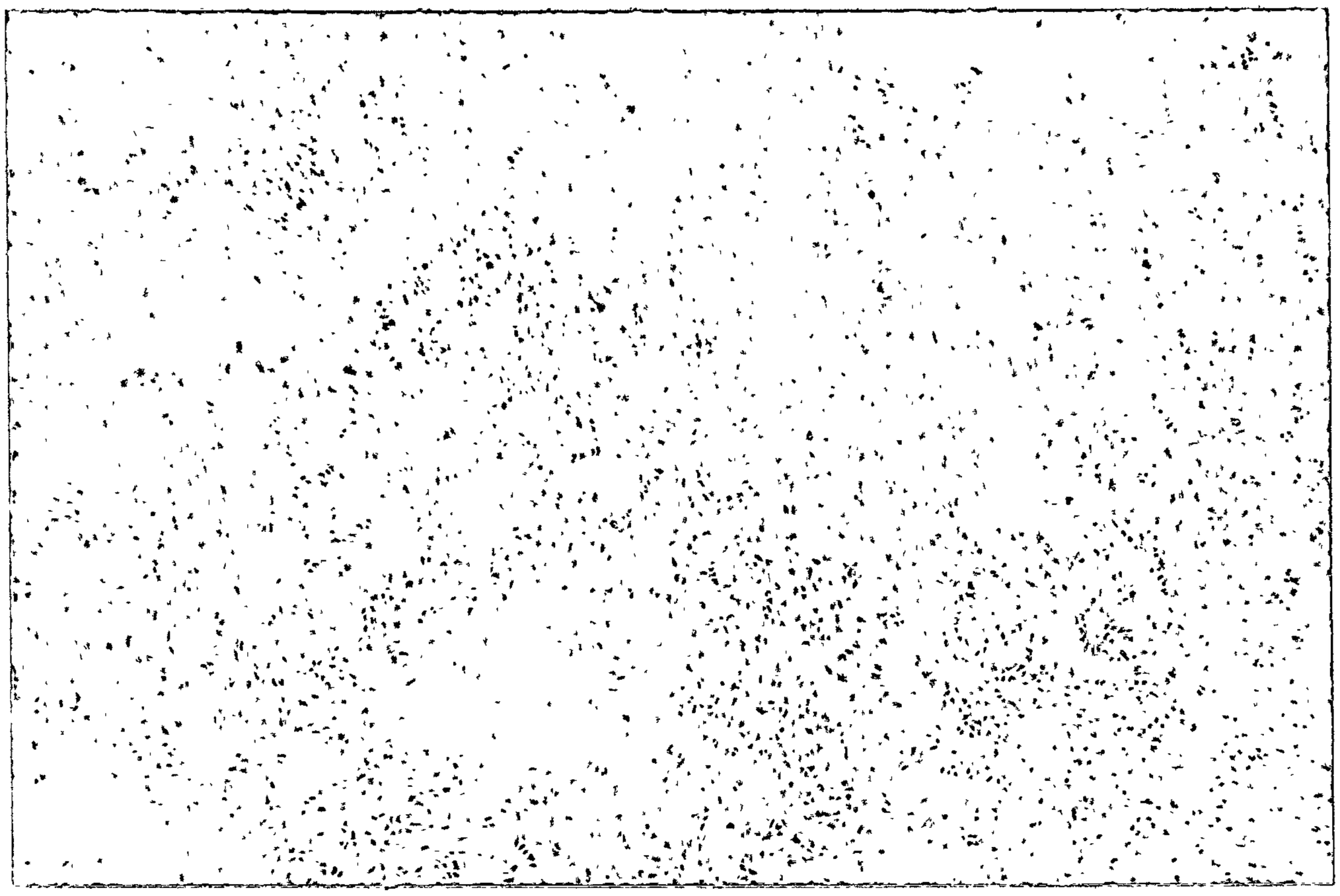
All the tumor types show invasion and destruction of local tissues.The pattern of infiltration of adjacent tissue by neoplastic epithelium is variable.Some tumours have a broad front of invasion.In others, separate islands or individual cells are found in advance of tumour growth,and there is a variable lymphocytic and plasma cell infiltration in the supporting stroma, possibly representing a reaction to tumour antigens, tumour necrosis and ulceration.

Spreading by lymphatics or blood vessels may follow. Tumour infiltration along nerve

Figure 1.2 The histological pattern of a well differentiated oral squamous cell carcinoma (stained with Haematoxylin and Eosin) (100x).

Figure 1.3 The histological pattern of a moderately differentiated oral squamous cell carcinoma (stained with Haematoxylin and Eosin) (100x).

Figure 1.4 Moderately differentiated oral squamous cell carcinoma showing a mitotic cell in the centre (stained with Haematoxylin and Eosin) (400x).



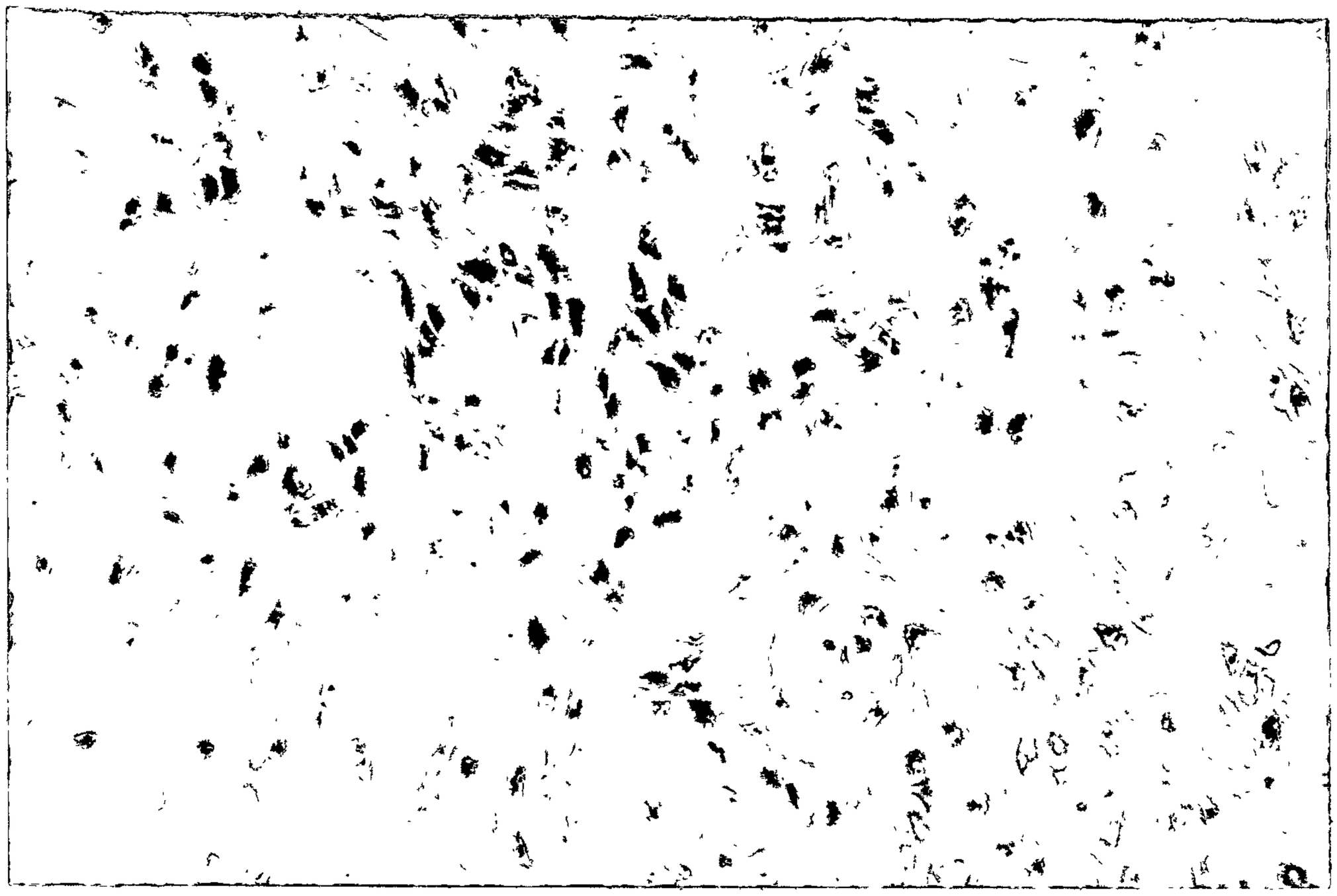


Figure 1.5 The histological pattern of a poorly differentiated oral squamous cell carcinoma (stained with Haematoxylin and Eosin) (200x).

bundles including the inferior alveolar nerve in the mandible and muscle fibres may extend a considerable distance entailing the need for wide excision margins.

1.2.1 Bryne malignancy grading

The cells at the deep invasive margins of oral squamous cell carcinomas and other cancers, often show characteristics other than those of superficial parts of the tumour . For this reason Bryne *et al.* (1992) introduced a malignancy grading system of only the deep invasive margins of oral squamous cell carcinomas, which proved to be of a high prognostic value.The system scores five morphological features: degree of keratinization, nuclear polymorphism, number of mitoses, mode of invasion, and plasma-lymphocytic infiltration ,the latter taken as an indication of host response which was not evaluated in Broder's (1941) classification. Each feature is given a score from one to four and summed to give a malignancy score.

Willen et al, (1975) observed in gingival squamous carcinomas that higher grading features are found in the invading front.This was personally confirmed in the present study.

Most malignant human neoplasms consist of heterogeneous cell populations with probable different biological behaviours (Fidler, 1990). The cells within the superficial parts of the tumour may not be representative therefore and grading of this area of the carcinoma may be unreliable for predicting the clinical behaviour of the tumour (Bryne *et al.*, 1989).An incisional biopsy therefore, may not produce a sample representative of the whole tumour.

Several other studies have also concluded that a better evaluation of invasive and metatstatic behaviour of cancer cells, may be gained within the invasive margins of different tumours (Prime *et al.*, 1985. Kearsely, 1990)

This present research investigated the clinical value of combining invasive cell grading with the presence of P53 in the tumour and at the invasive front of the tumour.

1.3 Clinical staging of disease.

The extent of spread of a tumour before treatment or staging is a key consideration in predicting the outcome.The most widely used system for staging of tumours of the oral cavity has been the TNM system (T, tumour size; N, node involvement; M, presence of distant metastases). It classifies the size of the primary lesion, extent and distribution of metastases to regional lymph nodes and the presence or absence of distant metastases, as proposed by the Union Internationale Contre Le Cancer (UICC) (see table 1.4).

Several pre-therapeutic classifications have been employed differing to some degree. Platz *et al* (1982), analysed various classifications and showed that no one classification produced distinctly separable prognostic categories.Other factors such as the site, inclusion of the extent of infiltration of adjacent structures and histological size,should also be taken into account (Smith , Pindborg , Binnie 1990). Rapidis *et al* (1977) believed that in addition to the more conventional TNM criteria the site and histopathology should be included with the staging (table 1.5) course of the disease and should correlate with expected survival.They concluded that the presence or absence of confirmed lymph node metastases and metastases had the greatest prognostic effect.

Other modifications of the methods used to code each tumour by its TNM category are available.The N status for example may not only depend on clinical examination and radiology but be determined also with the aid of aspiration cytology.Shah *et al*. (1976) showed that patients with a higher T status, a higher N status and thus a higher stage of disease , did poorly in terms of local and regional control of disease.White and Byers (1980) showed that primary lesions less than 4cm in greatest dimension , treated with radiotherapy or surgery can be controlled locally. Extracapsular extension of disease in cervical lymph nodes and involvment of multiple nodes at different levels put patients

into a high risk category (Shah *et al.*,1976) .The importance of the status of the regional lymph nodes, was also confirmed by DiTroia in 1972 and Woolgar *et al.* in 1995.

1.4 Prognosis

The prognosis of OSCC (all stages , all grades) is poor (Boyle & Macfarlane *et al.*, 1990). Despite advances in ablative and reconstructive surgery the mortality remains high, since a substantial proportion of patients with controlled locoregional disease develop systemic metastases (Vikram *et al.*, 1984) or second (metachronous) malignancies (Carr & Langdon 1989).Soame and Southam (1992), also conclude that blood borne metastases may occur late in the clinical course of the disease but many patients die before distant metasases are apparent.

1.4.1 Site

There are documented differences in incidence, tumour behaviour, treatment, outcome and complications based on the site of oral cavity lesions(Teichgraber & Clairmont 1984). Tumours more posterior in the mouth tend to have a worse prognosis possibly due to late diagnosis and rich lymphatic drainage favouring metatstatic spread.

1.4.2 Other factors

Females tend to have a better prognosis than males, and the elderly tend to fare less well with surgery and other treatment (Soame & Southam 1992).

For patients without lymph node metastasis, the survival probabilities at one year, two

years and five years were 95%, 86% and 68% respectively. For patients with metastasis, the corresponding survival probabilities were 71%, 52% and 44% respectively,showing the critical importance of early diagnosis of the primary tumour. The prognostic significance of histologically involved regional lymph nodes, their number and extracapsular spread (Snow *et al.*, 1982,Woolgar *et al.*, 1995) is greater than that of the characteristics of the primary tumour (Hibbert *et al.*, 1983).

Mcguirt *et al.*, (1995) also showed an increased survival of 100% of patients who were disease free after three years, as a result of elective neck dissection supporting a more aggressive treatment of the clinically negative neck.

In regard to the overall diagnosis and management of patients with oral squamous cell carcinomas , Smith and Pindborg (1990) conclude that further methods are needed to predict growth capacity and malignancy of the tumour.

Table 1.4. Clinical staging of malignant neoplasm of the oral cavity based on the TNM system (Smith & Pindborg 1990, Soames and Southam 1992).

Tumour

T1	Greatest diameter of primary tumour 2cm or less
T2	Greatest diameter of primary tumour >2cm but not >4cm
T3	Greatest diameter of primary tumour >4cm
T4	Massive tumour >4cm with gross local invasion

Nodes

N0	No clinically positive nodes
N1	Single ipsilateral node not greater than 3cm diameter
N2	(a) Single ipsilateral node >3cm but not >6cm diameter
	(b) Multiple ipsilateral nodes not >6cm diameter
	(c) Bilateral or contralateral nodes not >6cm diameter

Metastasis

M0	No distant metastases
M1	Distant metastases

Clinical staging

Stage 1	T1	N0	M0
Stage 2	T2	N0	M0
Stage 3	T3	N0	M0
	T1,T2,orT3	N1	M0
Stage 4	T4	N0 or N1	M0 or
	any T	N2 or N3	M0 or
	any T	any N	M1

Table 1.5. The STNMP Classification for Intra-Oral Carcinomata (Rapidis *et al* 1977)

	S (site)		**T (tumour)**		**N (node)**
S1	Lip-skin	**T1**	<20mm diam	**N0**	no palpable nodes
S2	Lip-mucous membrane	**T2**	20-40mm diam	**N1**	equivocal node
S3	Tongue	**T3**	40-60mm diam		enlargement
S4	Cheek		And/or extending beyond the	**N2**	Clinically palpable
S5	Palate		primary region.		homolateral regional node(s)
S6	Floor of mouth		And/or through		not fixed.
			adjacent periosteum.	**N3**	as N2 but fixed
S7	Alveolar process		And/or through the capsule.	**N4**	Clinically palpable
S8	Antrum	**T4**	>60mm in diam		contralateral or bilateral nodes
S9	Central Ca. Of bone		And/or extending to involve		not fixed
			adjacent structures.	**N5**	as N4 but fixed.

	M (metastasis)		**P (pathology)**
M0	No distant metastasis	**P0**	Hyperkeratotic lesion with atypia
M1	Clinical evid. Of distant metastases	**P1**	Carcinoma-in-situ.
	without definite histological and/or	**P2**	Basal cell carcinoma
	radiographic confirmation.	**P3a**	Verrucous Carcinoma
M2	Proven evidence of metastases beyond	**P3b**	Well differentiated SCC
	the regional nodes	**P3c**	Moderately differentiated SCC
		P3d	Poorly differentiated SCC

Arithmetic values given to each factor:

T1	0	N0	0	M0	0	P0	0	S1	4
T2	10	N1	10	M1	30	P1	5	S2	6
T3	20	N2	20	M2	40	P2	5	S3	8
T4	35	N3	30			P3a	5	S4	10
		N4	40			P3b	10	S5	12
		N5	40			P3c	15	S6	14
						P3d	20	S7	16
								S8	18
								S9	20

Stage I 0-30
II 31-50
III 51-70
IV 71-155

1.5 Carcinogenesis

Oral squamous cell carcinoma is a malignancy of oral lining epithelium thought to arise from a stepwise series of changes at the genetic level (Speight and Morgan ,1993).The sequential progression in carcinoma is from normal cell to minimally altered initiated cell, to autonomously proliferating cell, to invading cell to metastasizing cell and further (Locker 1995). Changes in the structure and behaviour of the tissue eventually become evident clinically and microscopically.It is generally accepted that a series of discrete events takes place which eventually combine to form an invasive neoplasm (Speight and Morgan ,1993).Fearon and Vogelstein (1990) investigated the transformation of adenomatous polyps to carcinoma showing chromosomal deletions and oncogene mutations.Field (1992) supports the implication that aetiological factors such as tobacco and alcohol damage cells at the DNA level which involves the oncogenes and tumour suppressor genes.The cells undergoing mutations forewarning uncontrolled growth, may progress to oral cancer.

1.5.1 Precancerous lesions

In 1978, the World Health Organisation (WHO) defined the term precancerous lesion as a morphologically altered tissue in which oral cancer is more likely to occur than in its apparently normal counterpart.The term precancerous condition is defined as a generalised state associated with a significantly increased risk of cancer.In both definitions part of the mucosa is more likely to become malignant,it does not mean all precancerous lesions are committed to malignant transformation.

Table 1.6. Classification of precancerous lesions and conditions(Pindborg , 1980).

Precancerous Lesions	*Precancerous Conditions*
Leukoplakia	Syphilis
Erythroplakia	Sideropenic dysphagia
	Submucous fibrosis
	Lichen planus
	Discoid lupus erythematosus
	Actinic keratosis.

Leukoplakia is a clinical definition for a white patch or plaque that cannot be rubbed off and cannot be characterised clinically or histologically as any other disease (WHO,1978).Axell *et al* (1984) added to this definition the qualification that it is not associated with any physical or chemical causative agent except the use of tobacco.Leukoplakia therefore is established by exclusion.The histology may range from keratosis of the epithelium, to varying degrees of disordered epithelial maturation, proliferation and epithelial dysplasia (Kramer,1980).Pindborg (1980) showed that epithelial dysplasia is present in a range from 15% to 54% of oral leukoplakias.Silverman *et al.* (1976) reported that a proportion of lesions undergo malignant change varying from 0.1% to 10%. Several other studies including Kramer *et al.* (1978), established that a patient with leukoplakia is at a greater risk of developing cancer than a person with clinically normal oral mucosa.

Erythroplakia is defined as a red patch that cannot be characterised clinically or histologically as due to any other condition (WHO,1978).Erythroplakia has a higher malignant potential than leukoplakia (Shafer & Waldron ,1975), and is the most common early clinical sign of invasive carcinoma (Mashberg *et al.*, 1973).Erythroplakia contains

more severely dysplastic epithelium and is more likely to show invasive carcinoma than leukoplakia, which usually contains mild dysplasia or non-dysplastic epithelium (Shafer & Waldron ,1975).

1.5.2 Dysplasia

Dysplasia can be considered to represent a combination of disordered maturation and disturbed cell proliferation.

Table 1.7 Epithelial changes which may be seen in epithelial dysplasia (Speight and Morgan ,1993. WHO 1978).

Disorderly maturation:
irregular hyperplasia and/or atrophy
keratosis/parakeratosis
drop-shaped rete processes
irregular stratification and disturbed cell polarity
low level keratinisation in single or small cell groups
reduced epithelial cell cohesion
cell pleomorphism
Disturbed cell proliferation:
loss of basal cell polarity
basal cell hyperplasia
increased nuclear-cytoplasmic ratio
enlarged nucleoli
nuclear hyperchromatism
high- level mitoses
anisonucleosis
abnormal ('bizarre') mitoses

From a histological examination a subjective estimate is usually made of the degree of dysplastic change in the epithelium.This is usually expressed as mild, moderate, severe dysplasia or carcinoma in situ.In the present study , a standardised assessment avoiding subjective error of the epithelium adjacent to the tumour margin was attempted by using a WHO photographic reference (1978).There is a strong subjective element in the diagnosis of dysplasia, especially in the mild to moderate categories (Kramer,1980). Carcinomas may subsequently develop in lesions showing only a slight degree of dysplasia while lesions with severe dysplasia have persisted with little change for years (WHO 1978).Several studies have documented the fate of dysplastic lesions ; a portion became malignant and increased in severity,some regressed and some showed no change at all (Mincer et al. ,1972) (Gupta et al., 1980).In general terms , it is considered that the degree of dysplasia is linked to the degree of probability of the developement of malignancy.It is well understood that severely dysplastic lesions are least likely to regress, and wherever possible, all lesions with more than slight degrees of dysplasia should be removed (WHO, 1978).The clinical features and the histological evidence of dysplasia can be interpreted in terms of stage of progression to malignancy but reliable indicators for predicting spread or inevitable transformation to cancer is lacking.

1.6 Tumourigenesis of oral squamous cell carcinoma

Rapid development of molecular biology has lead to the recognition that cancers arise as the result of the accumulation of genetic alterations interfering with the normal control of cell growth and differentiation (Syrjanen *et al*, 1993).

Two main types of genes have been implicated in the malignant phenotype: proto-oncogenes and tumour suppressor genes (Langdon. & Partridge , 1992).Other genes include those coding for deoxyribonucleic acid (DNA) repair.

1.6.1 Proto-oncogenes

Proto-oncogenes are present in the normal human genome (Varmus ,1984) and encode growth associated proteins which become activated in response to various mitogenic signals. As a result of somatic mutation, proto-oncogenes become activated as oncogenes to cause dysregulation of growth and differentiation. They therefore enhance the probability of neoplastic transformation (Syrjanen *et al*, 1993).

Seemayer & Cavenee (1989) illustrate the three manners in which oncogenes may be involved in neoplasia :-

1. Juxtaposition of *c-myc* genes next to enhancer segments inducing tumours eg.transgenic mice and the induction of lymphoma.
2. By point mutation giving a net effect of an altered gene product eg. *ras* mutation in human colorectal adenocarcinoma.
3. Conferring of metatstatic potential to a neoplasm eg.protein kinases confer metastatic potential to fibroblastic cell lines.

The role of *ras* and *myc* oncogenes has been investigated in oral squamous cell carcinoma. The incidence of *ras* mutations appears low.The *myc* oncogene however is reported at higher levels of expression in advanced disease , perhaps contributing to altered growth control in late stage oral squamous cell carcinoma (Langdon & Partridge, 1992).Other molecular mechanisms intervene in a small number of human cancers in which viral genes have causal roles eg. Burkitt's lymphoma and the Epstein Barr virus, cervical cancer and human papillomaviruses, to promote disease (Friend *et al*, 1988).Single oncogenes acquired in target cells by mutation or viral infection are however not sufficient to convert cells into a full blown tumour (Land *et al* ,1983), and it is accepted that multiple independant genetic changes act collaboratively to orchestrate the full range of neoplastic traits, explaining multistep carcinogenesis (Friend *et al*, 1988).

1.6.2 Tumour suppressor genes

In contrast to proto-oncogenes , tumour suppressor genes (TSG) are normal cellular genes which when inactivated lead to a disturbance of cell proliferation and development of neoplasms (Syrjanen *et al* ,1993).Tumour suppressor genes encode proteins that have the ability to suppress cell division. Mutations therefore lead to loss of negative regulation of cell growth.Normal cells have receptors detecting growth-inhibiting and differentiation-inducing factors and will continue to respond to mitogenic signals but lose responsiveness to signals that would usually urge the cessation of growth (Friend *et al*., 1988).

Tumour suppressor genes play a more important role than oncogenes in the development of neoplasia (Langdon& Partridge ,1992).One such well characterised tumour supressor gene is the p53 gene whose protein acts in the nucleus of the cell and is involved in the regulation of DNA transcription (Lane & Crawford , 1979).Knudsen (1993) predicts that the loss of tumour suppressor gene function requires two hits, one on each allele of a diploid cell.The probability of two such mutations is low and suppressor gene inactivation should therefore occur only at late stages of progression of sporadic tumours.In hereditary cancer, individuals may already carry one inactivated allele so that a single hit can inactivate the other allele.Cancer predisposing genes may act by affecting the exogenous precarcinogens to be converted to carcinogens that can damage the cellular genome.The effect may interfere with the repair of damaged DNA, allowing genetic predispositions which may alter the immune surveillance system or alter regulation of normal cell growth (Friend *et al* 1988).In hereditary cancers of which approximately 50 are known, inactivation of the normal allele of the suppressor gene is said to be the earliest event in tumour progression.Such hereditary cancers include retinoblastoma and sarcomas,Wilms' tumour, neurofibromatosis,polyposis coli,breast cancer and in the rare Li Fraumeni syndrome,where the p53 tumour supressor gene has been well characterised. p53 gene mutations are also the most common genetic abnormalities found in non familial human cancers (Locker,1995).

1.7 Tumour markers

In addition to the refinement of methods for analysing the pattern of tumour architecture and morphological indicators of tumour cell differentiation, attention is also being turned to modern immunological and genetic methods to establish properties of tumour tissue and prognosis.

The usefulness of single tumour markers in oral and other types of cancers as diagnostic tools and indicators to monitor the progress of the disease or evaluate treatment effectiveness has been well discussed in the literature (Kelly ,1988).The understanding of genetic alterations carries with it potential therapeutic implications.Chromosome alterations at 11q 13 for instance, may involve the expression of many genes, including glutathione-s-transferase (GST).The expression of this gene is reflected in circulating GST blood levels, and its levels may be determined to predict chemotherapeutic responsiveness (Schantz ,1993).

Little is known about the antigenic makeup of human carcinomas, though in several carcinoma types, antigens have been described that are present on tumour cells but are absent from normal tissue eg. Epstein-Barr virus (EBV) are expressed on Burkitt's Lymphoma cells .Patients with this disease often have antibodies against EBV antigens (Klein , 1975). Autologous serological typing has identified antibodies to tumour cell surface antigens in patients with melanoma , leukemia , and renal cell carcinoma (Carey *et al* , 1993).Similarly other studies have performed serum levels of six tumour markers in patients with oral squamous cell carcinomas :-

Carcinoembryonic antigen (CEA)

Squamous cell carcinoma antigen (SCCA)

Immunosuppressive acidic protein (IAP)

Alpha-feto protein (AFP)

Ferritin (FER)

Carbohydrate antigen 19-9 (CA 19-9).

These studies concluded that the three tumour markers CEA, SCCA and IAP were of considerable diagnostic value especially if used in combination assay for screening patients for the early diagnosis of metastases in other organs (Kurokawa *et al* , 1993).Serum antibodies (tumour - specific antigens) however, could not be found in many patients.Attempts at identifying characteristic squamous carcinoma antigens and those unique to tumour cells has also largely been disappointing (Carey *et al*, 1993).

Cell-cycle associated antigens such as the proliferating cell nuclear antigen (PCNA) as markers of cell proliferation have also been evaluated.This marker shows a strong correlation between suprabasal expression and dysplasia or carcinoma in situ (Coltrera *et al* ,1992).Cytokeratin 19 (CK19), a cytoskeletal protein within the suprabasal squamous epithelium, has also been assessed as an indicator of moderate to severe dysplasia and carcinoma (Rheinwald *et al* 1989).Conflicting results have been reported as to the sensitivity or specificity of CK19 as a marker of premalignancy in oral epithelium.Coltrera *et al* (1992) recommended that it should not be used to distinguish hyperplasia from dysplasia.

Table 1.8. Potential markers of diagnostic or prognostic potential in oral cancer (Speight & Morgan ,1993).

Marker	Specific target	Diagnostic/prognostic value in Oral cancer and precancer
Viruses	Human papillomavirus	Unproven
	Herpes group	Unproven
Oncogenes	*Ras* group	Mutations probably non-specific Western and far Eastern differences.
	myc	Level of expression may relate to metatastatic potential?
Tumour suppressor genes	p53	Mutant form the most widely expressed.
Proliferation markers	Ki67,BrdU,AgNORs,PCNA	No simple relationship with behaviour.
Aneuploidy	Flow cytometry	Aneuploidy may be associated with stage of carcinoma.
Intermediate filaments	Simple keratins	Expression in severe dysplasia and associated with loss of differentiation in carcinoma.
Epithelial surface antigens	Blood group antigens	Loss in conjunction with invasion.
Basement membrane components		Deficiencies associated with loss of differentiation in carcinomas.
Cell adhesion molecules	Integrins	Loss of integrins associated with poorly differentiated carcinomas.

At present it is unclear as to which tumour marker will have the greatest diagnostic value for identifying a lesion committed to malignant transformation at the earliest possible stage, or the most prognostic value for the successful management of oral cancer.

1.7.1 P53 as a tumour specific marker

Mutation stabilises p53 protein and extends its half-life in tissues from 6 - 20mins for the wild - type protein to up to 6hours . The detection of p53 by immunohistochemistry (IHC) has been assumed therefore to denote a mutation (Lane & Benchimol 1990). Missense mutations often increase the half-life and quantity of the p53 protein, allowing its recognition by IHC (Lane ,1994).Mutations which result in deletion or truncation of the protein (nonsense and frameshift) do not cause protein accumulation (Hall & Lane 1994).IHC will be inefficient in detecting tumours with high proportions of such mutations.Tumours with deletions with both p53 alleles will be negative by IHC (Sasano *et al.*,1992).Wild-type p53 may also bind to a papilloma virus which may degrade it or form a complex with the mutant protein (Scheffner et al., 1990).

Hall *et al* (1991) using diagnostic cytopathological aspirates from neoplastic and non-neoplastic lesions, concluded that the presence of p53 immunoreactivity can be used to infer that a cell is neoplastic since p53 immunoreactivity was not identified in any patient in whom there was no morphological evidence of neoplasia. This was also confirmed by Ogden & Cowpe *et al.*, (1994) in oral smears.They noted a limitation however, in that only 40% of oral carcinomas showed positivity to p53 staining, compared with 94% in the study conducted by Hall *et al.*, (1992).Thus p53 mutations may be insufficient or unecessary for the development of OSCC. Secondly ,additional aids are required in the cytological or histological diagnosis of p53 protein negative tumours.Studies involving the diagnosis of oral cancer using conventional exfoliative cytology alone had false negative rates approaching 60% (Dabelsteen *et al.*, 1971).Therefore, combined with morphology, p53 immunostaining of protein can be of assistance in the detection of neoplastic cells.

p53 immunostaining has been regarded as an inexpensive method used to detect p53 mutations, compared with polymerase chain reaction (PCR) amplification and sequence specific oligonucleotide hybridisation for the detection of specific p53 mutations (Sidransky *et al.*, 1991).Immunohistological demonstration of p53 is also easily controlled and can be applied in routine pathology laboratories, nearly all of which use immunohistological methods in diagnosis (Hall *et al.*, 1991)(Coltrera 1992).It seems that screening of tumours using molecular biology techniques such as polymerase chain reaction , followed by single strand conformational polymorphism and DNA sequencing remains essential to detect all these varieties of p53 mutation.

1.8 p53 , the tumour suppressor gene

1.8.1 The discovery of p53

The p53 nuclear phosphoprotein was originally discovered in extracts of transformed cells reacting with antiserum from animals with tumours induced by Simian virus 40 (SV 40)(Lane *et al* 1979).The protein formed an oligomeric complex in transformed cells with the large T antigen which has a potent capacity to transform cells. Large quantities of p53 were detected in a variety of tumour derived and transformed cells in culture.The protein was found to have a much longer half life than in non-transformed cells (Reich *et al.*, 1983).

p53 clones were then isolated that could immortalise cells in culture and cooperate with *ras* oncogene to transform rat embryo fibroblasts in culture, so the gene p53 was classified as an oncogene (Eliyahu *et al.*, 1984). It was only until these p53 DNA clones were found to be mutant forms of p53 , that further studies discovered its tumour supressor gene activity, negatively regulating the cell cycle , requiring loss of function mutations for tumour formation (Levine 1992).The study by Donehower *et al.* (1992) highlighted the role of p53 in tumour formation using chimaeric mice. Although

developing normally , homozygotic mice null for the p53 gene are susceptible to the spontaneous development of tumours.They concluded that the loss of a normal p53 allele is sufficient to predispose animals to all types of tumours but that the gene was not required for normal growth and development.

1.8.2 Wild type p53 (wt p53)

Human tumour cells transfected with a wild type p53 gene grow at reduced rates in culture (Baker *et al.*, 1990) and have decreased tumourigenicity , indicating a suppression of the tumour phenotype (Chen , 1990).Finlay *et al* (1989) gave evidence that p53 can act as a TSG by supressing tumour formation indúced by several oncogenes in a murine model system. Although p53 mutation together with another activated oncogene ras, can transform cells (Hinds *et al* , 1989, Jenkins *et al* , 1985) , the wild type p53 actively inhibits transformation (Martinez *et al* 1991).

1.8.3 P53 gene and gene product

The p53 gene encompasses 16-20 kilobases of DNA on the short arm of human chromosome 17 at position 17p 13.1 (Miller *et al.*, 1986). It is composed of eleven exons and the p53 gene product has five highly conserved regions amoung amino acid residues in a cross- species comparison (Soussi *et al.* ,1990).

The p53 gene product is a 393 amino acid nuclear phosphoprotein about 53 kilodaltons in molecular weight. Lane & Crawford (1979) identified the p53 protein because it formed a tight complex with the SV 40 large T antigen , and was co-immunoprecipitated with anti -T antibodies from extracts of SV 40-transformed cells.

The p53 protein was found in very low quantities in normal cells ,almost undetectable by immunohistological techniques, because of its short half life of 6-20 minutes (Levine *et al* ,1991) , and its site of action in the nucleus is thought to regulate the replication of DNA (Langdon & Partridge ,1992). Larger quantities of p53 (5 -100 fold) could however, be detected in transformed cells in culture and in human tumours (Syrjanen *et al* .,1993).Mutation somehow allows stabilisation of the protein and extends the half - life up to 6 hours (Lane & Benchmmol 1990)(Levine *et al* 1991), thus the ability to detect mutant p53 in tumours was suggested as probably being synonymous with the presence of a mutation.

1.8.4 P53 and human cancer

Mutations and allele loss of the p53 gene are associated with tumours from a wide variety of human organs and tissues including :- lung, breast ,colon , oesophagus , liver, bladder , ovary , brain and haematopoietic (Hollstein *et al* 1991) , ie. all major histogenic groups :- epithelial , mesenchymal , haematopoietic and lymphoid tumours, and those of the central nervous system (Chang *et al* 1993).Mutations in the p53 gene are now the

most frequently observed genetic lesions in spontaneous human cancers. More than half of human malignancies tested so far have been shown to overexpress the p53 protein (Bartek *et al* 1991). In addition , p53 mutations are associated with the inherited cancer susceptibility Li-Fraumeni syndrome (Malkin *et al.*, 1990).Affected individuals often inherit a point mutation in highly conserved regions of the p53 gene, primarily in exon 7. It is presumed that these germline mutations predispose affected individuals to cancer development, particularly after the normal p53 allele is somatically lost or mutated.

The incidences of p53 mutations vary greatly between tumour types ,geographical distribution as well as from author to author. The reason for these variations remain unclear (Syrjanen 1993).Burns *et al.* (1993) have sequenced the p53 coding region of 14 squamous cell carcinoma lines (SCC), including 8 of 9 cell lines expressing high levels of p53 protein. In all cases where elevated levels of p53 protein were detected, mis-sense mutations , or inframe deletions within the coding region were found, thus confirming that elevated levels of p53 protein is a good indicator of p53 mutation in cancers of the head and neck region.

1.8.5 Molecular analysis of p53 mutation

The mutation of p53 has been implicated in inherited and sporadic forms of malignancies in humans. 75% to 80% of colon cancers show a loss of both p53 alleles. Few colon cancers show the deletion of one allele of the p53 gene or a point mutation. The point mutations are usually missense, giving rise to an altered protein (Vogelstein 1990 , Nigro *et al.*,1989).The mutations at the normal p53 locus are recessive to the wild type p53 allele, contributing only to tumourigenesis when the wild-type allele is inactivated through errors in mitosis or recombination leading to loss of normal chromosome or via mutations.This supports the wt-p53 gene as being a tumour supressor gene (Levine 1992).Point mutations occupy numerous positions in the human p53 gene in the tumours examined. (Hollstein et al 1991b).Most are missense mutations giving rise to an altered protein and are not randomly scattered , but are clustered between amino acid

residues 130 and 290 (out of 393 residues).Most of these mutations are localised in four regions of the protein , residues 117-142 , 171-181 , 234-258 and 270-286 , which are highly conserved among several different species.There are mutational 'hot spots' affecting residues 175 , 248 and 273 in colon and lung tumours.The frequency and distribution of these 'hot spots' differ among cancers from different tissue types.In gastric cancer, mutation of the 245th codon of p53 involved replacement of glycine (GGC) by serine (AGC) and constituted the mutational hot spot. In particular, 3 of 4 mutations at codon 245 occured in early gastric cancer (Uchino *et al.*, 1993). Hollstein *et al* (1991) state that 98% of p53 gene mutations in different cancers have been found in exon 5-8. It is unknown why differences exist .It may be due to different mutagens , different cell environments , or different selectivities for promoting cell growth.It is clear however, that different alleles have different properties (Levine 1992). Mutations in different locations of p53 may lead to distinct biological effects.The allele mutant for residue 175 is 3-10 fold more efficient than the mutant for residue 273 in co-operating with *ras* to transform primary rat cells in culture (Levine *et al.*, 1991). Fearon and Vogelstein (1990) have postulated a genetic model in humans depicting the interaction of oncogenes and onco-supressor genes in colon carcinogenesis incorporating p53 mutations and deletions as occurring around the transition from adenoma to carcinoma.These findings also imply that the mutations induce a global conformational switch in the p53 protein (Oren 1992).The conformation of p53 with mutations between amino acids 135 and 175 is different from that of the wild type protein as indicated by its reaction with monoclonal antibodies (Yewdell *et al.*, 1986) and by binding of heat shock protein hsc 70 (Hinds 1990). Some mutants are able to transactivate a test gene whereas others cannot (Fields *et al.*, 1990).Thus different mutant alleles of the p53 gene have different biological and biochemical properties.This may perhaps mean that cancer patients with different p53 mutant alleles might have different prognoses.

1.8.6 Loss of p53 function and the tumourigenic process.

The suggestion that the loss of p53 function triggers the tumourigenic process is mainly supported by studies with Li-Fraumeni patients (Malkin *et al.*, 1990) and with mice (Donehower 1992).Li-Fraumeni syndrome (LFS) is a familial disposition to cancer , characterised by multiple early onset malignancies.Members of many LFS families inherit germline mutations in one allele of the p53 gene.In the tumours ,expression of the remaining wild type p53 allele is completely lost (loss of heterozygocity) resulting in sufficient loss of p53 function to initiate tumourigenesis.Therefore the presence of one mutant p53 allele is sufficient to initiate a neoplastic process. Mutation of the p53 gene can occur as a result of DNA damaging agents such as UV light (Maltzman & Czyzyk 1982) and has been found in sun related SCC's in both humans and animals, as well as in normal skin exposed to recreational doses of solar mimetic UV light (Hall *et al.*, 1993).

1.8.7 Stability of mutant p53 protein and overexpression in cells

Nuclear accumulation of p53 protein in different tumours has been shown to usually reflect p53 gene mutations, but other mechanisms could also be responsible. These include the formation of stable molecular complexes of p53 with a variety of viral proteins as well as with peculiar host proteins, such as heat shock protein (hsp 70) or MDM2 proteins (Scarpa *et al.*,1993).

1.8.8 Viral influences and p53

Some viruses such as the adenovirus E1b and SV40 large T antigen through oncogenic complexing may lead to p53 overexpression (Lane & Crawford 1979). HPV E6 also forms complexes with wild type p53 protein (Werness ,Levine *et al.*, 1990) , and HPV types 16 and 18 (involved in 90% of cervical carcinoma and possibly oral carcinoma)

can bind to p53 (Scully 1992).The viral oncogene products were soon shown to actually inactivate p53 when E6 protein was able to bind to and promote the proteolytic breakdown of p53 via the ubiquitin proteases system, hence targeting the degradation of the p53 protein (Scheffner *et al.*, 1990) and unlikely to increase p53 expression. This feature highlights the fact that p53 genes via binding of their products, are targets for oncoproteins of DNA tumour viruses, perhaps resulting in mutation.

p53 mutations have been documented in HPV negative cervical carcinoma cell lines and SCC's .Wild type p53 has been found in HPV positive cervical cell lines and SCC's (Crook *et al.*,1991).This data has lead to the hypothesis that wt-p53 cell suppressor function is annulled in human cervical carcinomas either by mutations in the HPV negative cases , or as a consequence of their complexing with HPV E6 in the HPV positive cases.Crook *et al* (1991) suggest that p53 appears to play a role in the regulation of entry into S-phase of the cell cycle and that interference with its function by complexing with E6 and subsequent degradation , may lead to dysregulation of the cell cycle , which may in turn be associated with chromosome instability and aneuploidy, a regularly observed consequence of high-risk HPV infections.

1.8.9 Heat shock protein and p53

Heat shock proteins (hsc 70) lead to an increase in p53 by complexing , and extending the half life of p53 protein in the cell. Such increases could result from changes in phosphorylation , binding to other cellular proteins , or oligomerisation of p53 , all of which have been reported to occur.The synthesis of another gene product involved in the induction or stabilisation of p53 protein could also be important (Finlay *et al.*, 1988).

1.9 The regulation of p53

Study of the regulation of p53 function has shown that the protein is controlled by at least three general mechanisms :-

1. Post-translational regulation of the p53 protein half life (Maltzman & Czyzyk 1984).

2. By binding of specific viral and host proteins (Lane & Crawford 1979) (Scheffner *et al.,* 1990).

3. By a form of allosteric regulation brought about by post-translational modification (Hupp *et al.*, 1992).

1.9.1 Conformational regulation of p53

The production of a conformation-specific antibody Pab246, for wild-type p53 , provided a tool for the comparison of the tertiary or quaternary structure adopted by wild-type in comparison with mutant p53 protein. Many tumour derived mutants of p53 exhibit a loss of reactivity with Pab 246, but react with other conformation specific antibodies.This implies that mutations do occur at a considerable distance from the epitope of the Pab246 and induces a global conformational switch in the p53 protein. This alteration is correlated with the loss of ability to exert antiproliferative effects. Milner (1991) suggests that wild type p53 may not always be locked in the wt-p53 conformation, because an hour after stimulation of mouse fibroblasts , the majority of normal wt-p53 can be found in mutant conformation.Also, the preponderance of mutant p53 suggests that the protein may possess an inherent tendency to switch between wild and mutant conformations (Milner & Medcalf , 1991), hence the conformational hypothesis for the regulation of wt-p53 activity.This assumes that the switching from wt-p53 to an alternative conformation occurs in response to signals such as growth factors,

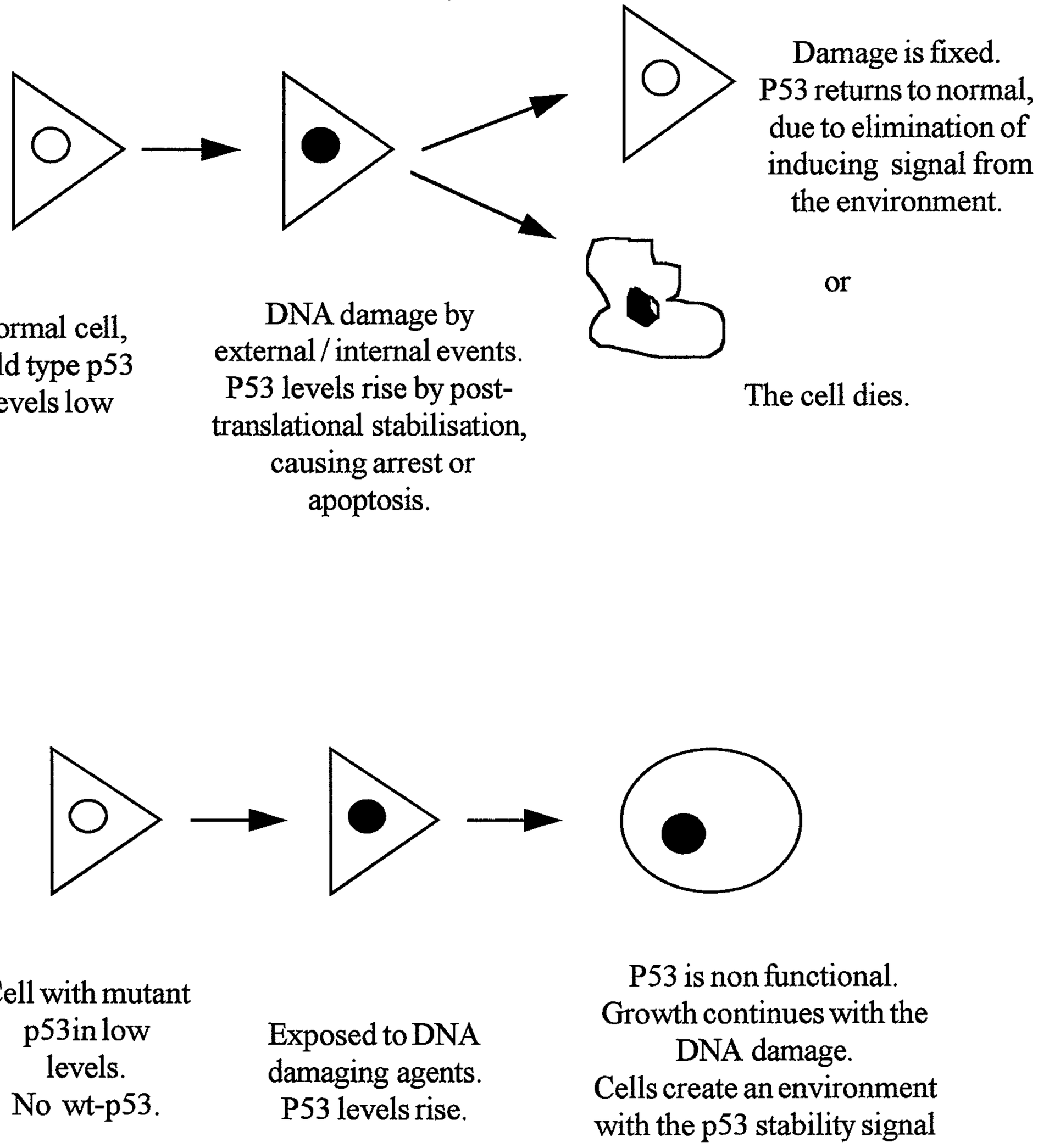
inducing p53 to the mutant conformation to inactivate the antiproliferative capacity of wt-p53 and allowing cell proliferation to proceed and growth restrictive conditions, where p53 maintains its wild type conformation to block cell cycle progression.

1.9.2 Environmental model of the stability of p53

Although another primary antibody ,Pab 240, recognises an epitope localised to the central region of the p53 protein which is hidden by the folded structure of the normal protein and exposed at the surface of mutant protein appearing to make it more stable in cells and accounting for its accumulation (Lane, 1994), the critical factor allowing p53 accumulation is the environment of the cell.The tumour cell environment is created when normal cells are exposed to DNA damaging agents and that mutant p53 is not always stable and wt-p53 not always unstable.The main evidence in support of this is derived from the study of fibroblasts from LFS individuals.In these people the otherwise normal heterozygotic cells encode both wild type p53 and mutant p53 protein, and the mutant proteins do not accumulate to high levels and are unstable like wild type p53.One particular family had a strong dominant transforming mutant p53 and the tumours from these individuals showed intense p53 staining and loss of the wild type allele, implying that some other event aside from mutation is needed for stability.Lane (1994) postulates that this event relates to what normally triggers stability of wt-p53 in cells exposed to DNA damage. p53 is stable in tumour cells because the cell is in a permanent state of damage. Normally wt-p53 is stabilised and accumulates to cause cell growth arrest or to correct the damage causing the induction or programs the cell to die.Stimulated fibroblasts show localisation of p53 to the cell outside the nucleus and entering it only at S-phase.It may well be that while the cell proliferates , p53 is actively excluded from the nucleus during the rest of the cell cycle.This could be achieved by the interaction of other proteins, or an affinity for a cytoplasmic anchor (Gannon *et al* 1991) , and may be mediated by a conformational switch from suppressor to promoter mode. Cells therefore with stable wt-p53 do not accumulate , but the cells expressing only mutant p53 can not

overcome the damage causing the induction of stability in p53, and continue to divide, containing high levels of stable mutant (see figure 1.6).The event triggering stability of p53 is therefore of key interest.

Figure 1.6 **<u>Hypothesis for the stability of mutant p53 in tumour cells.</u>** (Lane, 1994)



1.9.3 The dominant - negative model of mutant p53

According to the dominant negative hypothesis, the action of mutant p53 , although devoid of any biochemical activity , will interfere with the function of co -expressed wt-p53 and render it practically ineffective.The net effect of mutant p53 will be a reduction in cellular wt-p53 activity. Experimental systems using cultured cells are transformed when transfected with mutant p53 with no loss of endogenous wt-p53 alleles (Martinez *et al.*, 1991). Fagin *et al.* (1993) studied the high prevalence of mutations of the p53 gene in poorly differentiated human thyroid carcinomas.They found that neoplasms showing loss of one p53 allele and mutation of the other are common and can be considered to have complete impairment in p53 function. A number of tumours were also identified as having a mutant allele co-expressed with wt-p53 , consistent with a dominant negative effect of the defective protein.They found that the p53 mutations almost exclusively occurred in poorly differentiated thyroid tumours and cancer cell lines suggesting that p53 inactivation may confer aggressiveness on these tumours and loss of differentiated function.

The ways in which a dominant negative effect may be exerted , includes either a direct interaction between the mutant and wild type molecules and a competition for targets.The possibility of direct interaction is reflected in mutant p53 forming tight complexes with the endogenous wild type p53 in transformed and immortalised cells (Levine *et al.*, 1991). In a cell free system , wt-p53 polypeptide is found to adopt the aberrant conformation of the mutant partner (Milner *et al.*, 1991).If the final association product is a dimer , an equimolar concentration of wt-p53 and mutant p53 would decrease total wt-p53 activity by 75%. If p53 actually assembles into tetramers the residual wt-p53 activity is even lower.The dominant negative model implies that the mutation of one allele of p53 along with the other allele giving rise to wt-p53 protein, will confer a strong selective growth advantage on cells and that partial deficiency in p53 function conferred by mutation in one allele predisposes the cell towards genomic instability.

In living cells , a 1:1 ratio of mutant and wild type p53 is insufficient for the abrogation of total wt-p53 activity, of which a minor fraction remains active (Johnson *et al.*, 1991). Indeed in transfection experiments , the transforming effects of mutant p53 are only elicited when the mutant protein is in vast excess.This is still the subject of debate but it appears that the mutant allele may contribute to tumour progression and even promote neoplastic change independent of its interaction with endogenous wt-p53 (Oren, 1992), since when mutant p53 cDNA was transfected into cells not expressing p53, the mutant protein was expressed and the ability of the cells to produce tumours in animals enhanced (Wolf *et al.*, 1984).There was no endogenous wild type function to eliminate and mutant p53 must have acted to stimulate cell growth and tumour formation.There is evidence for both dominant loss of function mutations in transformed cells and gain of function mutations in tumourigenesis assays.Overexpression of wild type p53 protein in a cell with mutant p53 protein, suppresses transformation, cell growth, and tumourigenic potential.The ratio of mutant to wild type could be critical in regulating cell division (Finlay *et al.*, 1989 , Mercer *et al* 1990, Chen *et al.*, 1990).

1.9.4 Regulation of protein of p53

MDM2

The MDM2 gene was originally identified as a dominant transforming oncogene present on a 'mouse double minute' chromosome.Oliner *et al.* (1992) described the human homologue of MDM2 and mapped it to the long arm of human chromosome 12, and show that it can also bind and regulate p53.Initial results have suggested that in those tumours where MDM2 is amplified ,no mutations in p53 are found, so that high levels of MDM2 may , like the DNA tumour virus oncoprotein, Simian virus 40 large T antigen, adenovirus E1b and papilloma virus E6, inactivate the tumour suppressor activity of p53 by complexing to it. Amplification of MDM2 production therefore may have the same functional effect as mutation of the p53 gene.It also seems likely that other protein partners exist since the different viral oncoproteins and MDM2 bind to p53 in

different ways, and MDM2 binds also to mutant p53.

1.9.5 Allosteric regulation of p53

Wild type p53 protein is potentially dangerous to the host cell since it can trigger apoptosis and growth arrest. It is not surprising that p53 activity is under tight control. p53 is regulated by modification of its stability and binding to other proteins as well as tight allosteric regulation.The p53 protein can bind in a sequence-specific manner to DNA and through a highly charged N-terminal domain, can act as a specific transcription factor controlling the expression of growth arrest genes such as GAADD-45 (Kastan *et al.*, 1992) and WAF -1 (El -Deiry *et al.*,1993).Activators can bind to the p53 protein molecule and work by inducing some subtle conformational change in the protein. So far, studies have shown that activators work by neutralising an inhibitory function of the C-terminus (Hupp *et al.*, 1993). It may therefore be possible to rescue the wild type p53 activity of some mutant p53 proteins with small allosteric affectors targeting the C terminus.

1.10 Epithelial kinetics

Oral epithelium is classified as being a 'steady state renewal' type tissue.This means that there is a steady appreciable level of cell loss counterbalanced by a steady level of cell replacement. The epithelium lining the oral cavity, comprises different categories of keratinocytes.Some are differentiated cells performing the function of the tissue which eventually become senescent , die and are shed.There are potentially proliferative cells which are in a dormant quiescent state (Go) , and there are proliferating cells progressing in their cell cycles.

Stem cells in adult tissue are cells with extensive self maintenance capacity.They are

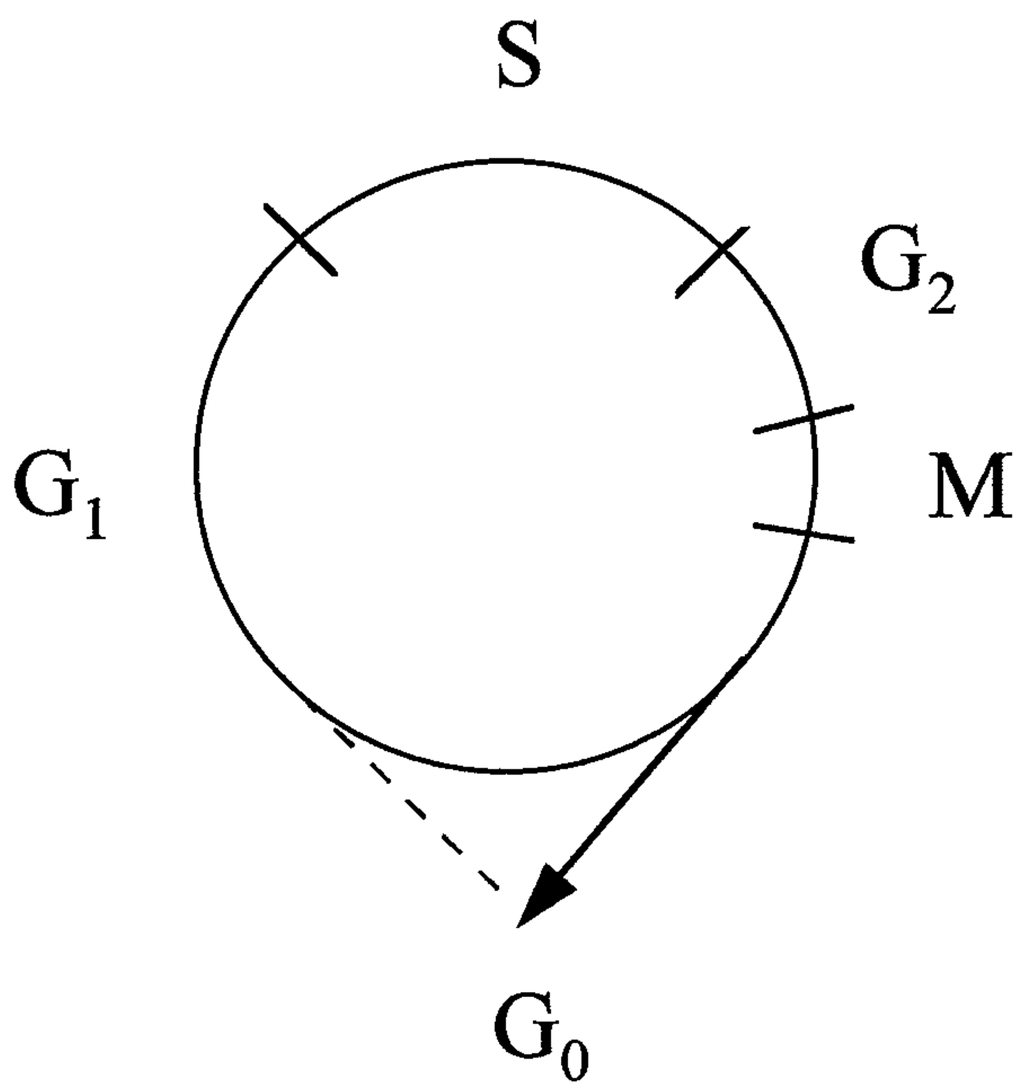
ultimately responsible for all cell replacement in the tissue for the life span of the individual.There are indications that the cell cycle duration of stem cells is slower than that of other proliferative cells, or that they spend appreciable periods of time in Go.Stem cells provide an output of cells which feed into other cell compartments.It is not clear how stem cell numbers are maintained , but for 'steady state renewal' tissue there may be a few stem cells feeding directly into a simple transit population or a dividing transit population which itself feeds a simple transit compartment.For most surface epithelia, it is now becoming evident that wherever large numbers of cells are to be continually replaced in order to maintain the thickness of the epithelial layer,the dividing transit population feeding a simple transit compartment is the hierachical scheme applicable.Transit cells derived from a few stem cells, undergo amplifying cell divisions before maturing to a point beyond which divisions no longer occur and are eventually shed.In epithelium, the stem cell population is located in the basal layer of cells .Evidence suggests that only a small fraction (10%) of the basal cells function as stem cells of clonogenic cells (Potten ,1992). Studies on the rete ridges of rat gingival epithelium suggest that stem cells lie at the base of the rete ridges , which inevitably means that there is some movement of cells along the basal lamina towards the top of the dermal peg. Lauker & Sun (1983) found that the cells at the deep epidermal positions are morphologically less differentiated than those at the top of the dermal peg.

Oral mucosa consists of a stratified squamous epithelium.The progenitor or stem cells lie in the basal and parabasal positions in the epithelium.The amplifying transient group of cells lie above the basal layer where greater cell division takes place.Above this layer lies the maturing cells of the spinous and granular layers, where more terminal differentiation is occuring.In non-keratinised epithelium the granular cell layer is absent and the superficial cells are flattened with elongated nuclei and the superficial keratinised regions of orthokeratinised epithelium consist of cornified squames.

1.10.1 The cell cycle

The cell cycle consists of four main phases designated G_1 , S , G_2 and M.The S phase denotes the onset and completion of DNA synthesis and doubling of genetic material. The M phase denotes the onset and completion of mitosis.The periods between the two phases are G_1 and G_2.The G_1 phases separates S phase from the preceding mitosis and G_2 follows the S phases where there is inactivity prior to the next mitosis (see figure 1.7).The original observations made by Howard and Pelc in 1953 led to the concept of the cell cycle.It was later revised by Patt and Quastler in 1963 to include the Go component, where proliferative genes are normally repressed but may become activated for the cells to rejoin the cell cycle. Later studies demonstrated at a molecular level , cell proliferative control by way of regulatory genes (Dunphy and Newport 1988).In epithelium, dividing cells at each division, progress closer to their terminal division and differentiation.There is variability in the progress made by cells through the cell cycle and eventually progression may cease altogether and the cells enter the quiescent Go phase (Potten 1992).

Figure 1.7 The Cell Cycle (Voorhees *et al.*, 1976)



1.10.2 How p53 works in the regulation of cell growth

In trying to understand why wt-p53 expression is so often abrogated during tumour development , the cellular actions of the protein need to be defined.

The normal physiological role of p53 seems to be in regulation of the cell cycle.The p53 protein is phosphorylated in a cell cycle dependent pattern by cdc 2 kinase (Sturzbecher *et al.*, 1990), a regulatory kinase required for mitosis in mammalian cells, with maximal levels of p53 phosphorylation reached during mitosis (Bischoff *et al.*, 1990).In the cell, p53 is spatially regulated , accumulating in the cytoplasm during G_1 and migrating to the nucleus at the beginning of the S-phase.If the damage were not repaired before initiation of S-phase , the use of a damaged DNA template during replication could propagate mutagenic lesions that might contribute to cellular transformation (Kastan *et al.*, 1991), and may cause the cell to be prone to neoplastic development. p53 protein levels increase after DNA damage in different tissue types with wt-p53 genes and following different DNA damaging agents.Transformed cells when transfected with wild type p53 gene, arrest in the G_1 phase of the cell cycle (Diller *et al.*, 1990). Mercer *et al* (1990) also demonstrated the role of p53 protein in G_1 arrest by demonstrating the effect of wild type p53 genes transfected into various tumour cell lines inducing G_1 arrest. This arrest of replicative DNA synthesis after DNA damage is to provide ample time for the cell to repair DNA lesions before S-phase and/or mitosis.Hence the p53 gene is thought to function as an inhibitor of cellular replication.Kastan *et al* (1991) confirmed the inhibition of the DNA synthesis as an active physiological response to DNA damage, and that wild type p53 protein participates in G_1 arrest following DNA damage.It also appears that either loss of expression of wild type p53 or overexpression of mutant p53 can result in an abnormal cell cycle in response to radiation exposure.After the exposure of cells to ultra violet radiation (UV) , non lethal doses of gamma irradiation , and actinomycin D , the cells with wt-p53 genes rapidly increased in levels of p53 protein and remained in G1 arrest , but the cells containing mutant p53 did not (Lu & Lane 1993).Thus the cell cycle regulation of p53 protein may contribute in maintaining genetic

stability in the circumstance of a DNA damaging event.

It is possible that mutations in the p53 gene may be required in the development of UV-induced SCC since p53 mutations have been found in sun related SCC in humans and animals (Kanjilal *et al.*, 1993). These mutations appeared to be an early event. Human actinic keratosis is potentially a precursor lesion of SCC.

1.10.3 P53 and apoptosis

The p53 gene has also been shown to play an important role in inducing apoptosis , a mechanism to prevent the replication of damaged DNA. An increase in the wt-p53 levels are associated with an increase in transcription of p53 response genes with the induction not only of growth arrest but also apoptosis (Lane , 1994).In a murine p53 negative myeloid leukemia cell line , activation by wild type p53 protein leads to rapid cell death with the distinct features of apoptosis (Yonish-Rouch *et al.*, 1991).Lowe *et al* (1993) and Clarke *et al* (1993) demonstrated the same results on cells with the wt-p53 gene. Studies on the p53 null allele in thymocytes illustrated extraordinary resistance to the induction of cell death following irradiation , when compared to normal thymocytes (Clarke *et al.*, 1993).They also showed that p53 is a required response of the cell to DNA damage but not for the induction of apoptosis by other pathways, since the same thymocytes retained susceptibility to apoptosis when stimulated by glucocorticoids and calcium - ionophore.

Cell deletion by apoptosis is a well documented developmental process.Its role in embryogenesis and in fully developed mamalian tissues is vital (McGee *et al.*, 1992). Donehower *et al* (1993) however demonstrated that wt-p53 did not play an important role in the normal apoptotic process.They showed that null mice who were lacking the p53 gene, were eventually able to develop normally. In fact it was only after a period of time , that such mice seemed to be prone to develop spontaneous tumours and to be

susceptible to exogenous carcinogens.They therefore concluded that although p53 induced apoptosis is not a pathway required in development , it is possible that disturbances in the normal pathway promote the proliferation of tumours.

If the p53 pathway in a cell is inactivated by mutation, allelic loss, binding with other proteins, or by other mechanisms is damaged ,the cell will survive and divide with no p53 checkpoint.The cells are able to form clones of genetically damaged cells and are susceptible to further damage through which neoplastic clones may emerge.Studies by Merrit *et al* (1994) further elucidate the role of p53 induced apoptosis by radiation exposure of gut epithelial cells in the crypts of large and small bowel.They noted the appearance of apoptotic cells preceded by increases in the levels of p53 protein in the cells.They also noted that variable cells in the region showed variable sensitivities to the radiation and sites of p53 protein accumulation. The stem cells in the small bowel readily showed p53 protein and apoptosis. Compared to the large bowel , the response was less precise,possibly explaining the increase in sensitivity to carcinogens in this region of the bowel.It appears then that the loss of p53 function may allow cells to survive illegitimately , perhaps providing the cells with a strong selective advantage and facilitate the establishment of a neoplastic cell population.

Although the mechanism by which p53 induces apoptosis is unclear , the possibility that other tumour suppressors being involved in the control of cell death and survival now appears more likely. p53 mediated apoptosis is inhibited by interleukin -6 (IL-6)and it has been suggested that IL-6 may be necessary for survival of cells (Oren , 1992). Lu and Lane (1993) also postulate that other gene products may be essential for the p53 response to DNA damage and may lie at the heart of p53 tumour suppressor gene activity.

1.10.4 P53 and cell differentiation

That wt-p53 could induce cell differentiation, was suggested by the partial differentiation of normal pre-B cells in culture and their further differentiation in vivo when injected into mice, whereas mutant p53 cells did not differentiate.In normal haematopoietic cell maturation , the levels of p53 were seen to increase.This implies that at least in certain cell types ,the loss of p53 contributes to tumour progression by arresting the cells in a more immature and continuously self renewing state (Shaulksky *et al.*, 1991).This proposition may also be supported by the presence of p53 mutations almost exclusively in poorly differentiated thyroid tumours and thyroid cancer cell lines suggesting that inactivation of p53 may confer these neoplasms with aggressive properties , and further loss of differentiated function (Fagin *et al.*, 1993).

1.10.5 Biochemical functions of p53

The biochemical basis for the tumour supressor activities of wt p53 has still not been established unequivocally.Studies do indicate that wt p53 has to be present in the cell nucleus in order to exert its antiproliferative functions (Martinez *et al.*, 1991).Investigations have been done using DNA virus model systems. It has been proposed that p53 possibly binds to specific sequence elements that control the initiation of cellular DNA replication and directly represses initiation (Kastan *et al.*, 1991).Another possible action of wild type p53 is as a transcriptional regulator.The p53 protein possesses a potent transactivation domain that can function very efficiently when fused to a heterologous DNA binding domain.This property is abolished by many p53 mutations associated with neoplastic processes (Fields *et al.*, 1990). The ability of a protein to act as a promoter-specific transcriptional activator usually requires the selective binding of the protein to defined DNA elements. Indeed wild type p53 was found capable of sequence - specific binding to DNA (Kern *et al.*, 1991).Tumour derived p53 mutants failed to do so.In addition to its ability to positively regulate specific promoters , p53 can also act as a transcriptional repressor.Wild type p53 has been shown

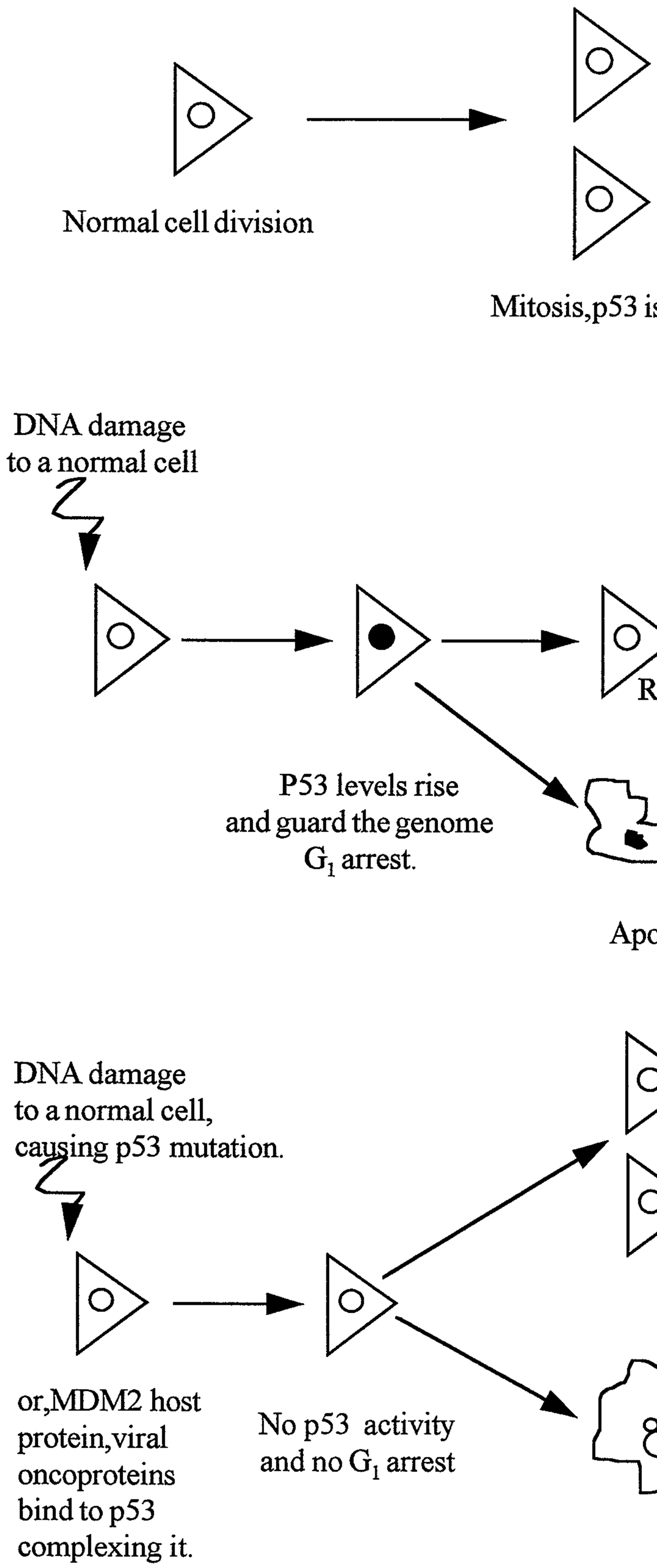
to down-regulate proliferating cell nuclear antigen (PCNA) , mRNA and to interfere with the induction of *c-fos* mRNA during serum stimulation (Mercer , 1991) (Ginsbeg. *et al.*, 1991). Wt-p53 protein selectively down regulates ,by repressing transcription from various promoter regions in DNA synthesis. One specific gene proven to be down modulated is a factor for DNA polymerisation thus demonstrating that p53 protein is actively involved in supression of S - phase activity. Transcriptional repression by p53 could represent a direct inhibitory effect of the protein on the transcription machinery rather than merely a secondary consequence of p53 -mediated growth arrest.Attempts to characterise the associate proteins of p53 with which it interacts may hopefully prove informative.

1.11 Model for p53 function

Lane D.P. (1992) found that normal p53 acts as a ' molecular policeman ' monitoring the integrity of the genome. If DNA is damaged , p53 accumulates and switches off replication to allow extra time for its repair.If the repair fails , p53 may trigger cell suicide by apoptosis (Yonish *et al.*, 1991).Tumour cells in which p53 is inactivated by mutation , or by binding to host or viral proteins , cannot carry out this arrest.They are therefore genetically less stable and will accumulate mutations and chromosomal rearrangements at an increased rate , leading to rapid selection of malignant clones.

The model of p53 function represented in figure1.8, is consistent with the p53 null mice who develop normally but have a high incidence of tumours (Donehwer *et al.*, 1992), with those who have LFS (genetic instability) , and with the success of radiation and chemotherapy treatments where cells with no normal p53 function are more susceptible to the killing ellects of DNA damaging agents, and similarly at lower doses are more susceptible to the mutagenic effects of these agents. Although cells can survive without p53 it plays a central role in the control of the cell cycle progression and perhaps differentiation and in the midst of DNA damaging agents , programmed cell death. p53 function is both biochemical (as a specific transcription factor) and biological (as a G_1 checkpoint control for DNA damage).Investigations on how the pathways interact and with further exposure of unknown oncogenes research may eventually gain aids for the future management and treatment of cancer.

Figure 1.8 **Model for the function of p53.** (Lane, 1992)



1.11.1 Possible future therapeutic manipulation of p53

Various aspects of gene and gene product research have exposed a number of different pathways to enhance the management and treatment of tumours.The binding sites for example of viral and cellular proteins on the p53 gene are being analysed using antibodies, mutational analyses and synthetic peptide approaches.This has allowed identification of antibodies that block and even reverse the protein interactions. Such agents may act as models for the development of therapeutic compounds , especially the E6 - p53 interaction which may restore p53 function to tumour cells in a specific manner (Lane,1994).

Modifications to the current use of radiation and chemotherapy in the treatment of cancer may well be imminent as our understanding how p53 functions progresses. It seems an appealing option to induce p53 using a non-toxic agent , hence arresting normal cells and treating the tumour cells with high doses of a conventional agent increasing its therapeutic effectiveness.

Studies on the allosteric effects of certain chemicals on the C-terminus of the p53 mutant protein indicate that some of its wild type activity can be restored. This has potential implications for tumour management. Current investigations are being made undertaken in collaboration with pharmaceutical industries for effective allosteric agents.

Many reports describe the successful reconstitution of wt-p53 expression in cells with no p53 expression at all and in those cells with mutant p53. Often wt-p53 resulted in cessation of cell proliferation, accumulating in G_1 phase of the cell cycle. Excess of wt-p53 activity leads to programmed cell death (Yonish *et al.*, 1991). In vitro the wtp53 had a mild effect on growth, cells continued to proliferate but at a reduced rate. The reasons for the variety of response are not clear but perhaps by reintroducing a functional copy of the normal p53 gene into tumour cells , reversal of the malignant phenotype may result. Although gene therapy is only a future prospect, appropriate in vivo wt-p53 expression vectors are being considered. More understanding of the

triggering of p53 accumulation, signals, targets for p53 function , and other gene and protein controls are still required.New ideas will no doubt provide improved treatment of cancer , prevention and diagnosis in the years to come.

1.12 Detection of p53

Strong evidence exists in the finding that abnormalities of p53 represent the most common molecular change in human cancer.Such abnormalities can be detected in a number of ways.Chromosomal changes can be detected directly by fluorescence in- situ hybridisation or indirectly by conventional restriction fragment length polymorphism (RFLP) analyses.Mutations can be demonstrated by sequencing of the commonly mutated exons or inferred by single-strand conformation polymorphism (SSCP) analysis (Hall & Lane , 1994).The abnormalities of the protein can be investigated by immunochemical methods , including immunohistochemistry which is strongly supported by Iggo *et al* (1990), because p53 mutations are not restricted to a single site. They argue that an immunohistochemical rather than a nucleic acid based approach will be most straight forward a means to identify p53 mutations and that there is a clear association between increased p53 protein stability and mutation.Brambilla *et al* (1993) also suggest that immunohistochemical studies of p53 in lung cancer represent a rapid, relevant and efficient approach to estimate p53 mutations.Flow cytometry techniques as used by Kastan *et al* (1991) are another way of detecting the p53 protein in tumour cells. A number of groups are using enzyme - linked immunosorbent assays (ELISAs) for p53 protein which are based on non-competitive immunoassay principles using monoclonal and polyclonal anti-p53 antibodies.The advantage over immunohistochemistry is that in these procedures, p53 must bind to two different antibodies instead of the single p53-specific antibody used in immunohistochemical methods, thus giving rise to increased antigen specificity.It is also claimed that the numerical cut-off values using quantitative ELISAs make it more objective, however the lack of an accepted standard preparation for calibration purposes is a current limitation

(Diamandis & Levesque, 1994).As well as this , the draw back is its inability to take account of variations in the proportion of malignant cells present in the sample, so that although accurate quantification of cellular protein in cells is desirable it still remains problematic.(Wynford -Thomas 1994).

Melham *et al.*, (1995) conducted studies in two independent laboratories using the immunohistochemical technique , polymerase chain reaction and DNA sequencing, on the same specimens in assessing the status of p53 mutations in tumours.They concluded that assessment of mutation by sequencing was considered the gold standard. This technique also has its limitations in that not all mutations exist in commonly examined exons 5-9 and some occur in intronic regions. Baas *et al* (1994) also commented that the analysis of p53 mutations at the molecular level is cumbersome , time-consuming , and generally not suitable for routine use.

1.12.1 Immunohistological detection of p53

The immunohistochemical method of demonstrating the presence of abnormal p53 protein in tumour cells also provides information about differences in p53 expression between cells , the intracelluar location of p53 , which may vary for different mutations of the gene (Shaulsky *et al.*, 1990) and the conformation of important regions of the p53 protein.The variability in staining between samples and among cells within the same sample was concluded by Bodner *et al* (1992) to be related to differences in growth conditions and proliferative activity in the samples, and that the levels and localisation of the p53 protein are dependent on the cell cycle (Reich & Levin , 1984).This is also supported by Bodner *et al* (1992) where cells even with high levels of p53 protein expression did not demonstrate immunoreactivity to the p53 protein and was not caused by loss of specific epitopes.In pancreatic adenocarcinoma, p53 gene mutations are frequent genetic abnormalities, often associated with the immunodetectable nuclear

accumulation of p53 protein and the proportion of cells varied between 5% and 80% of the neoplastic cells. Scarpa *et al* (1993) found that immunohistochemical and genetic analyses gave concordant results in 50% of cases of pancreatic cancer.The reported frequency of positive p53 staining carcinomas of the lung has varied between 40% and 82% (Iggo *et al* 1990).Bodner *et al* (1992) showed that only half of all p53 mutations in lung cancer stained for p53, although all the strongly staining cell lines had missense mutations in exons 5-8. Hollstein *et al.* (1991) also affirms that 98% of p53 gene mutations in different cancers have been found in exons 5-8. However Bodner *et al* (1992) showed that negative staining was also found in a number of cell lines with deletions , splicing mutations , nonsense and missense mutations outside exons 5-8.Therefore it appears that the probable level of p53 mutations in many tumours could be much higher than predicted by immunocytochemistry alone.The idea that there is in general , a correlation between detectable expression of the p53 protein and neoplasia comes from retrospective and descripitve studies of archival pathological material (Bartek *et al*., 1991). Baas *et al* (1994) showed strong evidence to support this in a series of colorectal cancers in which the molecular pathology of p53 was precisely defined.They showed a close correlation between overexpression of p53 protein and mutation of the p53 gene , and that most anti-p53 antibodies give the same result.

Rodrigues *et al* (1990) observed p53 overexpression by immunohistochemical means in 50% of colorectal cancers, and Bartek *et al* (1990) detected p53 protein in 20% to 50% of breast cancers. The occasional strongly positive cells in a tumour do not seem to correlate with obvious molecular abnormality of p53 .Baas *et al* (1994) suggested that this represented the normal working of the p53 system where wild type p53 accumulates in response to spontaneous genetic errors which occur at higher frequency in tumours than in normal tissue. Hall and Lane (1994) state that p53 overexpression in tumours or normal tissue may be an appropriate response to some external or internal cellular stimulus. The biochemical basis for the various patterns of p53 phenotype staining is still not fully resolved. Strong staining in the majority of cells is frequently associated with mutation. Elevated levels of p53 protein have been found in 50% to 60%

of head and neck squamous cell carcinomas and the positive immunohistochemical demonstration of p53 was found to correlate with increased proliferation and dedifferentiation within the same tumour with a poor outcome (Fields *et al.*, 1993). Boyle *et al.* (1993) also assert that p53 mutations detected by immunohistochemistry increase with the progression of head and neck cancers. Barnes *et al.* (1993) , showed that differences in p53 phenotype are of real prognostic significance. The work of Visokarpi *et al.* (1992) using proliferating cell nuclear antigen to determine the proliferation rate , revealed that an increased cell proliferation rate occured only in tumours with a high level of p53 immunoreactivity. In fact patients with tumours showing strong p53 immunostaining had a significantly worse prognosis than those with p53 negative tumours in their study, and that intense immunostaining was one of the best prognostic factors in prostatic carcinoma and indicated a twelve fold elevated risk for death. Quinlan *et al* (1992) found that the accumulation of p53 in cancer cells also correlated with a poor prognosis in stages I and II tumours of the lung.

Interestingly , the study of p53 mutations in different carcinomas has shown that their nature and site differ depending on tumour type. Liver cancers of high risk areas mostly have mutations in codon 249, and 50% of colon cancer mutations cluster at three specific codons 175, 248 and 273. Burns *et al.* (1993), noted that many of the mutations or deletions detected in squamous cell carcinomas of the tongue occurred within a region (codons 144 - 166), which also had been reported as a hot spot for non-small cell lung carcinoma (Mitsudomi *et al.,* 1992). Gusterson *et al* (1991) also recorded one of two tongue SCC's to be mutated around this region. In general it is believed that the type of mutation reflects the mutagen involved since specific mutational spectra are associated with individual mutagens. The adenine phosphoribosyltransferase locus spontaneously point mutates and is predominantly represented as G to A transitions whereas chemically induced mutations are predominantly G to T transversions.In addition different cell types may show intrinsic differences in susceptibility to malignant transformation by p53, suggested by the fact that transgenic mice expressing mutant alleles of p53 have a high incidence of lung adenocarcinomas (Iggo *et al.*, 1990).

Where there is no positive detection of p53 staining in a tumour , similar to that seen in normal tissue, this may reflect a very low level of p53 protein or in some cases the deletion of both alleles of the p53 gene (Bennett *et al.*, 1991).The lack of p53 protein accumulation may also reflect a specific effect of the particular mutation failing to stabilise the protein , or could be due to a transcriptional or post- transcriptional defect (Scarpa *et al.*, 1992). HPV types 16 and 18 are known to bind and degrade the p53 protein (Scheffner 1990) .This is relevant to oral SCC since HPV 16 and 18 are known to occur in the oral cavity (Maitland *et al.*, 1987).

To obtain meaningful results with immunocytochemistry experimental conditions such as variations in fixation (which has been implicated as a factor responsible for discrepancies in the immunoperoxidase procedures), the antibodies used and pretreatment of the sections must be carefully standardised (Hsu Su-ming *et al.*, 1981).The possibility of subjective error should be taken into account and the mechanisms underlying the stabilisation of the p53 protein should be considered.

The effect of antigen retrieval techniques can markedly alter the detection thresholds and Hall and Lane (1994) have suggested that the numbers of cells labelled may be more meaningful than the intensity of staining per se. The immunohistochemical detection of an antigen will be influenced by many variables including the :- absolute level of the antigen, affinity of the antibody, duration of incubations, sensitivity of the detection system and the methods of fixation.

These factors and the other complexitites involved in p53 immunohistochemistry such as the interpretation and quantification of staining, provide difficulties but p53 seems to have potential. It is of value in pathology and may possibly be relevant in diagnosis, prognosis and prediction of tumour progression, but more research is needed.

1.13 Aims of research

The treatment of oral squamous cell carcinoma requires optimal individualised therapy from both a curative and post-therapeutic functional point of view. Reliable prognostic factors must be pinpointed enabling prediction of the risks of recurrence and the probability of survival.This research was undertaken to shed further light on the significance of a relatively new and exciting genetic discovery applicable to the majority of cancers affecting mankind.

1.13.1 Hypothesis

p53 mutations may be distributed widely in the epithelium in oral mucosa in patients with oral squamous cell carcinoma as a "field change" and may predispose the patient to multiple discrete primary cancers or local recurrences .It is hypothesised that p53 mutations in the mucosal margins of specimens of surgical resections of primary oral squamous cell carcinoma have prognostic significance, i.e. they might predispose patients to local recurrences or might be more common in patients who subsequently developed new primary oral cancers.

This hypothesis was tested as follows :-

1. That p53 aberrant protein expression detected by immunohistochemistry indicates the presence of p53 mutation.

2. That there is an increased rate of local tumour recurrence and lower survival rates for surgically treated primary oral squamous cell carcinomas with positive p53 staining :-

    (a) at the resection margin of the tumour

    (b) in the tumour

3. That p53 positivity might be associated with epithelial dysplasia at the resection margin.

4. That p53 positivity in the tumour may be related to the tumour grade (degree of differentiation).

5. That there may be an association between other prognostic factors such as the site of the primary tumour, local and distant tumour recurrence and survival .

6. That tumour depth is another prognostic factor that may predict lymph node involvement.

7. That the histological grading (Bryne index) was correlated with tumour staging (STNMP), tumour size (T) and tumour depth.

8. That p53 immunoreactivity associated with tobacco smoking and alcohol abuse may also be shown in clinically normal mucosa from otherwise healthy controls.

9. That p53 immunohistological detection might be improved by microwave antigen retrieval in histological sections.

10. That the detection of p53 in the same stained section is reproducible with a low subjective error.

Chapter Two

MATERIALS AND METHODS

2.1 Immunohistochemical means of detecting mutant p53 protein.

One important aspect of research into p53 function in human tumours has been the examination of messenger RNA, or protein expression.The determination of protein levels in tumors has been the subject of the majority of studies (Dowell and Hall, 1995).Immunohistochemistry assumes that protein formation and content represents the endpoint of gene expression and is the mechanism by which gene function is expressed and effected.

2.2 Selection of primary oral squamous cell carcinomas.

Fifty patients with oral squamous cell cancer were originally identified and selected from archival material in the department of Anatomical Pathology,ICPMR,Westmead Hospital.Twenty of these patients had to be excluded from this study due to having received treatment before the final excision of the tumour,with either chemotherapy or radiotherapy or had had previous surgical excision of the primary tumour.

The 30 remaining cases of patients with primary oral squamous cell carcinomas presenting to Westmead Hospital from 1980 onwards had treatment by surgical excision and had not received radiotherapy or chemotherapy and were selected for the study.

All tissue used had been fixed in 10% formol saline, and routinely processed for paraffin embedding.The archival paraffin embedded tissues of these patients were obtained in the form of multiple blocks per resection.Tissue blocks from the excision margins were chosen by examining haematoxylin and eosin stained slides.

2.3 Preparation of the sections.

Two 4μm thick paraffin sections per slide were dried in room air overnight on poly-L-lysine coated glass slides followed by baking for 60mins at 60^{0}C in an oven. The sections were dewaxed in a series of three changes of xylene, taken through graded solutions of absolute, 95% and 70% alcohol, washed in tap water and rinsed with phosphate buffered saline (PBS) which has a pH approximately equal to 7.3.The sections were ringed with a binding pen (Dako)[1].

[1]DAKO (Australia) Pty.Ltd.12 Lord St Botany NSW 2019 Australia.

2.4 Primary antibodies to p53 used in this study.

Pab DO-7

The antibody DO-7 is an anti-human monoclonal IgG2b kappa antibody developed specifically for use with paraffin embedded human tumour material for the detection of p53 protein.The antibody binds to the N-terminal portion of the protein between amino acids 37 and 45 (Vojtesek et al, 1992).This antibody ,which is stereo-chemically distinct from another anibody ,1801, recognises both wild type and mutant human p53.It was used at an optimal working dilution of 1:100 established by prior experiments on a known p53 positive oral sqamous cell carcinoma.The diluted antibody was applied to the ringed section in one half of the slide while the ringed section in the other half was treated with phosphate buffered saline acting as a negative control.The slides were then incubated at 25^0C for 1 hour.

Pab 1801

This primary antibody is a human specific IgG1 monoclonal antibody that recognises an epitope in p53 between amino acids 32 and 79 (Banks et al , 1986).This is reacts with normal and mutant forms of p53. Pab 1801 was recommended for use only on frozen sections, Cattoretti et al. (1993) however demonstrated a high concordance (96%) between fresh frozen and formalin-fixed ,paraffin-embedded tissues in ovarian and breast cancers.

This antibody was used at a working dilution of 1:40 established as described above for DO-7 antibody.The diluent consisted of 94 units of PBS, 5mls of normal goat serum, 1G of bovine serum albumin, and 0.05G of sodium azide.The same protocol for slide preparation was used as for DO-7,except that the primary antibody 1801 was incubated with the section at 42^0C for 1 hour. Application of 1801 antibody was made to the section on one half on each slide and the section on the other half was kept moist with PBS as a negative control.

Table 2.1 The comparison of two antibodies to p53

	Antibody	
	DO-7	**1801**
Nature	monoclonal	monoclonal
Subclass	IgG2b kappa	IgG1
Reactivity with:		
wild type p53	+	+
mutant p53	+	+
Specificity	a.a. 37-45	a.a. 32-79
Staining pattern:		
nuclear	+	+
cytoplasmic	+	+

2.5 Secondary antibody and chromagen.

Following the incubation of slides with primary antibody, the sections were washed in running tap water,immersed in 3% hydrogen peroxide for 5mins,and rinsed in water.The secondary antibody, a biotin-conjugated goat anti-mouse antibody (Tago)[2],was then applied and used at a dilution of 1:250 to detect the bound primary antibody incubated with the section for an hour at room temperature.The sections were again washed in tap water and rinsed with PBS.Peroxidase-conjugated streptavidin (Tago) at a dilution of 1:4000 was applied at room temperature for one hour.After washing and rinsing, the sections were treated with diaminobenzidine as the chromagen.The sections were then washed and counterstained with haematoxylin and blueing solution.They were then dehydrated in alcohol and xylene and mounted with coverslips and Eukitt[3].

2.6 Microwave antigen retrieval for immunohistochemistry.

Neutral buffered formalin remains the most popular method for tissue fixation.Formalin is not always the best fixative for preserving antigenicity of tissues for immunohistochemical study however, because of crosslinking between formalin and proteins.Techniques using microwave treatment have produced marked improvements in the immunostaining for many antigens in formalin-fixed paraffin sections (Shi *et al.*,1991). It is hypothesised that at tertiary or quaternary structural levels ,cross linking of proteins occur.Heating tissue sections above 100^0C, high energy microwaves may break these links, unmasking the epitopes.

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[2]TAGO IMMUNOCHEMICALS distributed by HAEM Pty.Ltd. P.O.Box 174 Camberwell Victoria 3124.

[3] EUKITT LOMB Scientific, 7 Koonya Crescent Taren Point NSW 2229 Australia.

Ten cases were randomly selected from the 31 patients for further analysis using the microwave technique.Tissue sections 4μm thick were floated and placed on poly-L-lysine coated glass slides.The sections were deparaffinised and rehydrated to distilled water.The slides were then transferred in to a glass coplin jar with sodium citrate buffer (pH 6.0), covered with vented gladwrap and heated in a 800 watt microwave oven for two five minute cycles at full power.They were allowed to stand for 20mins undisturbed.The slides were removed , sections ringed using a binding pen and primary antibody applied. These were incubated with the primary antibody DO-7 as described above.

2.7 Positive and negative controls.

Positive and negative control cells were used to validate the staining technique in each experiment. The positive control comprised cells subcultured from one of eleven colorectal adenocarcinoma cell lines,SW480, with known p53 mutations at codon 273 in exon 8 (CGT to CAT) and codon 309 in exon 9 (CCC to TCC) (Goyette & Cho , 1992) (see figures 2.1 and 2.3). The negative control cell line ,SAOS-2, is derived from a primary human osteogenic sarcoma, and is null for p53 (see figure 2.2).

These cell lines were obtained from the Children's Medical Research Institute by courtesy of Dr Roger Reddell, and cultured in T75 tissue culture flasks and Dulbeccos modified Eagles Essential Medium (DMEM) supplemented with 10% foetal calf serum and an antibiotic (gentamicin).The flasks were incubated at 37^0C in a 5% CO_2 atmosphere.After incubation the flasks were brought to a sterile hooded bench, and tapped together with added trypsin and growth medium to detach the cells.The fluid contents were pipetted into a test tube and all cells resuspended.The cells were centrifuged and the supernatant growth medium removed.The pellet of cells was resuspended and counted in a haemocytometer counting chamber.When a concentration

Figure 2.1 Positive control SW480 cells stained with PabDO-7 in the conventional way (100x).

Figure 2.2 Negative control SAOS cells stained with PabDO-7 in the conventional way (100x).

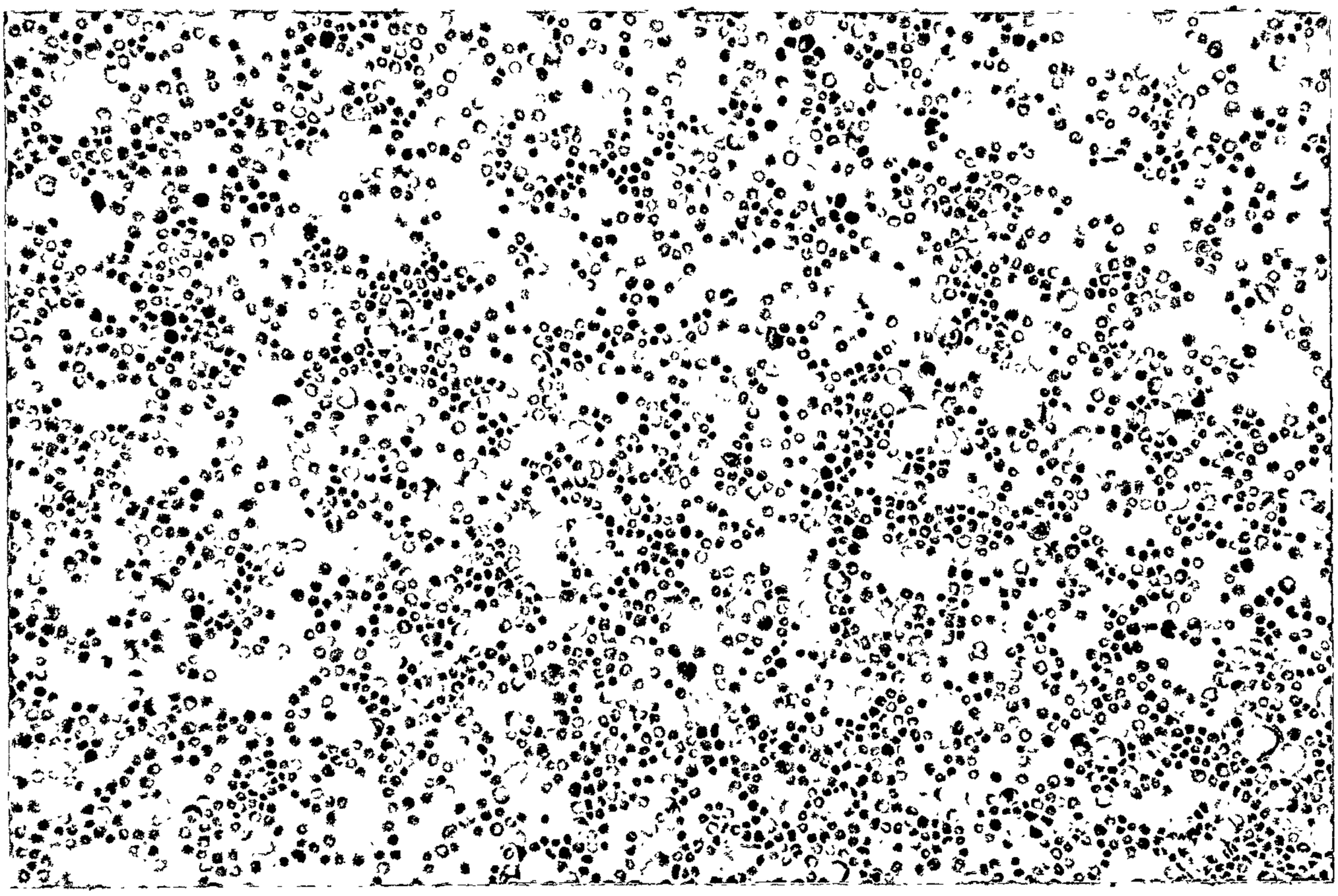
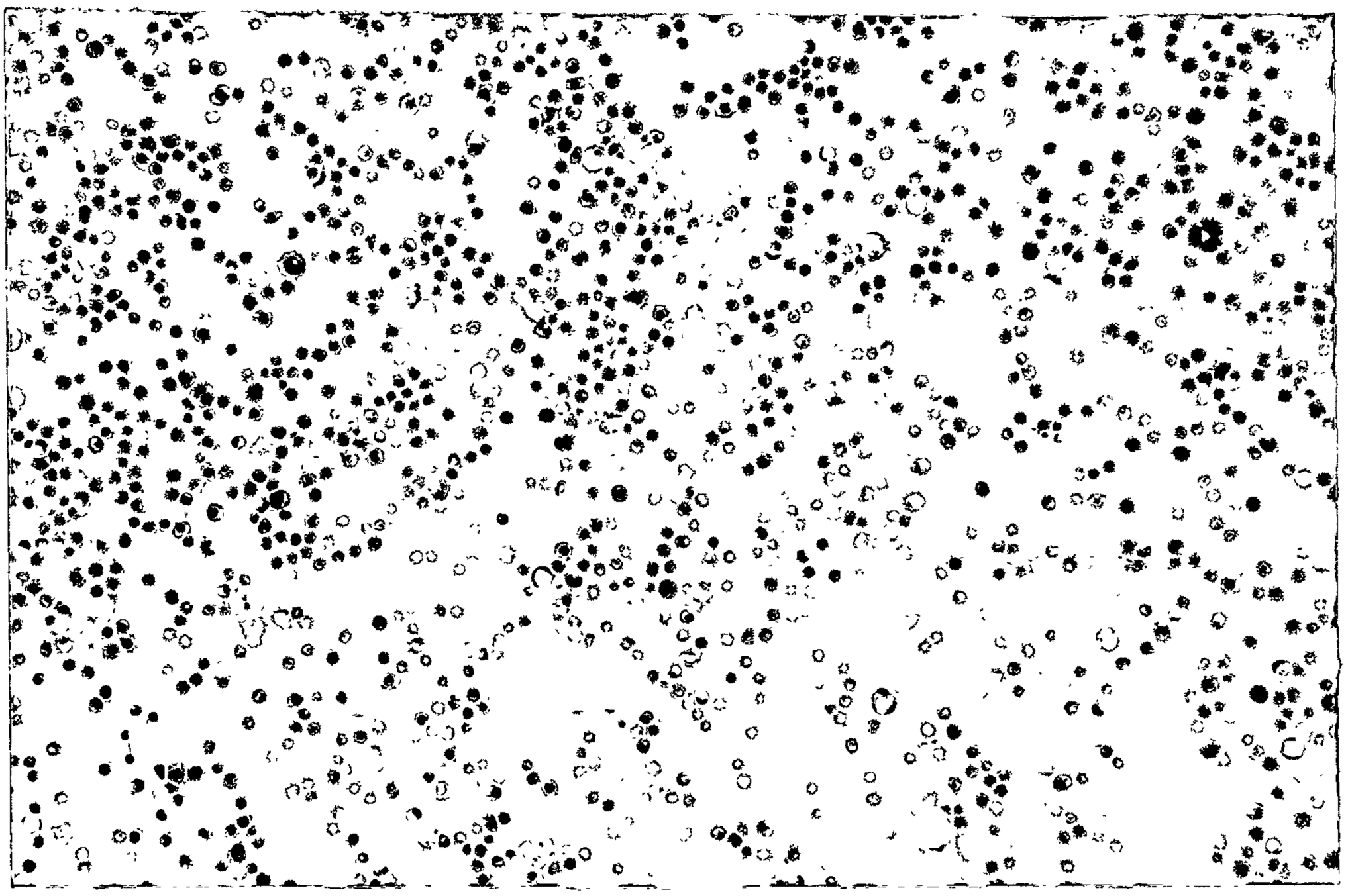


Figure 2.3 Positive control SW480 cells stained with PabDO-7 using the microwave technique (100x).



of ten million cells per ml was achieved , the cells were cytocentrifuged onto histology slides. Spinning was at low speed (120 revolutions) for 2 minutes, the slides removed and the cells briefly allowed to dry in air. The cells were then fixed in 20^{0}C methanol for 10mins, removed and placed in a sealed container for storage at -70^{0}C. When ready for use, the slides with both controls were defrosted, soaked in acetone for 10 mins and then ringed with a Dako pen.Immunohistochemistry for p53 was then carried out starting with the application of the primary antibody as detailed above in section 2.4.

2.8 Clinically normal human oral mucosa.

Sections of specimens of redundant clinically normal oral mucosa harvested from patients undergoing minor oral surgery were made available by Dr Alex Jones from a separate ethically approved study.Those specimens had been fixed overnight in neutral buffered formalin and routinely processed for paraffin embedding. Four micrometer thick sections were prepared and stained using both primary antibodies DO-7 and 1801, as described in sections 2.4 - 2.7. A brief history of the alcohol consumption (if any) and smoking habit of each patient was available.

2.9 Clinical and medical data.

From their hospital files, the following information was tabulated for the 30 patients under scrutiny:- age at diagnosis, gender, location of oral cancer, clinical staging, history of tobacco and alcohol intake, any recurrence at the primary site or other sites, regional spread and outcome resulting in terms of survival time, time to local recurrence or death.The presence or absence of any other significant medical illness was also noted.A tumour was regarded as being recurrent , if it recurred in the same site as that of the

primary tumour within twelve months of the surgical or radiotherapy treatment. Hazardous alcohol intake was defined as :- 4 units or more per day for men and 2 units or more per day for women (1unit of alcohol = 8 - 10 grams of alcohol) (RACP, 1992).

2.10 Evaluation of histological sections.

All immunohistochemical sections were evaluated by light microscopy.The positive and negative controls were first examined after staining, to verify satisfactory performance in the immunohistochemistry process.The tumour sections stained with primary antibodies DO-7 and 1801, were then examined for the detection of p53 positivity within the primary tumour and at the tumour resection margin.Tumour or adjacent normal epithelium was regarded as p53 positive if 5% or more of the cells had a positive reaction ,appearing as a brown stain in the cell nucleus (Kaur et *al.*, 1994).

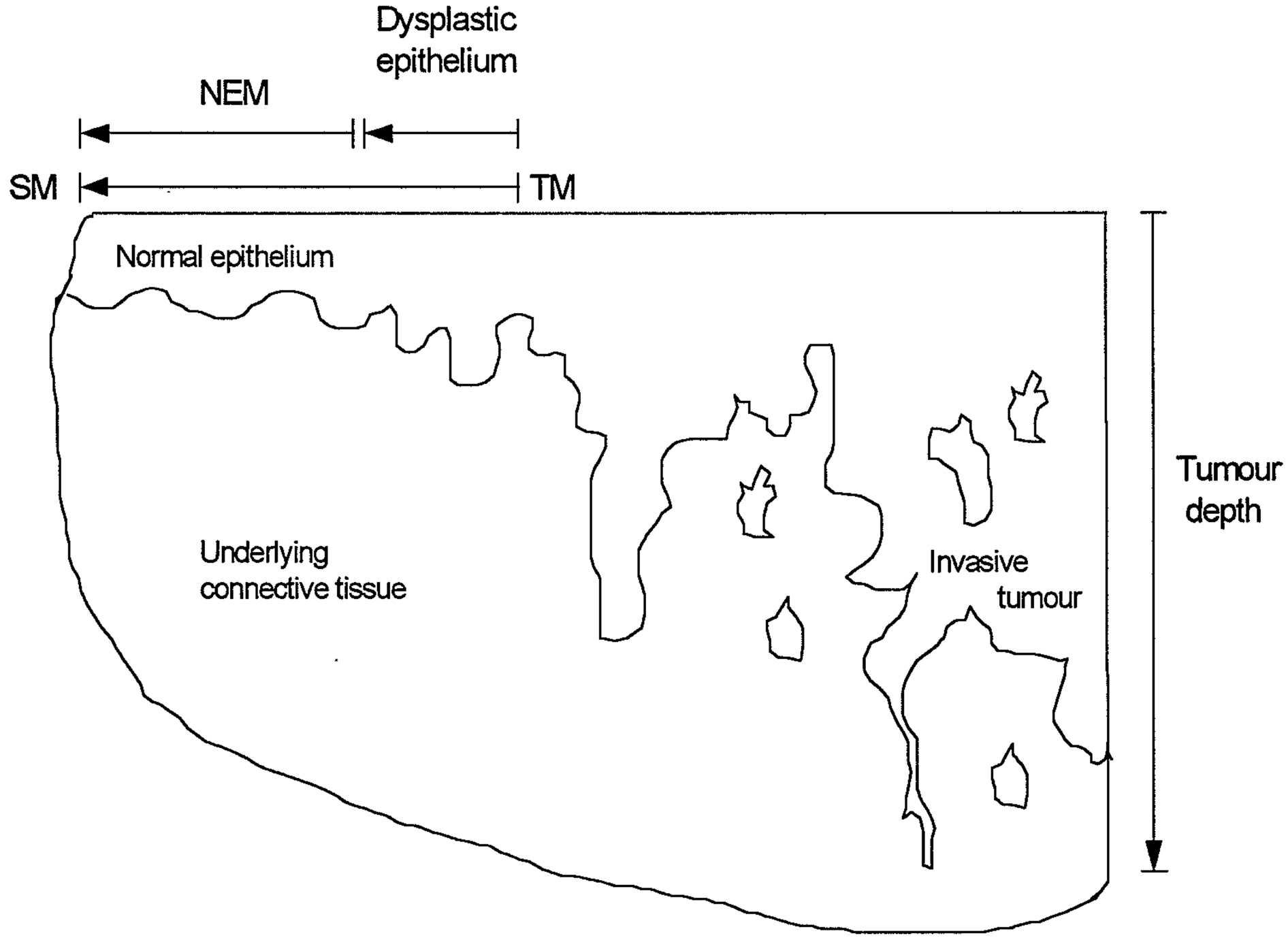
The epithelial resection margin adjacent to the tumour in all thirty patients was assessed using photographic aids (WHO ,1978) to consistently identify slight,moderate , or severe dysplasia within the marginal tissue.The length of dysplasia was measured using an ocular micrometer and converted to represent a percentage of the margin between surgical and tumour margins. The pattern and intensity of staining were compared for the primary antibodies DO-7 and 1801 for all 30 patients.The ten specimens of redundant clinically normal oral tissue were also evaluated histologically for the detection of positive or negative p53 immunostaining.

Measurements of the distance between the tumour margin (TM) and surgical margin (SM) (recognised by the coloured ink originally applied at "cut-up"), the normal epithelial margin (NEM) i.e. non-dysplastic margin and the surgical margin, and the tumour depth (TD), were standardised by using the same microscope and ocular micrometer (see figure 2.1). These readings were calculated using microscopic objectives and accounting for magnifications:

At **4x** objective, 0 to 81 on the micrometer corresponded to an actual **2mm** distance.
At **10x** objective, 0 to 100 on the micrometer corresponded to an actual **1mm** distance.
At **20x** objective, 0 to 100 on the micrometer corresponded to an actual **0.49mm** distance.

The ten patients selected to evaluate the microwave technique for antigen retrieval for staining of their specimens with Pab DO-7 were also assessed in exactly the same way.

Figure 2.4 Diagrammatic representation of histological measurements.



NEM = normal epithelial margin
SM = surgical margin
TM = tumour margin

2.11 Reproducibility of the study.

To monitor consistency in the observations, a reproducibility test was undertaken which involved examining for p53 positivity and measuring NEM to the SM,TM to the SM,and the TD (as mentioned in section 2.10), of ten p53 immunohistochemically stained slides which were chosen at random and coded by a laboratory officer.

2.12 Malignancy grading.

Haematoxylin and eosin stained sections of the thirty patients with primary squamous cell carcinomas graded by an experienced pathologist[4], according to Bryne's modifications (Bryne *et al.* , 1981) to the Anneroth system of malignancy grading for oral squamous cell carcinoma (Anneroth and Hansen , 1984). The most abnormal fields in the deep invasive margin of the tumours were graded.The four morphological features:- the degree of keratinisation, nuclear polymorphism, pattern of invasion and lymphoplasmacytic infiltration, were graded from 1 to 4 and the score for each variable was added to provide a total malignancy score for each tumour.Scores from 4-8 ,9-12 and 13-16 , were accorded a low, intermediate and high malignancy grade respectively (see table 2.2).

A separate classification (Broder's, 1941) based upon the proportion of highly differentiated cells with in the tumour was also recorded from the examination of the haematoxylin and eosin sections.The tumours were classified as either well, moderately, or poorly differentiated oral squamous cell carcinomas.

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[4]Professor Murray Walker, Dept of Oral pathology and Oral Medicine, Westmead Hospital Westmead NSW Australia.

Table 2.2 Bryne Index Scoring forOral Squamous Cell Carcinoma

Malignancy grading of deep invasive margin of biopsy.

Degree of keratinisation :	Score
Highly keratinised (>50% of the cells)	1
Moderately keratinised (20-50% of the cells)	2
Minimal keratinisation (5-20% of the cells)	3
No keratinisation (0 - 5% of the cells)	4

Nuclear polymorphism :	
Little nuclear polymorphism (>75% mature cells)	1
Moderately abundant nuclear polymorphism (50-70% mature cells)	2
Abundant nuclear polymorphism (25-50% mature cells)	3
Extreme nuclear polymorphism (0-25% mature cells)	4

Pattern of invasion :	
Pushing well defined infiltration borders	1
Infiltrating solid cords, bands and /or strands	2
Small groups or cords of infiltrating cells (>15)	3
Marked and widespread cellular dissociation in small groups and/or in single cell (n<15)	4

Lymphoplasmacytic infiltration :	
Marked	1
Moderate	2
Slight	3
None	4

4-8 : low grade

9-12 :intermediate grade

13-16 :high grade

Grade = Total Score =