

4.5.3. Function of p53 in normal tissue

4.5.3.1. p53 down-regulates PCNA mRNA

Studies of the age incidence of cancer indicate that multiple alterations are required in the genetic material for the development of many tumours (Fearon and Vogelstein, 1990). Molecular and cytogenic studies now provide convincing evidence supporting this proposal (Vogelstein, Fearon, 1988). The variable order of appearance of these genetic alterations suggests that their cumulative number, rather than order of occurrence, may be more important for tumour progression (Fearon and Vogelstein, 1990, Vogelstein et al, 1988).

Tumour progression, mediated by genetic mechanisms, presupposes that the requisite recombination events occur at reasonable frequencies. It is therefore striking that some chromosomal alterations that are commonly detected at late stages of tumour progression, such as gene amplification, do not occur at measurable rates in normal tissue (Tlsty, 1990). This observation implies that there is normally a control which functions to prevent the occurrence of gene amplification, and by inference, other types of chromosome rearrangements. Further, such controls are probably lost during tumour progression. It has been proposed that mutations that increase genetic instability could provide selective advantages during tumour progression by increasing the rate of generating phenotypic variants that are better able to survive (Nowell, 1982)

Mutations in those genes which have multiple functions could contribute to the ability of cells to undergo chromosomal rearrangements such as gene amplification. Genes participating in DNA synthesis or repair can engender genetic instability when mutated (Cleaver, 1989). Alternatively, mutations in genes encoding proteins that monitor completion of different cell cycle stages can also profoundly affect the frequency of

genetic rearrangements (Yin et al, 1992). The p53 tumour suppressor gene is just such a gene.

Mercer et al (1991) demonstrated that wild-type p53 protein selectively downregulates various promoter regions in DNA synthesis. One specific gene that has been proven to be down-modulated is a factor for DNA polymerisation thus demonstrating that p53 protein is actively involved in suppression of S-phase activity. The mechanism by which the p53 protein acts is believed to be via dimerization and formation of oligomers which then bind to DNA. Mutant p53 proteins can bind to and inhibit the DNA-binding ability of wild-type p53 protein, presumably through alteration of the dimers' 3-dimensional conformation. In contrast to other tumour suppressor genes, eg the Rb gene, most p53 mutants are dominant over the wild-type protein (dominant negative effect).

Mutations documented in nonepidermal malignancies show characteristic patterns. In colon cancer, G:C to A:T transitions predominate and are believed to be due to endogenous spontaneous mutations at inherently susceptible sites. However, in lung and liver cancers, G:C to T:A transversions are predominant, which are mutations rarely seen in colon cancer (Lee, 1992). This type of mutation is known to be induced by benzo (a) pyrene and aflatoxin B1, clastogens which are implicated in the carcinogenesis of lung and liver cancers, respectively. Thus careful study of the type of p53 mutations involved can implicate certain exogenous mutagens in a particular carcinogenesis model. In view of this data, it could be hypothesised that many of the oral tumours with mutations in the p53 gene, are similar to the transversions found in the lung ie G:C to T:A, due to the presence of a similar carcinogen, conveyed in tobacco smoke (Lee, 1992, Suzuki et al, 1992)..

4.5.3.2. Regulatory proteins of p53

One of the main goals in understanding the function and role of p53 has been to find other proteins which regulate the p53 protein.

In the mouse the MDM2 gene was originally identified as a dominant transforming oncogene present on a 'mouse double minute' chromosome. Oliner et al (1992) have shown that the human homologue of MDM2 can bind to p53, and have also mapped it to the long arm of human chromosome 12. Their work has demonstrated that some neoplasms with a high level of expression of the MDM2 protein show no mutations of p53. This suggests that the tumour suppressor activity of the p53 gene is inactivated by the amplification of MDM2 (Oliner et al 1992), which is the same functional effect as mutations of the p53 gene.

4.5.4. Place of p53

Wild-type p53 functions as a cell cycle control protein, in that progression from G1 to S phase is often blocked in cells expressing high levels of this protein (Mercer et al, 1990, Martinez et al 1991). In many types of human cancers the p53 gene is mutated, and loss of heterozygosity for p53 often occurs during tumour progression (Lane and Benchimol, 1990, Levine et al, 1991). In mice, mutant p53 genes, or lack of wild-type p53 expression contribute to precocious development of various tumours (Lavigne and Bernstein, 1991, Donehower et al, 1992).

One hypothesis to explain the elevated risk and early age of cancer onset in humans and mice with germ-line p53 mutations is that they undergo genetic aberrations with

increased frequency owing to inactivation of wild-type p53 function through the formation of heterodimers with the mutant protein (Lane and Benchimol, 1990, Levine et al, 1991, Milner, 1991) (Kern et al, 1992). Yin et al (1992) suggest that it is the ratio of mutant to wild-type p53 protein, rather than the absolute level of wild-type protein, which is the critical determinant (Yin et al, 1992).

A possible explanation for the interaction between p53 expression, gene amplification and the control of cells moving into S phase is provided by the work of Yin et al (1992). The wild-type (wt) p53 is an essential component in controlling those cells which are to cross the G1-S boundary, and normal cells arrest in the G1 phase in the absence of sufficient nutrients (for example nucleotides). This seems to be due to the presence of wt p53. However in situations where there is an absence of wt p53 expression then the cells are unable to respond to the metabolic deficiency and cross the G1-S boundary, and replication errors occur (Yin et al 1992).

It therefore seems that the p53 gene acts in part as a feedback control of replication. When cells are damaged, whether it is due to irradiation, lack of metabolites, or other form of carcinogenic stimulus, the wt p53 switches the cell off (arrests it in the G1 phase), which allows repair. In situations where repair is not possible the cell may be programmed to die. Absence of the wt p53 allows inappropriate cell division and chromosomal rearrangements, amplification, and deletions may be the result. Yin et al (1992) were also able to demonstrate that the expression of wt p53 in transformed cells containing mutant p53, restores G1 control and reduced the chromosomal aberrations. Wild-type p53 has previously been described as the 'guardian of the genome' (Lane, 1992), - preventing the situation where mutated genetic material can be amplified. It would appear that wt p53 prevents a cell from moving into the cell cycle until the necessary genetic "house keeping" functions are completed.

4.5.5. P53 and low precursor pools for cell mitosis

Genes that participate in progression from G1 to S phase might also affect the genetic stability if their mutation permits entry into S phase in the presence of precursor pool levels, of materials such as nucleotides, that are insufficient to allow a complete round of DNA synthesis (Yin et al, 1992). This suggestion is based on the observation that various treatments that induce transient arrest of DNA replication through depriving the cell of certain nutrients, lead to increased rates of chromosome breakage and gene amplification (Hahn et al, 1986, Morgan et al, 1986) This is consistent with reports that gene amplification can be initiated with chromosome breakage followed by the formation of acentric fragments harbouring the target gene (Hahn et al, 1986, Windle and Wahl, 1992) Thus, cells entering S phase with limiting precursors might accumulate partially replicated structures that, when broken, constitute precursors for gene amplification.

4.6. p53 overexpression in cells - unrelated to gene mutation.

4.6.1. Viral influence on p53 expression

Some viruses lead to p53 overexpression through viral oncogenic protein-complexing. These include adenovirus E1b and SV40 large T antigen (Lane and Crawford, 1979, Braithwaite et al, 1991) . However, viral oncogene-p53 complexes do not always result in increased expression of p53. The E6 proteins of the human papilloma viruses (HPV) encoded by HPV types 16 and 18, have been shown to target the p53 proteins for degradation, thus inactivating the proteins rather than altering its function

(Scheffner et al, 1990). Experimental work undertaken on reticulocyte extracts has shown that the E6 protein, type 16 or 18, binds to and promotes the proteolytic breakdown of p53 through the ubiquitin protease system. In degrading p53, E6 is causing a true loss of function, consistent with the role of the gene as a regulator of growth. In samples of cervical cancer cell lines as well as squamous cell carcinoma of the anus, positive for HPV, the p53 protein was normal but inactivated by HPV oncoproteins, whereas in HPV-negative cancers p53 was mutant (Crook et al, 1991).

The nature and position of a p53 mutation seems to be influenced by the cell or tissue type, and it is uncertain as to whether this is due to differences in the environment of these cells (eg mutagen exposure) or to selection of mutant alleles by different cancers.

4.6.2. Heat shock Proteins

It has been suggested that the presence of heat shock proteins, hsc70, leads to an increase in p53 (Finlay et al, 1988). It does this by binding to p53 and extending its half-life, and thus increasing the levels of p53 in the cell. (This in turn might contribute to the transformation of these cells). Yet the p53 protein which persists is in a mutated form. Thus qualitative changes in p53 contribute to quantitative increases in the p53 levels and it is possible that the p53-hsc70 complex may play a role in these quantitative changes by extending the half-life of the mutant p53 protein (Finlay et al 1988).

4.7. Model for p53

Lane (1992) suggests 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 (Lane, 1992).

This model provides both a biochemical function and a biological function for p53 (as a specific transcription factor and as a G1 checkpoint control for DNA damage). It explains how DNA viruses neutralise the action of p53, and is consistent with the observations of p53 null mice (Donehower et al, 1992), which develop normally but have a very high level of aneuploidy and mutational events in tumours, as well as the relative success and risks of conventional radiation and chemotherapy. Tumour cells without the normal safeguards of p53 are more susceptible to the killing effect of DNA damaging agents because they replicate through the damage; similarly they are more susceptible to the mutagenic effects of these agents (Lane, 1992).

4.8. Detection of p53

4.8.1. Is the detection of p53 synonymous with mutation ?

Various techniques are available which allow for the detection of protein within cells, and p53 is no exception. Rodrigues et al (1990) using a multiple analysis approach to the study of p53 in colorectal tumours (immunohistochemistry, immunocytochemistry, ELISA analysis, immunoblotting of p53, direct sequencing of p53 mutations from asymmetric PCR products, as well as chemical-mismatch-cleavage analysis) showed that a high level of p53 protein was present within colorectal tumour cells. Correlation with the presence of point missense mutations of evolutionarily conserved residues in 10 colorectal cancer cell lines was also demonstrated and Rodrigues et al (1990) argues that as the level of p53 in 'normal' tissue is usually undetectable, then when p53 is overexpressed, it indicates that the p53 gene has mutated. This study showed that there was a very high proportion of p53 mutations in the colorectal cell cancer lines which is consistent with other studies for breast cancer cell lines (Bartek et al 1990) and primary lung tumours (Iggo et al 1990). However, there are a number of tumours carrying p53 mutations which were not readily detected by immunohistochemistry. Nonsense mutations result in a premature stop codon occurring at random sites throughout the gene and are not detected by an immunohistochemical approach (Thor and Yandell, 1993).

The data on 17p allele loss suggests that although not all tumours are associated with p53 mutations, up to 70-80% of total malignancies are associated with mutations in the p53 gene (Rodrigues et al, 1990).

It is interesting to note that in premalignant conditions of the lower aerodigestive tract p53 has not been detected (Rodrigues et al, 1990), whereas it has been demonstrated in

the upper aerodigestive tract (Casson et al, 1991, Coltrera et al, 1992) and the gall bladder (Kamel et al, 1993).

4.8.2. p53 mutations and immunohistochemical staining patterns

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 intracellular location of p53, which may vary for different mutations of the gene (Shaulsky et al, 1990) and the conformation of physiologically important regions of the p53 protein, such as the SV40 large T antigen-binding sites (Yewdell et al, 1986) .. Takahashi et al, (1989 and 1990) and Lane et al (1990), have shown that some p53 mutations found in human tumours actually result in lack of expression of p53 mRNA and hence the p53 protein. There are several factors which can potentially regulate intracellular levels of p53 protein in tumour cells, including transcriptional and post-transcriptional regulation (Reich and Levine, 1984), degradation (Scheffner et al 1990), stage of cell cycle (Steinmeyer et al, 1990), protein binding (Deppert et al, 1987) the presence of introns (Hinds et al, 1989, Lozano and Levine, 1991) and stabilisation (Reihnsaus et al, 1990)

Bodner et al (1992) endeavoured to compare the type of p53 mutation as determined by PCR and SSCP, and the detection of the p53 protein by immunocytochemical means. The analysis showed a broad spectrum of mutations including homozygous deletions, single base pair deletions, nonsense mutations, splicing mutations, and missense mutations. The majority of the missense mutations occurred within exons 5-8, and half of these were mutations at locations involving arginine.

Bodner et al (1992) also showed that negative or marginal staining was found in a number of cell lines with deletions (not necessarily homozygous deletions), splicing mutations, all the nonsense mutations and missense mutations outside of exons 5-8. Missense mutations within exons 5-8 were the only class of mutations that demonstrated positive immunocytochemical staining. Missense mutations in locations involving arginine were associated with particularly strong staining. In the positive cases the main form of staining was nuclear, although in some of the cell lines cytoplasmic staining was also found, usually accompanying the nuclear staining. The staining with the primary antibody PAb 1801 was noted to be a coarsely granular, non-specific, immunoprecipitate in the nucleus and cytoplasm.

Bodner et al (1992) had originally predicted that all nonsense mutants in exons 4, 6, 8, and 10 would express epitopes that would be detected by some of the monoclonal antibodies used. However this was not the case and suggested that the lack of staining was not simply caused by loss of specific epitopes. Even in the cell lines with high levels of p53 protein expression not all cells in the specimen demonstrated immunoreactivity to the p53 protein. It should be remembered that the levels and localisation of the p53 protein, are dependent on the cell cycle (Reich and Levine, 1984) Therefore variability in staining between samples and variability among cells within the same sample may be related to differences in the growth conditions and the proliferative activity in the samples.

The study of Bodner et al (1992) showed that only about 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. Therefore, it appears that the probable level of p53 mutations in many tumours could be much higher than predicted by immunocytochemistry alone. (A statement previously made by Rodrigues et al 1990). In fact Bodner et al (1992) go on to suggest that the effectiveness of this technique may indeed, depend upon the type of tumour being analysed

4.8.3. Analysis of cell lines without p53 mutations

The majority of authors indicate that p53 nuclear staining is not present in clinically normal tissue. Examples of this involve gastric mucosa (Uchino et al, 1992) and lung (Pavelic et al, 1992).

Minimal staining of normal tissue, and cell lines demonstrating either negative or marginal staining was noted by Bodner et al (1992).

4.8.4.. Immunohistochemical means of detecting mutant p53 protein

Several methods have been used to assess p53 expression in human tumours - DNA, RNA or protein. Of these the examination of the protein levels has been suggested as the best approach (Harris, 1990). This is assumed on the basis that protein content is the end point of gene expression and is the mechanism by which gene function is expressed and effected.

4.8.5. p53-Antibody Profile

The prevalence of p53 mutations in different human neoplasms, as determined by immunochemical means, is demonstrated in the following table.

Table 4.1 Profile of p53 expression in human neoplasms, using immunohistochemical techniques.

Author /Ref	Site	Stage	Cases (n)	p53 + ve %	Antibody used
Purdie et al (1991)	Colorectal	Carcinoma	86	47	1801
		Adenoma	46	09	
Ostrowski et al (1991)	Breast	Carcinoma	90	36	1801
Davidoff et al (1991)	Breast	Intraductal	15	13	1801
		1o carcinoma	35	17	
		1o + metastasis	22	50	
Cattoretti et al (1988)	Breast	Carcinoma	200	15-40	421/1801
Bartek et al (1991)	All sites	Malignancies	212	76	CM1
Gusterson et al (1991)	Head and neck	Sq cell ca	47	34	1801 JG8, CM1
Field et al (1991)	Head and neck	Sq cell ca	73	67	421/1801
Vojtesek et al(1992)	All sites	All	92	60	D07
Kamel et al (1993)	Gall bladder	Adenocarcinoma	30	14	CM-1
		Dysplasias	9	2	
Allred et al(1993)	Breast	Carcinoma	700	362	240/1801

Sq cell ca = squamous cell carcinoma

Adeno =Adenoid

1o=primary

p53 +ve % = the number of positive cells per hundred cells

4.8.6. Antibodies to p53 used in study

4.8.6.1. PAb CM-1

This is a polyclonal antibody which was raised against full-length human p53 and recognises both end terminal regions of the p53 protein- the N-terminal (similar to PAb 1801) (Banks et al 1986) and the C-terminal.

CM-1 is also able to detect the protein in archival histology specimens fixed in formalin and embedded in paraffin.

Immunoblots of whole cell extracts showed that the CM-1 antibody recognised a single species of 53 kD present in extracts from p53 mutant-expressing cell lines. The band precisely co-migrated with the p53 bands detected with other monoclonal antibodies, whereas the CM-1 did not react with any band in an equivalent immunoblot of the p53-negative cells (Midgley et al, 1992)

Effect of fixation and processing on immunohistochemistry

Midgley et al (1992) showed that CM-1 gives excellent results in tissues fixed in formal saline. A number of factors were shown to reduce the intensity of the p53 reaction, and these included prolonged fixation periods (over 48 hours) in formal saline and the presence of calcium in the fixative. These were both shown to be deleterious. Heating of the sections normally used to aid their adherence to the slide was also harmful, and the recommended protocol included the use of poly-L-lysine -coated slides.

Staining patterns

The primary antibody (PAb) CM-1 was shown to have a number of staining patterns (Midgley et al 1992). The most striking was an intense nuclear positive staining confined to the tumour cells and present in the majority (>65%) of the tumour cells. This stain was excluded from condensed chromosomes and showed some variation in intensity from nucleus to nucleus. A second pattern of staining reaction was characterised by a slightly less intense stain and fewer of the nuclei were positive. The third form of presentation was a very weak positive stain. The final presentation was a negative result. It was noted that cases of carcinoma *in situ* often demonstrated a staining pattern where the cells at the outer margin of the lesion stained most intensely. Two other staining presentations were notable. Some sections displayed cytoplasmic staining, either in addition to or without nuclear staining, and others showed an intense reaction in single nuclei scattered throughout the tumour (not as pairs or clusters).

4.8.6.2. PAb 1801

Antibody PAb 1801 is a human specific monoclonal antibody that recognises an epitope in p53 between amino acids 32 and 79 (Banks et al, 1986) This is directed against normal and mutant forms of p53 It is an IgG1 class of antibody

While PAb 1801 is recommended for frozen sections (Cattoretti et al, 1988) due to the risk that the epitope would be destroyed by formaldehyde fixation, a high concordance between fresh frozen and formalin fixed, paraffin embedded tissues of the order of 92-96% has been demonstrated by Kerns et al (1992) in two common epithelial cancers

(ovarian cancer, and primary breast cancer). [This assay included incubation with antibody, and avidin-biotin complex at 42°C -this permitted completion of immunostaining in 5 hr rather than two days].

4.8.6.3. PAb DO-7

The antibody DO-7 is a monoclonal antibody developed specifically for use with paraffin-embedded human tumour material for the detection of p53 protein. The antibody binds to the denaturation resistant N-terminal portion of the protein between amino acids 1 and 45, most probably between amino acids 37 and 45 (Vojtesek et al 1992) This antibody is stereically distinct from 1801. It recognises both wild-type and mutant human p53. Vojtesek et al (1992) indicates that the staining is essentially the same as that obtained by CM-1 polyclonal anti-p53 antibody.

The antibody itself is an IgG2b class antibody.

Table 4.2 Comparison of three antibodies to p53

Nature	CM-1	1801	D07
	Polyclonal	Monoclonal	Polyclonal
Reactivity			
wild-type	+	+	+
mutant	+	+	+
Immunoreactivity inhibited by prolonged fixation	+	+	+
Specificity	N-terminal & C-terminal regions	aa 32-79	aa 37-45
Staining pattern.			
nuclear	+	+	+
cytoplasmic	+	+	-

+

 positive for the particular characteristic

-

 negative for the characteristic

aa

 amino acid

4.8.7. Significance of variations in staining patterns

Bartek et al (1990) showed that there is a simple relationship between mutation of the p53 gene and the 'high' level of expression of the p53 protein in tumours. It appears to be caused by an increase in stability of the mutant p53 proteins in the environment of a tumour cell (Reihnsaus et al, 1990).

The use of antibodies to probe for the presence of the p53 protein is well known, as is the characteristic variation in the staining intensity between cells. PAb 1801 (recognising a denaturation resistant epitope between amino acids 32 and 79) also shows a wide range of responses within the nuclear staining (Rodrigues et al, 1990). The latter also demonstrated a degree of cytoplasmic staining, together with the report that there was an heterogeneous staining pattern, but only a fraction of the tumour cells actually stained positive. A similar staining pattern has also been described for melanomas, carcinoma of the testis, breast and pancreas (Bartek et al, 1990, Bartkova et al, 1991, Barton et al, 1991) Here again in a number of cases, the positive staining was restricted to a subpopulation of the tumour cells.

The various patterns of staining observed possibly represents the various phenotypes of the p53 gene. However the biochemical basis of these is not fully resolved. They are all specific to the area of the tumour and therefore must represent some tumour-specific alteration in the control of p53 expression- except for the null phenotype. (Midgley et al, 1992) The pattern of staining in which fewer than 30% of the cells are positive has been reported by Gannon et al, (1990) as being present in tissue which possibly possess a normal p53 gene, and perhaps the high level of p53 may represent transcription activation (Reich et al, 1983). Midgley et al (1992) suggests that the cytoplasmic staining can occasionally be found in cultured cells when the nuclear transport signal is absent or its activity is counteracted by binding to the adenovirus

E1b oncoprotein (Zantema et al, 1985) or perhaps by heat shock protein 70 binding (Bartek and Bartkova et al, 1990; Bartek, and Iggo et al, 1990; and Gannon and Lane, 1991) also present the situation where single occasional cells are positive even in cell lines. This may represent an epigenetic event or a lethal phenotype, since it does not seem to be present in daughter cells. The final situation where there is no positive stain in a tumour, similar to that seen in normal tissue, may just reflect the very low level of p53 or in some cases the deletion of both of the alleles of the p53 gene (Bennett et al, 1991)

4.9. Material and Methods

4.9.1. Subjects and cell lines

The archival material used in this study came from subjects described previously in Section 3.2.1.

In addition a human osteosarcoma cell line, Saos-2 (Chen et al, 1990) was chosen because it had no endogenous p53 owing to the deletion of the gene (Masuda et al 1987). These cells had been grown on a monolayer culture and removed with a solution of trypsin, to form a suspension. This was then placed in a cytopsin tube and spun down to form a pellet which was embedded in an agar block. The agar block was then fixed in formalin and processed in the routine manner, and finally embedded in paraffin - the cell line having been processed in similar manner to the tissue biopsy specimens.

These human osteosarcoma cells, Saos-2, formed the negative control substrate for the immunohistochemical study.

4.9.2. Immunohistochemical techniques

4.9.2.1. Preparation

All material used in this study had been fixed in 10% formal saline, for a varying length of time ranging from 6 to 24 hours and routinely processed for paraffin embedding.

4 µm sections were floated onto poly-L-lysine coated slides and then allowed to dry overnight at room temperature. Sections were dewaxed in two changes of xylene and two of absolute ethanol. Endogenous peroxidase was blocked by immersing the sections for 10 minutes in methanol : PBS (5:1, v/v) containing 0.03% hydrogen

peroxide. The sections were then washed in tap water and immersed in PBS. Two changes of PBS were used for all washings between applications of the staining reagents. Non-specific binding was blocked with 20% goat serum in PBS.

Phosphate buffered saline (PBS: pH 7.2) was used throughout the immunostaining schedule as the diluent buffer and for rinsing.

4.9.2.2 The primary antibodies

The optimal dilution, incubation time and temperature for incubation were determined for each antibody, in a pilot study by a chequerboard design series of experiments.

4.9.2.3. Secondary antibody

All sections, where a monoclonal antibody was used as the primary antibody, were subsequently incubated with biotinylated secondary anti-mouse antibody (ref 2.11.2). The secondary antibody used for the primary polyclonal antibody (CM-1) was goat anti-rabbit (Tago) All further procedures are as described in section 2.11.2

4.2.3. Positive and negative controls

A control specimen from a known p53 positive primary intra-oral squamous cell carcinoma of the tongue (which reacted strongly with all three antibodies) was used to standardise the procedure, with negative adjacent normal epithelium, as further negative control tissue included in the same section.

Controls for each case included omission of the primary antibody and reacting sections with diluent buffer only. In addition to this, a negative control slide was added which was the p53 null allele cell line - Saos-2.

4.9.4. Assessment of immunohistochemical staining

Stained sections were assessed using three standardised slides as the determinants for intensity. The evaluation was also carried out on two separate occasions, 6 weeks apart, and the results compared.

Positive and negative controls were included for each case (as per 4.9.5.).

The tissue block from patient No. 3 gave a strong positive staining response in the tumour cells, but not in the normal adjacent epithelium. These results were identical and reproducible for the three antibodies. This tissue block was chosen as the positive control tissue for immunohistochemical staining involving all the three antibodies.

Staining was scored semi-quantitatively according to frequency, intensity and location.

The score for frequency of cells staining was: 1 = <10% of the cells stained; 2 = 10-50%; 3 > 50%. The intensity of staining was semi-quantitated as: 0 = no staining,

1 = weak staining; 2 = moderate staining; and 3 = strong staining. The location was scored as: N (nuclear) or C (cytoplasmic). Reference slides previously assigned each of these scores were kept for recalibration of the observer.

4.9.5. Malignancy grading

Bryne et al (1989), in his modification to Anneroth's system of malignancy grading for oral squamous cell carcinoma (Anneroth and Hansen, 1984), demonstrated that patients with 5-10 malignancy points had a significantly better prognosis than those with more than 10 points.

Bryne's modifications (Bryne et al, 1989) to the original grading system were :-

1. only the most anaplastic fields in the most invasive areas of the tumours should be graded.
2. the morphologic parameter stage of invasion was omitted.
3. the remaining five morphologic features were graded from 1-4, and the score for each variable were added to provide a total malignancy score for each tumour. Carcinomas with a total score of 5-10 points (all stages) carried a 5-year survival rate of 58%, whereas only 18% of patients with tumour scores >10 points survived 5 years.

The present analysis involved the assessment of all the oral SCC included in the first part of the study. The H & E stained sections of the carcinomas were assessed on two separate occasions (8 weeks apart) by the same investigator. Where there was a variation in the scores then a mean score value was used.

4.10. Results

4.10.1. Primary Antibodies for p53 protein detection

A series of dilutions and incubation times and temperatures was undertaken for each of the three antibodies.

The optimal respective dilutions and incubation times determined by the chequerboard series of experiments were as follows:-

Table 4.3. Optimal dilution and conditions determined for primary antibodies for immunohistochemical detection of p53 and PCNA

Antibodies	Antigen specificity	Dilution	Incubation time	Incubation temperature
PAb CM-1	p53 wild & most mutant	1:500	1 hr	Room temp.
PAb D07	p53 wild & mutant	1:100	1 hr	Room temp
PAb 1801	p53 wild & most mutant	1:200	1 hr	42°C
PAb PC 10	PCNA	1:100	1 hr	Room temp

PAb = primary antibody

(All the antibodies were obtained from Novocastra Laboratories Ltd, UK. CM-1 is a rabbit polyclonal antibody, D07 is an IgG2b class murine monoclonal antibody, and 1801 is an IgG1 class murine monoclonal antibody.)

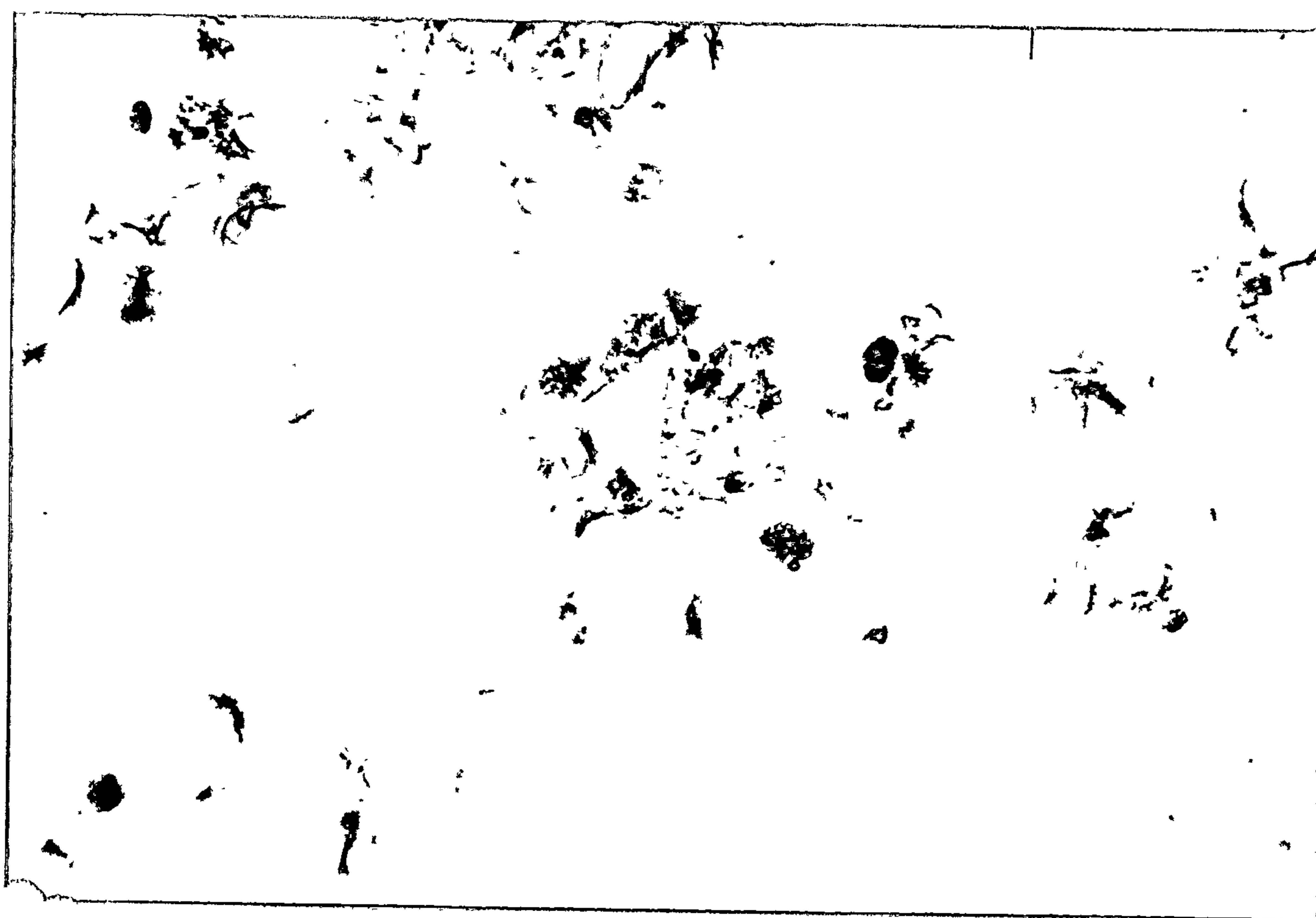
4.10.2. Positive and negative controls

The Saos-2 cell line (Fig.4.1) which had lost both alleles for the p53 gene, demonstrated negative immunoreactivity to all three antibodies. Meanwhile, the positive control carcinoma (see section 4.2.4.), demonstrated intense nuclear reactivity in response to all three antibodies.

Background staining

It was noticed on occasions that stromal tissue appeared positive with antibodies CM-1 and 1801, whereas there was never any stromal staining with the D07 antibody. The stromal tissue affected was usually endothelium, vascular smooth muscle, fibroblasts reacting with PAb CM-1 antibody while occasionally plasma cell and lymphocytic infiltrates were positive with PAb 1801 antibody. The stromal staining associated with PAb CM-1 was frequently nuclear while that associated with PAb 1801 was always cytoplasmic. In both situations this was considered to be background staining, because it defined a region that was not in agreement with the other antibodies, and was disregarded.

Figure 4.1. Saos-2 cell line demonstrating binding by PAb PC-10, which was absent in response to antibodies to p53. (This section demonstrates an abnormal nucleus, with bizarre nuclear forms. The cytoplasm has been largely lost due to preparative artifact.)



4.10.3. Detection of p53 in clinically normal oral mucosa

A total of 74 biopsies were obtained from 30 patients to analyse the distribution of p53 in clinically normal mucosa from patients undergoing treatment for periodontal disease or removal of teeth.

The monoclonal antibodies D07 and 1801, as well as the polyclonal antibody CM-1 were used to probe for the presence of p53 protein.

Positive p53 expression was found in 8 biopsies, from 5 (16.7%) of the 30 patients with clinically normal mucosa.

The specimens which contained positive p53 protein were taken from multiple oral sites, both anterior and posterior. Where multiple biopsies existed more than one site from a patient was positive as in cases 3 and 4. These presented in both a cluster form and as single cells. The positive areas were generally small and isolated. This material is presented in Table 4.4 and illustrated in Figure 4.2 a,b,and c.

Figure 4.2.a Clinically normal tissue (pseudoepithelial hyperplasia) demonstrating a raised growth fraction compared to that of the other clinically normal control biopsies (subject was a smoker, age 40years).

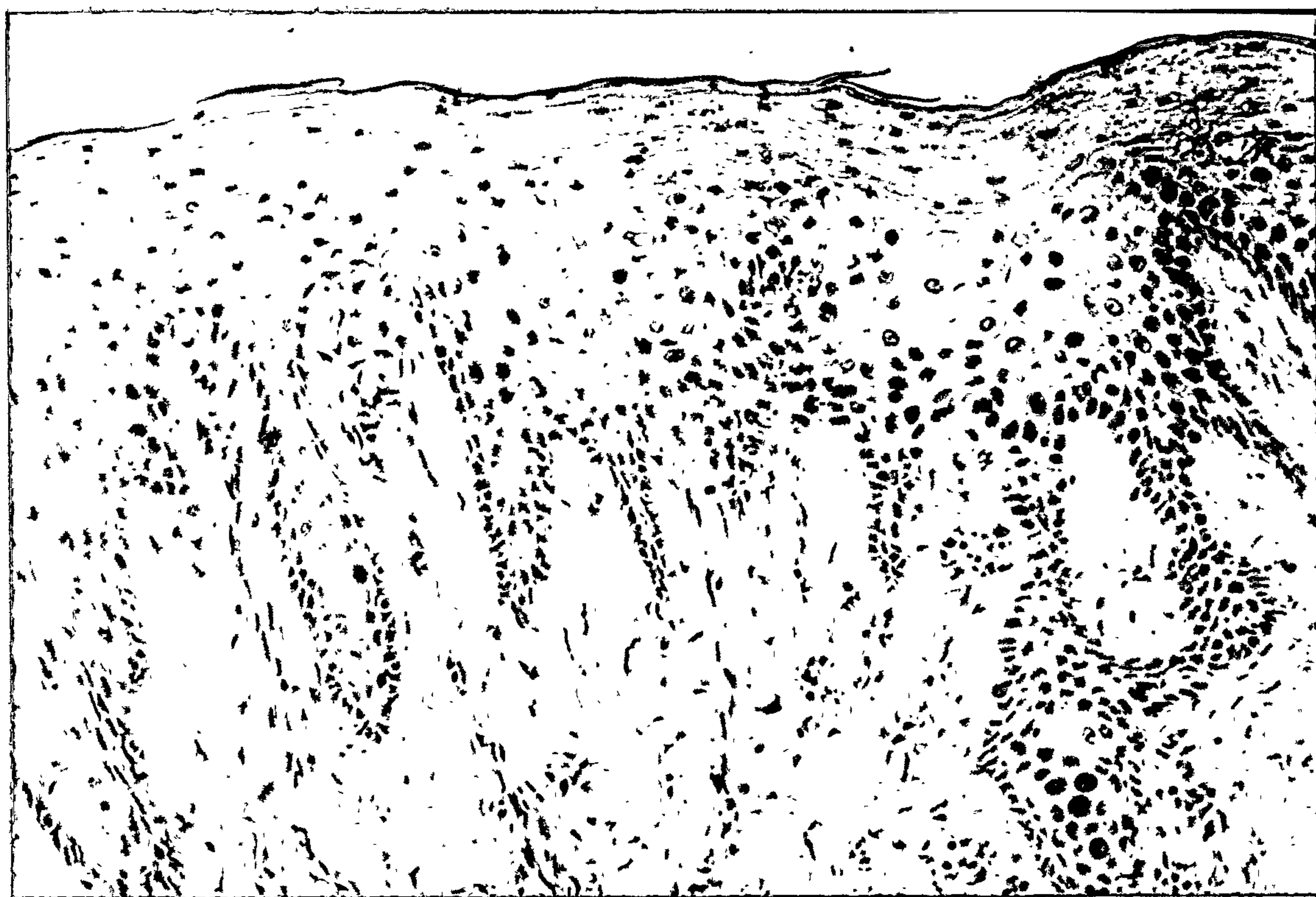


Figure 4.2.b. Isolated cluster of cells demonstrating immunopositivity to p53 antibody
(x 260)



Figure 4.2.c. Isolated single cells demonstrating immunopositivity to p53 antibody
(x 260)



Table 4.4. p53 presentation in clinically normal mucosa

Subject	Sex	Age	Biopsy site	Tobacco Habit	p53 +ve staining			Pattern of staining.		
					CM-1	D07	1801	Basal	Supra	Cluster
1	M	24	38	+	+	-	-	+	-	-
2	F	20	48	+	+	-	-	+	-	-
3a	M	42	46	+	+	-	-	+	-	+
3b	-	-	48		+	+	-	+	-	-
3c	-	-	37		+	-	-	+	-	-
4a	M	68	46	+	+	-	-	+	+	+
4b	-	-	47		-	+	-	+	-	-
4c	-	-	48		+	+	+	-	+	+
5	M	54	48	+•	+	-	-	+	-	-

• = Patient has not smoked for 3 years

NB Case No.2 demonstrated positivity in single cells only in the section

Biopsy site = a topographical representation of the mouth is found in Appendix A

The positive controls employed routinely for each experiment, namely an oral squamous cell carcinoma, had consistently positive tumour cells, and consistently negative adjacent oral epithelium in the same sections. Omission of the primary antibody was always associated with negative staining. Previously negative normal oral mucosa served as a further negative control.

Further serial sections of the p53 positive cluster regions (Fig 4.2.b.) were prepared to examine this unexpected finding more fully. Where a cluster of cells was examined these sections consistently showed the region of the biopsy to be positive, and the same basal or supra basal area, with the surrounding cells being negative. However, a single positive cell would frequently not present in the following serial section. The number of contiguous cells forming the cluster varied, but the average was 10 positive cells per section. Serial sections were taken to determine the total population of the cluster of cells in three dimensions. These cells persisted for 6-10 sections before disappearing - this was a gradual process, with one or two cells only in the last positive section of any

one cluster. Thus an estimate of the size of these clusters, based on the fact that each section is approximately 4µm thick and a keratinocyte is 20µm, and that the same positive cell could possibly feature in 5 sections, would be 15 -30 cells.

There was no background staining, the positive stain was restricted to the nuclear region (with no cytoplasmic staining) in the monoclonal (D07) and minimal in the polyclonal (CM-1) antibody.

Patient No. 2 demonstrated only a single positive cell in one section and none were seen in subsequent serial sections.

There was a strong association between those specimens with supra-basal presentation of PCNA, and the presence of p53. All the p53 positive cells were observed in sections which had supra-basal staining of the PCNA. However the converse that all sections with supra-basal staining were positive for p53 antigen, was not true.

No correlation between periodontal indices and p53 presentation could be investigated due to lack of periodontal data and infrequent immuno-reactivity amongst the clinically normal subjects.

The cluster presentation of the p53 antigen was restricted to subjects with a history of tobacco smoking, continuing up to the time of biopsy in cases 1-4, or discontinued 3years previously (case No.5) (Table 4.4). The age range of the positive patients was from 20-54 years. Patients who had ceased smoking for 10 years or more, were considered as non-smokers

4.10.4. Subjects presenting with an oral keratosis

Archival material was obtained from 15 subjects who had either lichen planus affecting the oral mucosa, or a keratosis affecting the oral mucosa or lips. These subjects had undergone biopsies for diagnostic purposes, and their clinical data and findings are presented in table 4.5.

Table 4.5. Data from patients with oral keratosis

	Subject	Sex	Age	Biopsy site	Tobacco Habit [†]	Growth Fraction (%)	p53 +ve staining *		Pattern of staining. individual cells		
							CM-1	D07	Basal	Supra	Cluster
Keratosis	1	F	64	56	+	5	+	-	-	+	-
	2	F	39	49/50	+	7	-	-	-	-	-
	3	F	56	49	+	2	+	-	+	-	-
	4	M	74	14	+	8	-	-	-	-	-
	5	F	41	13	+	5	-	-	-	-	-
	6	F	63	48	-	9	-	-	-	-	-
Lichen	1	M	42	19	-	7	-	-	-	-	-
Planus	2	F	54	18	-	10	-	-	-	-	-
	3	F	40	19	-	15	-	-	-	-	-
	4	M	54	55	+	9	-	-	-	-	-

† = Less than 20 cigarettes per day

* = Less than 4% of cells in basal region

Biopsy site is registered according to the topography of the mouth as indicated in Appendix A.

p53 was found in 2 subjects who were over the age of 50 years and who smoked tobacco. Although this is a very small sample, it is interesting to note that p53 was not identified in the tissue of subjects with keratosis of the lips even though this tissue is subject to actinic radiation and heat and tobacco smoke from the cigarette.

4.10.5. Study of clinically healthy oral mucosa in subjects from Nepal/India

- Presence of p53 in 'normal' tissue from subjects of Asian Origin

From a total of 20 biopsies were taken from 15 subjects 6 (40%) of the 15 had immunoreactive p53 protein (Table 4.6.). Unfortunately, as indicated above, no clinical data are available concerning the subjects from Nepal/India. Thus it is not possible to ascertain whether there is a similar association between tobacco, alcohol use, the other habits such as betel chewing, or age, and the presence of positive product for p53. Yet there was the same tendency for biopsy sections which demonstrated a suprabasal staining for PCNA also to have regions which stained positive for p53.

Table 4.6 Correlation between PCNA and detection of p53 in biopsies of clinically normal oral mucosa from Nepal/India (6 subjects out of a sample of 16 subjects demonstrated an immuno-positive p53 product).

Subject	Growth Fraction (%)	p53 positive staining				
				Location		Pattern
		CM-1	D07	Basal	Supra	Cluster
1	16	+	-	+	-	-
2	1	+	-	-	+	-
3	7	+	-	+	-	-
4	1	+	-	+	-	-
5†	20	+	-	+	-	+
6	x	+	+	+	-	-

* frequency less than 10%

† Fibro epithelial polyp

x Absence of immunopositive PCNA antigen bearing cells

+

- Absence of feature

4.10.6. Fibroepithelial polyp

Following the observations in a fibro epithelial polyp from Nepal, an immunohistochemical study using archival tissue retrieved from the ICPMR files, was undertaken in the manner previously detailed (4.9.2.). The results are presented in Table 4.7. and confirm the features that were highlighted in the single fibro epithelial polyp from a subject from Nepal (case 5). These features were firstly, a growth fraction (determined by PCNA) which was significantly higher than normal, with 30-50% of the basal cells showing a product.

Secondly, it consistently demonstrated the presence of p53 product. The positive cells were found in the basal layer, and occurred at a regular frequency (5-10% of basal cells). Both these features are unique to the deep epithelial downgrowths pervading the stroma and not the superficial epithelium.

Table 4.7.Correlation of growth fraction and presence and pattern of p53 positive epithelial cells in fibro epithelial polyps

Subject	Sex	Age	Biopsy site	Tobacco Habit	Growth Fraction (%)	p53 positive staining*				
						CM-1	D07	Location		Pattern
								Basal	Supra	Cluster
1	M	34	34	NS	30	+	-	+	-	-
2	F	2	62	NS	50	+	-	+	-	-
3	M	19	22	S	33	+	-	+	-	-
4	F	23	35	NS	28	+	-	+	-	-
5	F	46	23	S	x	+	-	+	-	-
6	F	28	24	NS	x	+	-	+	-	-

* Mean number of p53 positive cells in the basal region of the epithelial downgrowths was 8.5% (SD 6.5)

NS = non smoking

S = smoking

x = absence of immunopositive PCNA antigen bearing cells

+ = positive

- = absence of response

4.10.7. P53 and Oral Carcinoma

A total of 44 cases were selected for immunohistochemical investigation for p53 protein.

It was considered desirable to study more than one block from particular cases, where this was available. (This was carried out in four patients who had up to 5 separate biopsies.)

4.10.7.1. Characteristics of p53 staining patterns in oral carcinomas

Table 4.8 shows the variation in the distribution of the p53 immunopositive cells in the oral squamous cell carcinoma. The staining pattern found with the 3 antibodies tended to be different. The 'sporadic cluster' of reactive cells were the commonest finding, reflecting the possible heterogeneous nature of the tumours.

The staining patterns in the oral carcinomas was so varied and no pattern common to most of the tumours emerged. Only 33% of the biopsy specimens could be described as having any form of regular staining pattern in the tissue. In a number of the cases repeated biopsies had been carried out over a number of years (Cases 9, 18 & 20), and these also demonstrate a change in staining patterns in successive biopsies from the same region of the mouth.

10% or less of the total tumour cells sectioned were p53 positive in most cases (68%). It is important to note that the frequency of positive cells was similarly low in the clinically normal oral mucosal specimens (Table 4.4 in section 4.10.3).

4.10.7.2. Expression of mutant p53 protein in oral carcinoma

The expression of p53 mutant phosphoprotein was analysed in 44 cases of oral squamous cell carcinoma, using three different antibodies (PAb CM-1, D07, and 1801). Of the 44 cases, 30 (68%) showed positive product with at least one of the antibodies (Table 4.8). The reaction product, marking the presence of the antigen, was found in the nuclei. Positive nuclei were stained either homogeneously or in a granular pattern. In none of the carcinomas was there uniform staining of all neoplastic cells. Instead most were characterised by positively stained epithelial islands, or clusters of cells which were distributed widely in some cases and only sporadically in others.

Key to Table 4.8

Antibody PCNA = Primary antibody to PCNA
 CM-1= Polyclonal antibody for p53
 D0-7 = monoclonal antibody to p53
 1801 = monoclonal antibody to p53

Stain = the character and nature of the stain

N = nuclear; **C** = cytoplasmic

First numeral = the percentage of the basal cells stained **1** = 1-9%; **2** = 10-49%; **3** = 50-100%

Second numeral = intensity of stain in the nucleus on a scale of 1-3.

G = general epithelial presentation;

B = basal cells only;

P = rete ridge region;

R = regular;

S = scattered;

C = cluster

◇ = positive presentation

Pr.No	PCNA										CM-1										DO7										I801										Site
	Stain	G	B	P	R	S	C	gram	G	B	P	R	S	C	stain	G	B	P	R	S	C	gram	G	B	P	R	S	C	Differentiation												
1	N/3/3	0	0	0	0	0	0	CN/2/2	0	0	0	0	0	0	0	N/2/2	0	0	0	0	0	0	CN/2/2	0	0	0	0	0	Well	Tongue											
2	-							N/1/2					0	0	N/1/1					0	0		N/1/1				0	0	Poor	Floor											
3	N/2/2	0	0	0	0	0	0	CN/1/2	0	0	0	0	0	0	N/1/2		0			0	0		CN/1/2	0	0	0	0	0	Mod	Tongue											
4	N/2/2	0	0	0	0	0	0	N/3/3	0	0	0	0	0	0	N/3/2					0	0		-			0	0	0	Mod	Tongue											
5	N/2/2	0	0	0	0	0	0	N/1/2	0	0	0	0	0	0	N/1/1		0	0	0	0	0		N/1/2			0	0	0	Mod	H. palate											
6	N/1/2	0	0	0	0	0	0	N/1/1		0	0	0	0	0	N/1/2		0	0	0	0	0		N/1/3			0	0	0	Mod	Trigone											
7	N/2/3	0	0	0	0	0	0	N/1/3	0	0	0	0	0	0	N/1/2		0	0	0	0	0		N/1/2			0	0	0	Well	Tongue											
8	N/1/1	0	0	0	0	0	0	N/3/1	0	0	0	0	0	0	-					0	0		N/1/1	0		0	0	0	Mod	Tongue											
9a	N/2/2	0	0	0	0	0	0	N/1/2		0	0	0	0	0	N/1/2		0	0	0	0	0		N/1/1		0	0	0	0	Mod	Trigone											
9b	N/2/2	0	0	0	0	0	0	N/2/2	0	0	0	0	0	0	N/1/1			0	0	0	0		N/1/2	0	0	0	0	0	Mod	Trigone											
9c	N/2/2	0	0	0	0	0	0	-						N/1/1		0				0	0		-						Ca-in-situ	Trigone											
9d	N/2/2	0	0	0	0	0	0	-							-						0		N/1/2	0	0	0	0	0	Ca-in-situ	Trigone											
9e	N/2/2	0	0	0	0	0	0	CN/3/2	0	0	0	0	0	0	N/1/2	0	0	0	0	0	0		N/1/3	0	0	0	0	0	Mod	Trigone											
10	N/2/2	0	0	0	0	0	0	N/1/2		0	0	0	0	0	-								N/1/1	0		0	0	0	Well	Tongue											
11	N/1/1	0	0	0	0	0	0	N/3/1					0	0	-					0	0		-						Poor	Alveolus											
12	N/2/2	0	0	0	0	0	0	N/1/1					0	0	-					0	0		-						Well	Tongue											
13	N/1/1	0	0	0	0	0	0	-							N/2/2						0		N/1/1			0	0	0	Well	H. Palate											
14	CN/1/1	0	0	0	0	0	0	CN/1/1	0	0	0	0	0	0	-					0	0		-						Mod	S. palate											
15	N/2/2	0	0	0	0	0	0	N/1/1		0	0	0	0	0	-								-						Poor	Trigone											
16	N/2/2	0	0	0	0	0	0	CN/2/1	0	0	0	0	0	0	CN/1/2					0	0		CN/1/2	0	0	0	0	0	Mod	Trigone											
17	N/1/2	0	0	0	0	0	0	N/1/2					0	0	-								-						Mod	Floor											
18a	N/2/2	0	0	0	0	0	0	N/1/2	0	0	0	0	0	0	N/1/2	0	0	0	0	0	0		N/2/2	0	0	0	0	0	Mod	Tongue											
18b	N/3/2	0	0	0	0	0	0	N/1/2		0	0	0	0	0	N/1/1		0			0	0		N/2/2	0	0	0	0	0	Well	Tongue											
19	N/2/2	0	0	0	0	0	0	N/1/1					0	0	-					0	0		-						Mod	Buccal											
20a	N/2/2	0	0	0	0	0	0	CN/2/2		0	0	0	0	0	-								-						Severe dysplasia	Buccal											
20b	N/2/2	0	0	0	0	0	0	CN/2/1		0	0	0	0	0	-								-						Ca-in-situ	Buccal											
21	N/2/2		0	0	0	0	0	CN/1/1	0			0	0	0	N/1/1					0	0		N/1/1			0	0	0	Poor	Trigone											
22	N/2/2		0	0	0	0	0	N/1/2				0	0	0	-					0	0		N/1/1			0	0	0	Poor	S. palate											
23	N/3/1	0	0	0	0	0	0	N/1/2				0	0	0	-					0	0		N/1/2	0	0	0	0	0	Mod	S. palate											
24	N/2/2		0	0	0	0	0	N/1/1					0	0	-								-						Poor	Buccal											
25	N/2/2		0	0	0	0	0	CN/3/1	0			0	0	0	N/1/1					0	0		N/1/1		0	0	0	0	Mod	Floor											
26	N/2/2	0	0	0	0	0	0	N/1/1	0			0	0	0	N/1/1					0	0		N/1/1	0		0	0	0	Mod	Tongue											
27a	N/2/3	0	0	0	0	0	0	-							N/2/1								N/2/1			0	0	0	Mod	Buccal											
27b	N/2/2	0	0	0	0	0	0	CN/1/3		0	0	0	0	0	N/2/1					0	0		N/1/1			0	0	0	Mod	Buccal											
28	N/2/2	0	0	0	0	0	0	N/2/2		0	0	0	0	0	N/2/2					0	0		N/2/2			0	0	0	Mod	Floor											
29	N/2/2	0	0	0	0	0	0	CN/1/1				0	0	0	-								-						Well	S. palate											
30	N/1/2	0	0	0	0	0	0	-							N/1/1					0	0		-						Mod	Buccal											
31	N/2/2	0	0	0	0	0	0	-							N/1/2					0	0		N/1/2	0	0	0	0	0	Ca-in-situ	Buccal											

Differentiation = 'Well', 'Moderately', and 'Poorly' differentiated squamous cell carcinoma (as diagnosed by hospital histopathologists)

4.10.7.3. Staining patterns and frequency of detection of mutant p53 protein in oral carcinomas by the three primary antibodies for p53

The two antibodies PAb CM-1 and 1801 gave very similar staining patterns although these antibodies recognise different epitopes on the p53 protein (Table 4.2.). The PAb D07 demonstrated a positive product in 48% (21/44) of cancers, PAb 1801 55% (24/44) and PAb CM-1 demonstrated a positive product in 68% (30/44). (These differences were not statistically significant.)

PAb D07 identified a positive product in two carcinomas (5%) not detected by the other two antibodies.

Table 4.9. Numbers and proportions (%) of oral squamous cell carcinomas or carcinoma *in situ* reacting with three antibodies to p53 protein

	No.	CM-1	D07	1801	PAb x 3
Carcinomas - General	44	30 (68%)	21 (48%)	24 (55%)	16 (36%)
Poorly differentiated	15	6 (40%)	3 (20%)	4 (27%)	3 (20%)
Moderately well differentiated	15	14 (93%)	11 (73%)	12 (80%)	9 (60%)
Well Differentiated	10	7 (70%)	4 (40%)	5 (50%)	4 (40%)
		27	18	21	16
Ca-in situ	4	3 (75%)	3 (75%)	2 (50%)	1 (25%)

A tumour was recorded as being positive for p53 if more than 5% of the cells were positive.

PAb x 3 = response to all three antibodies.

4.10.7.4. Relation of p53 staining and Bryne's modification of Anneroth's malignancy score

Table 4.10 shows the comparison between the Bryne's modified score and the proliferation rate (as determined with antibody to PCNA), on tissue which is positive for abnormal p53 protein.

Positive p53 staining was present in both low grade and high grade tumours, as categorised by Bryne's index. The positive expression of p53 was more frequent, 17/24 (71%), in those tumours in the high grade category (Bryne index score ≥ 11 points), than in the lower grade (Bryne index ≤ 10 points) tumours (12/20, 60%) this difference was not significant ($p = 0.84$).

The intra-observer error between the two assessment periods at an 8 week interval was estimated as 8%.

Table 4.10 Correlation of Bryne's index with p53 positivity.

Modification of Anneroth's malignancy score (sum of 5 morphological features)	No.	p53 positivity		Deaths
		Positive	Negative	
5-10 points	20	12	8	10 (7)
> 10 points	24	17	7	18 (13)

The statistical analysis presented in Table 4.13 demonstrates figures based upon the Bryne index are almost significant, with patients with a score of >10 having an increased risk of dying (2.3 times that of those patients with the lower score)

($p = 0.066$). However the p53 positivity alone did not prove to be an important survival marker, with p53 negative patients having a greater risk of dying than the p53 positive patients, though these results were not significant ($p=0.95$). These results may be complicated by the fact that the cancers with a very high Bryne score (15+) often failed to demonstrate any immunoreactivity.

4.10.7.5. Positive p53 product in 'marginal' area around the carcinoma

In 42% (14/33) of the carcinomas where both the tumour and the epithelium were present in the same section, the adjacent overlying epithelium also demonstrated focal areas of cells immunoreactive with the antibodies to p53. The antigen-expressing cells were occasionally isolated but frequently they were found in clusters, where they were always adjacent to the basal region of the epithelium (in the progenitor compartment of the epithelium).

Frequently there appeared to be a reciprocal arrangement whereby p53 positive cells were located in either the basal or suprabasal region. However, in instances where clusters of positive cells were present, then both the basal and suprabasal region were involved.

Table 4.11.Presentation of p53 positive cells in epithelium adjacent to oral carcinomas

Patient	Sex	Age	Oral Habit*	PCNA (%)	p53 + ve cells [#]				
							Location		Pattern
					CM-1	D07	Basal	Supra	Cluster
1	M	51	+	30	1	1	+		+
2	F	72	+	30	1	-	+		
3	M	74	+	10	1	-		+	+
4	M	66	+	80	1	1		+	
5	F	53	+	40	2	1		+	+
6	F	53	+	15	1	1		+	+
7	F	53	+	30	1	1	+		
8	M	66	+	10	2	1		+	+
9	F	85	nil	40	-	2		+	+
10	F	85	+	20	1	-		+	+
11	M	63	nil	60	1	1	+		+
12	M	63	nil	25	1	1	+		
13	M	53	+	25	2	2	+		+
14	F	56	+	35	1	1		+	+

* Oral habits where present were at least 20 cigarettes per day with or without alcohol

1 = 1-10%; 2 = 11-50% of cells in the area affected

Staining pattern = reflects the region where the immunoreactive cells most frequently appeared, either in the basal layer or the supra basal area, and whether they appeared in clusters or as individual cells.

+ Positive for the feature described

- Absence of immunopositive response

Location = describes the region of the majority of the positive cells.

4.10.7.6. Deaths among patients with oral carcinoma

There have been 28 deaths during the period of the survey (1989-till the present 1993), with an average follow up time of 14 months. Two patients deaths were unrelated to their oral carcinoma. Only one patient below the age of 40 years presented with an oral squamous cell carcinoma. This is consistent with the national average of 1-3% of oral squamous cell carcinomas presenting at or below the age of 40 years.

Amongst the patients who died and who had tumours scoring >10 in the Bryne index, 75% (18/24) were also p53 positive, whereas for those which were ≤ 10 were p53 positive in 50% of cases (10/20), however once the survival time is also considered then these figures lose any apparent significance.

4.10.7.7. p53 expression in the epithelium adjacent to invasive carcinoma

The sections were re-examined to see the difference in the presentation of p53 positivity in the epithelium adjacent to a carcinoma (whether it was dysplastic or not), with the cells of the tumour itself.

The 44 oral carcinomas demonstrated a positive response in 32 sections (from a total of 79 biopsy specimens). This response was frequently present in both the mucosal epithelium and tumour. (A number of the biopsies specimens of oral carcinomas demonstrated minimal epithelium on the sections, and these were excluded.) The remainder possessed both epithelium and tumour in sufficient quantity to allow this

feature to be analysed. Most of the adjacent epithelium demonstrated varying degrees of dysplasia. The results are presented in Table 4.12.

Table 4.12. Distribution of cells which were immunopositive for p53

Tissue section	p53 positivity
	No. (%)
Adjacent epithelium	2 (6)
Only tumour cells	13 (41)
Adjacent epithelium and tumour in the same section	17 (53)

This study involved both smokers and non-smokers (non-smokers numbered only 5 patients). However there was no significant association between tobacco smoking and p53 expression in the adjacent epithelium, and this is probably due to the few number of non-smokers

Table 4.12 demonstrates that both the adjacent epithelium and the tumour itself can be positive for mutant p53. The carcinomas which featured p53 positivity in the dysplastic epithelium adjacent to invasive carcinoma were among those with a low malignancy score (5-10) according to Bryne's grading. (Bryne et al, 1989)

4.10.7.8. Statistical analysis of data from oral carcinomas

All variables were analysed independently against survival using the life-table method of Lee (1980) and the differences between the curves evaluated by the Mantel-Cox test (Kaplan and Meier, 1958). Statistical significance was considered when the P value was less than 0.05. In the second part of the study, variables with prognostic significance were compared using the regression model of Cox (1972) with the most significant variable being entered first.

Data analysis was facilitated by using a computer program- Statistical Package for Interactive Data Analysis (SPIDA)¹.

¹ Produced by the statistical computing laboratory of Macquarie University, Sydney, Australia

Table 4.13. Hazard ratio and life expectancy associated with histological grading, p53 expression, tobacco smoking, alcohol consumption, clinical staging and site in 44 oral squamous cell carcinomas.

Variable	Values taken	Hazard Ratio	95 % confidence interval	p-value	Median Life Expectancy (mths)
Bryne Index	≤10	1	-	-	-
	>10	2.3	(0.95,5.6)	0.07	17
p53	Negative	1	-	-	15
	Positive	0.97	(0.4,2.36)	0.95	27
Smoking	Non Smoking	1	-	-	-
	Smoking	2.15	(0.63,7.31)	0.22	26
Tumour size	T1	1	-	-	-
	T2,T3,T4	1.41	(0.88,2.24)	0.15	-
	T1+T2	1	-	-	-
	T2+T3	1.94	(0.76,4.94)	0.17	14.5
Node	N0	1	-	-	-
	N2	1.59	(1.00,2.53)	0.05	-
Sites	Retro molar	1	-	-	14
	tongue	0.39	(0.11,1.39)	0.12	34
	Palate	0.34	(0.1,1.21)	0.10	-
	Floor	0.95	(0.25,3.59)	0.94	10
	Alveolus	0.30	(0.04,2.63)	0.28	27
	Buccal mucosa	0.39	(0.08,1.21)	0.09	60
	Sites other than floor of mouth	1	-	-	34
	Retro molar	2.83	(1.20,6.65)	0.02	11
Alcohol	Non drinker	1	-	-	-
	Drinker	1.002	(1.00,1.01)	0.5	-
	<80 gm/day	1	-	-	28
	≥ 80 gm/day	1.23	(0.53,2.85)	0.64	13.5

Hazard ratio² = ratio of the instant where the probability of dying in one group is compared to another group which is used as the 'standard' for the comparison (this has a hazard ratio of '1'). It is an indicator of the relative risk of dying at any particular moment in time.

95 % confidence interval = if the test were applied to another 100 patients then 95 of these would have results which would fall in this interval.

² P. Armitage and G. Berry. Statistical Methods in Medical Research. 2nd Edition 1987, Blackwell Science Publications, Oxford, U.K.

The results of the univariate analysis show that significant prognostic indicators are found in the clinical involvement of contra lateral or bilateral lymph nodes, and the site of the primary carcinoma. Although there are trends in the other factors considered no significant prognostic effects could be shown by univariate analysis. This possibly reflects the small sample size. However, the 'hazard ratio' allows some interpretation of the remaining data.

The site of the tumour was a significant factor in the prognosis. Tumours of the retromolar trigone region and floor of the mouth had a poorer prognosis than any other location in the mouth. Patients with carcinomas at this site had three times the risk of dying from their disease than did patients who had tumours at other sites in the mouth

The presence or absence of involvement of nodes was significant ($p \leq 0.05$) with patients who presented with clinically involved nodes having a risk of dying 1.6 times that of patients without nodal involvement.

The other major risk factor was smoking, which had a relative risk of 2.2 for smokers, or those who ceased for a period of less than 10 years though this was not significant in this study. (A figure of ten years was an arbitrary figure. Doll et al (1976) calculate that a subject who has ceased tobacco smoking is at increased risk for at least 10-20 years, but this risk continues to increase in patients who continue to smoke, whereas it levels off if smoking is discontinued. Those who have quit the habit continue to be at a greater risk than those who have never smoked.

The size of the tumour also influenced the risk, although with the limited size of the study this did not achieve statistical significance. The abuse of alcohol was not shown to have any significance in the outcome of these oral carcinomas, possibly for the same reason.

It is surprising that the p53 values showed no difference in outcome, and in fact those staining positive for p53 tended to have a longer life expectancy than those which were

p53 negative. One suggestion for this is that the most dysplastic carcinomas generally failed to produce a nuclear product in response to any of the three antibodies to p53. Table 4.8 demonstrates that the most frequent demonstrator of immunoreactivity was the moderately well differentiated carcinoma. However these results may be a reflection of the fact that the mutation in the p53 gene is an early event in the development of oral carcinoma.

A multivariate analysis was undertaken on those variables which were significant or almost significant (Table 4.14).

Table 4.14. Multivariate analysis of data which was significant in the univariate analysis: site; node involvement and smoking.

Variable	Coefficient	Standard Error	p-value	Hazard ratio	95%confidence interval
Nodes	0.54	0.25	0.029	1.71	(1.06,2.77)
Site	1.24	0.52	0.017	3.47	(1.25,9.66)
Smoking	1.42	1.06	0.179	4.13	(0.52,32.68)
Nodes	0.42	0.24	0.083	1.52	(0.95,2.45)
Site	1.12	0.52	0.032	3.05	(1.1,8.49)

Nodes = contralateral and bilateral nodes.

Site = floor of mouth or retro-molar region

The inclusion of smoking as a variable for multivariate analysis had the effect of lessening the significance of the effect of nodal involvement and site. This therefore showed that smoking was not a significant contributory factor in this survival study.

4.10.8. Oral submucous fibrosis

4.10.8.1. Presence of p53 in OSF tissue

The specimens of OSF mucosa were screened for the presence of abnormal p53 protein using two monoclonal antibodies (PAb D07, and 1801), as well as a polyclonal antibody (PAb CM-1) in the method referred to in section 4.9.2.

The distribution of the p53 staining in the OSF tissue, is presented in Table 4.15. There appears to be no common clinical feature in the biographical data of patients with biopsies positive for p53, though the median mouth opening was greater for subjects who are p53 negative.

Figure 4.3 a & b demonstrate the increased growth fraction which was associated with the presence of abnormal p53 protein.

Unless otherwise indicated the results from the three antibodies were pooled, and oral mucosa reacting with at least one antibody to p53 was regarded as positive.

All cases of 'advanced stage' OSF had detectable p53 product (Table 4.15). All these biopsy specimens had been taken from the posterior buccal mucosa. Epithelial atrophy was also a feature in common. No other correlation between expression of p53 and stage of disease were apparent.

The distribution of p53 in all cases of oral submucous fibrosis seemed to be unique, in that only the basal layer of cells are immunopositive. This distribution of p53 positive cells found in OSF was not observed in any other tissues examined to date, neither the few cases in the clinically normal mucosa, nor in the oral squamous cell carcinomas..

Key to Table 4.15

Stage = the disease stage of oral submucous fibrosis

Habit = cig cigarette

pan the quid made from the areca nut, tobacco and other spices.

sup Supari = the areca nut.

NA Not available

Opening = Oral opening in mm

Dysplasia = the score from the Smith and Pindborg (1969) score / the number of dysplastic groups present in the tissue.

Epithelium = the histological presentation of the epithelium

Bx = the biopsy site as represented by Appendix A

Antibody

PC-10 = Primary antibody to PCNA

CM-1= Polyclonal antibody for p53

D0-7 = monoclonal antibody to p53

1801 = monoclonal antibody to p53

Stain = the character and nature of the stain

N = nuclear; C = cytoplasmic

First numeral = the percentage of the basal cells

stained 1 = 1-9%; 2 = 10-49%; 3 = 50-100%

Second numeral = intensity of stain in the nucleus on

a scale of 1-3.

G = general epithelial presentation;

B = basal cells only;

P = rete ridge region;

R = regular;

S = scattered;

C = cluster

◇ = positive presentation

CM-1																												DO-7										1801									
Slide	Sex	AGE	Stage	Habit	opening	dysplasia	Epithelium	Bx	PC 10	stain	G	B	P	R	S	C	stain	G	B	P	R	S	C	stain	G	B	P	R	S	C																	
1	M	60	Mod adv.	cig,pan,sup	30	25/9	Atrophy	19	80	N/1/1							N/1/1																														
2	M	30	Early	CIG	27	11\4	Hyperplasia	19	60	N/3/1							N/2/1																														
3	M	26	Advanced	sup	18	11\6	Atrophy	19	30	N/2/1							-																														
4a	F	53	Early	None	40	5\3	Normal	50	13	-							-																														
4b			Early/mod. adv	None	40	10\5	Hyperplasia	18	21	CT							CT																														
4c			Mod advanced	None	40	17\8	Hyperplasia	19	23	CT							CT																														
5a	M	30	Early/mod adv.	Sup	30	9\5	Normal	19	13	-							-																														
5b			Early	Sup	30	5\3	Hyperplasia	18	23	-							-																														
6a	F	55	Mod adv	pan,sup	35	9\5	Normal	20	36	N/2/2							N/1/1																														
6b			Early	pan,sup	35	11\6	Hyperplasia	40	26	N/1/1							-																														
7	F	25	Very early	pan	45	8\4	Normal	56	56	N/1/1							-																														
8a	F	22	Early/mod adv	sup reg	19	10\5	Atrophy	18	53	N/1/2							-																														
8b			Mod adv	sup reg	19	12\4	Atrophy	19	x	-							-																														
9	M	43	Early	cig reg	62	12\7	Normal	20	14	-							-																														
10a	F	28	Mod adv/adv	sup Occ	32	12\7	Normal	19	26	CN/1/1							-																														
10b			Early \mod adv	sup	32	10\5	Atrophy	18	20	-							-																														
11a	F	51	Early/mod adv	Sup	25	5\3	Hyperplasia	20	57	CN/1/1							-																														
11b			Early	Sup	25	8\5	Normal	19	14	CN/2/1							-																														
12	F	32	Mod adv.	sup	23	10\5	Normal	18	29	N/3/2							N/1/1																														
13	F	36	Mod adv	none	19	10\4	Atrophy	25	27	N/1/1							-																														
14	NA	NA	Early	NA	NA	8\4	Normal	NA	30	N/1/1							-																														
15	NA	NA	Mod adv	NA	NA	6\3	Atrophy	NA	52	-							-																														
16	NA	NA	Advanced	NA	NA	11\6	Atrophy	NA	18	N/1/1							-																														
17	NA	NA	Mod adv	NA	NA	9\5	Hyperplasia	NA	x	-							-																														
18	NA	NA	Mod adv	NA	NA	19\6	Atrophy	NA	x	CN/3/1							-																														
19	NA	NA	Mod adv	NA	NA	7\4	Atrophy	NA	1	N/2/2							-																														
20	NA	NA	Advanced	NA	NA	10\5	Atrophy	NA	9	N/1/2							-																														

Stage = more than one stage of the disease seem to frequently present CT = cut through

Figure 4.3.a the presentation of an increased growth fraction in OSF (PAb PC-10)(x 130)



Figure 4.3.b. The presence of abnormal p53 in OSF. (Note the pigmentary incontinence with melanin accumulation in the upper part of the lamina propria, presumably in macrophages) (x 130)

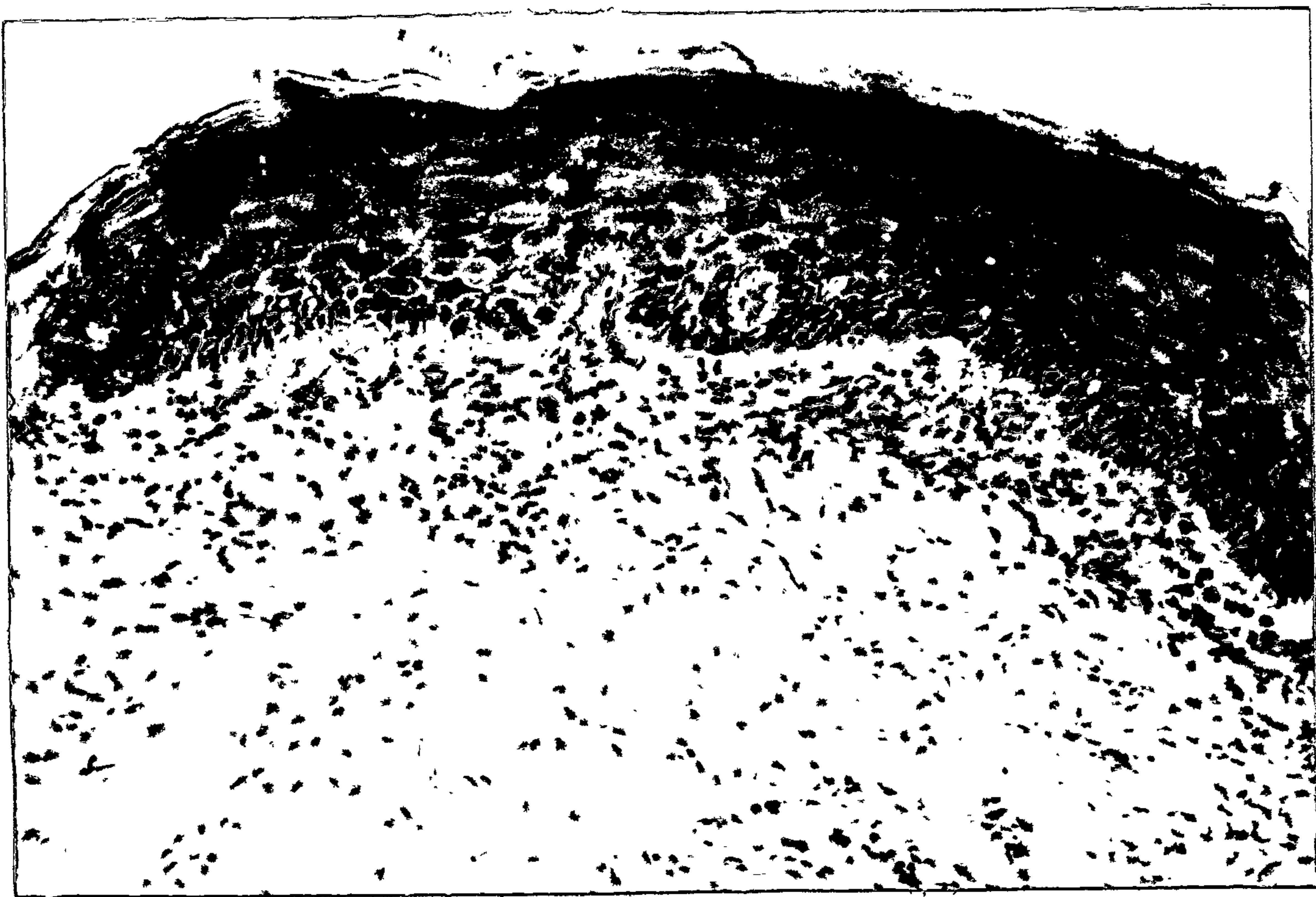


Figure 4.3.c. Abnormal p53 presentation in OSF (x 260)(Pab DO7)

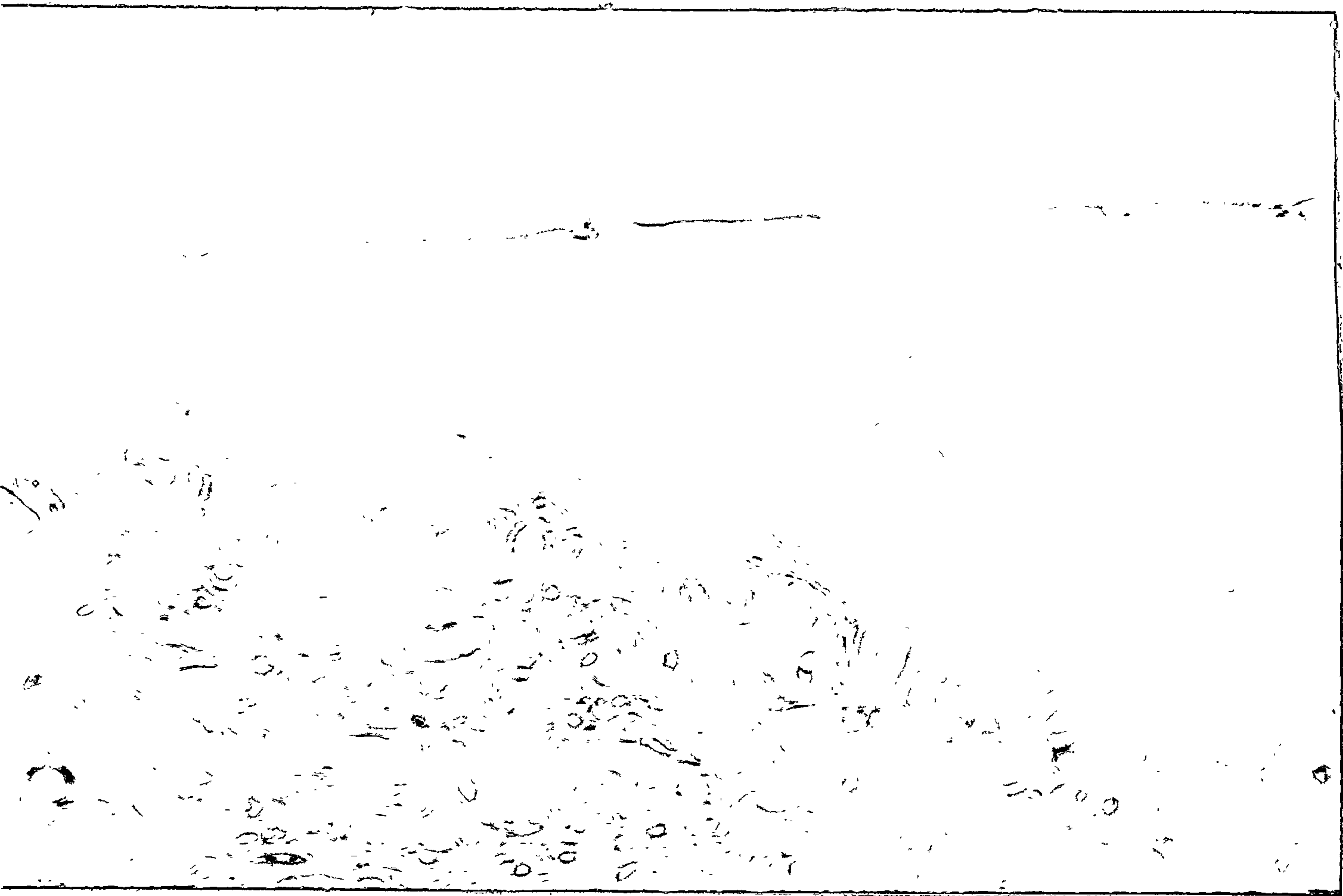


Table 4.16 Prevalence of positive p53 biopsies in patients with oral submucous fibrosis, grouped according to the clinical stage of the disease.(All positive presentations were included. Biopsy sections = 27, number of patients = 20)

	No. of biopsy sections	Very Early (%)	Early (%)	Moderately Advanced (%)	Advanced (%)
p53 Positive	14	0 (0)	4 (15)	6 (22)	4 (15)
Negative	13	1 (4)	4 (15)	8 (30)	0 (0)
	27	1 (4)	8 (30)	14 (52)	4 (15)
Chi test for early and advanced OSF p=0.43					

Table 4.17 Correlation of epithelial thickness and presentation of p53 positive cells in oral submucous fibrosis.(Biopsy sections = 27)

	No. of sections	Atrophic	Normal	hyperplastic
p53 positive	14	6 (22%)	4 (15%)	4 (15%)
Negative	13	4 (15%)	2 (7%)	7 (26%)
	27	chi test for atrophic and normal against hyperplastic p=0.18		

The data in Table 4.17 demonstrates the variation of frequency of abnormal p53 presentation according to the the number of cell layers in the epithelium. The notable feature was that p53 protein occasionally was found, irrespective of the number of epithelial cell layers in the OSF lesions.

The tissue was examined for signs of dysplastic change. Table 4.18 presents the degree of epithelial dysplasia and is based upon the scoring system of Smith and Pindborg (Smith and Pindborg, 1969). The division into 'moderate' and 'severe' was as follows with 'moderate' score being 3-5 and 'severe' ≥ 6 . This division was arbitrary.

It should be noted that clinically normal mucosa occasionally demonstrated a mild degree of atypia, with a score of 1 or 2 (according to the Smith and Pindborg classification). It should be noted that innocent lesions such as oral viral papillomas and other miscellaneous mucosal benign lesions may score even higher using this grading system.

A copy of the grading system of Smith and Pindborg (1969) is presented in Appendix C

Table 4.18 Comparison between the degree of dysplasia and p53 immunopositivity of the oral epithelium in oral submucous fibrosis.

	Total No. of biopsies	Mild to Moderate (1-5)	Severe (6+)
P53 Positive	13	7	6
Negative	12	10	2
Unknown	2	0	2
	27	chi test p = 0.35	

All the specimens of OSF which were examined, exhibited some degree of dysplasia, and there was no significant correlation between the degree of epithelial dysplasia and expression of p53. The specimen with the least cellular atypia fulfilled three of the criteria of Smith and Pindborg (1969), thus there were at least three types of cellular atypia present in the tissue sections.

Table 4.19 Oral Opening of patients with OSF - measured as maximal inter-incisal distance.

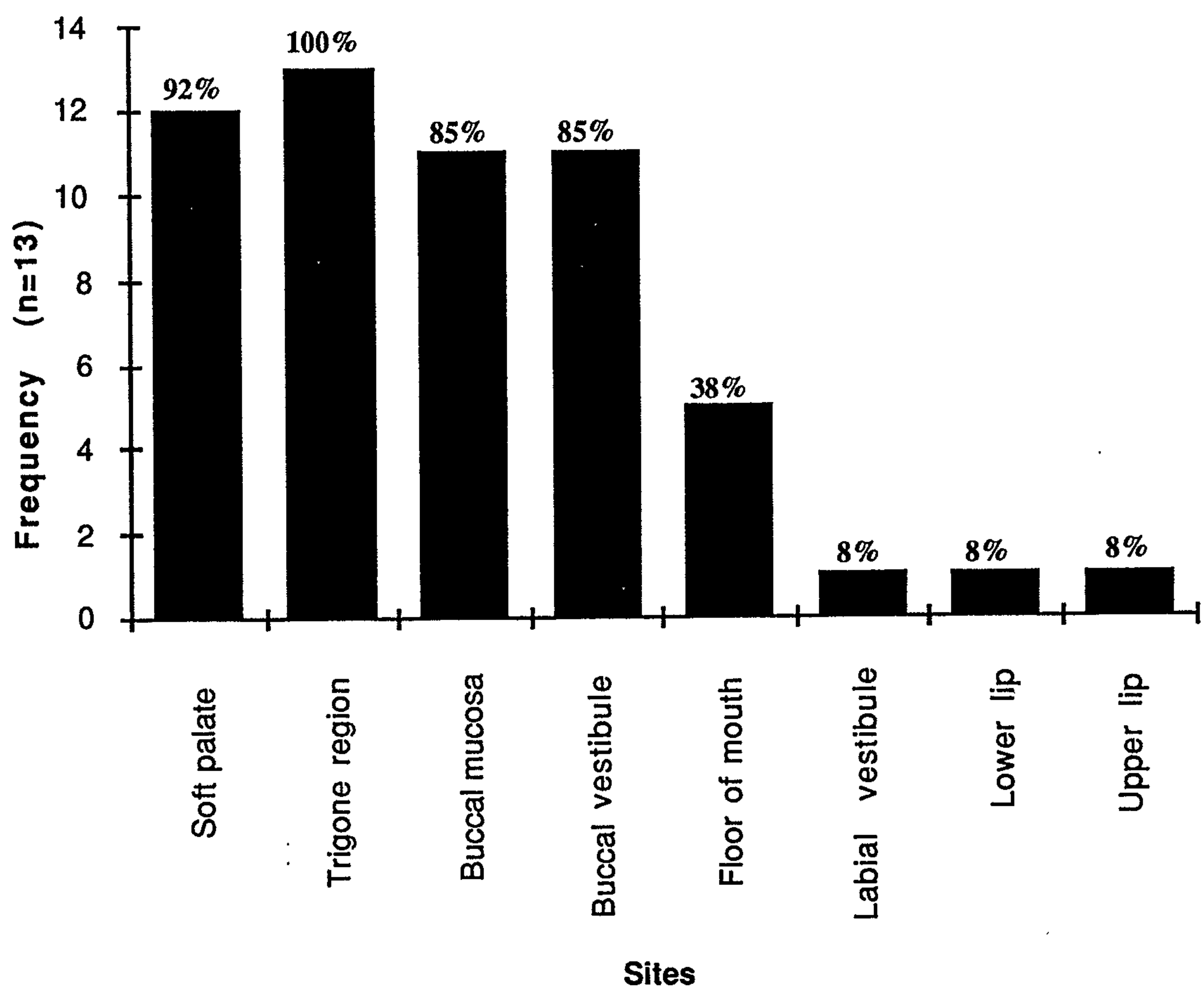
	No. of subjects	Normal (≥ 36 mm)	Limited (≤ 35 mm)
p53 positive	7	0	7
Negative	6	4	2
	13	chi test $p = 0.009$	

All patients with a restricted oral opening lacked any cause of limited opening other than OSF, and all demonstrated fibrous bands in the buccal mucosa on clinical examination, with subsequent confirmation of OSF histologically. The mean opening distance for the nine subjects with an opening of < 36 mm was 24.8 mm (SD=5.2). In 7 of these, p53 protein was found immunohistochemically in their oral biopsies, whereas it was not detected in any of the 4 biopsies from the patients with normal interincisal opening, and this was a significant difference ($p = 0.009$)

4.10.8.2. Regional variation within the mouth

Figure 4.4. demonstrates the distribution of the clinical signs of oral submucous fibrosis in the oral cavity from 13 patients. The most frequently involved site was the retromolar trigone area, followed by the soft palate region. While the literature suggests that this region is frequently affected, Bhonsle (1987) notes that there are regional variations in the presentation of this disease.

Figure 4.4 Intra-oral distribution of sites affected by oral submucous fibrosis



As the disease seemed to be progressive (usually being present in the retro-molar trigone and occasionally presenting anteriorly) it seemed reasonable to compare this region between patients. The buccal mucosa was a common biopsy site for all patients. The region of the premolars, below the occlusal plane had been chosen, as in the severe cases this was the most posterior point accessible for a biopsy. Hence biopsies from this part of the mouth were compared for: stage of disease; oral opening and the presence of p53 product.

Figure 4.4. shows that all the patients (13/13) had the posterior region of their oral cavity affected at a clinical level. Tissue samples which were positive for p53 were from subjects where the disease had progressed to involve the buccal mucosa. Among these, 85% (11/13) were affected, reacting with one of the antibodies, but 31% (4/13) responded with all three antibodies.

Most of the patients with clinically evident OSF and who expressed abnormal p53 antigen, either smoked tobacco or chewed the areca nut. It may be that the tissue affected are adjacent to where the tobacco or areca nut was held or that the region represents possible 'pooling areas' for oral these oral irritants.

Table 4.20. Correlation of sex and stage of the disease for the presentation of p53 positive tissue in tissue specimens from the buccal mucosa.

Sex	V. Early	Early	Mod Adv	Adv	Total
Female	2	0	4	1	7
Male	0	1 (1)	1 (1)	1	5

() = negative response to antibodies to p53

Only one patient (female) has been excluded from Table 4.15 and that was due to the fact that the biopsy block for the region corresponding to the buccal mucosa was 'cut through' and hence provided no data.

Where more than one biopsy specimen was present, for any one patient, then the biopsy demonstrating the most advanced stage of the disease was taken.

Table 4.20 demonstrates that for that region of the mouth which is commonly affected in all OSF patients from Nepal, namely the buccal mucosa, all the females, and 60% of the males were positive for the p53 product. This may only represent the region where the 'pan' is held or the 'betel nut' chewed. However, there two patients who state that they do not have an oral habit, and yet are positive for p53.

All biopsies were taken from regions which allowed clinically normal and diseased tissues to be examined. However the light microscopic examination of haematoxylin and eosin sections frequently failed to demonstrate any normal mucosa. Examination of the tissue for abnormal p53 demonstrated it in a widespread distribution throughout the biopsy specimen.

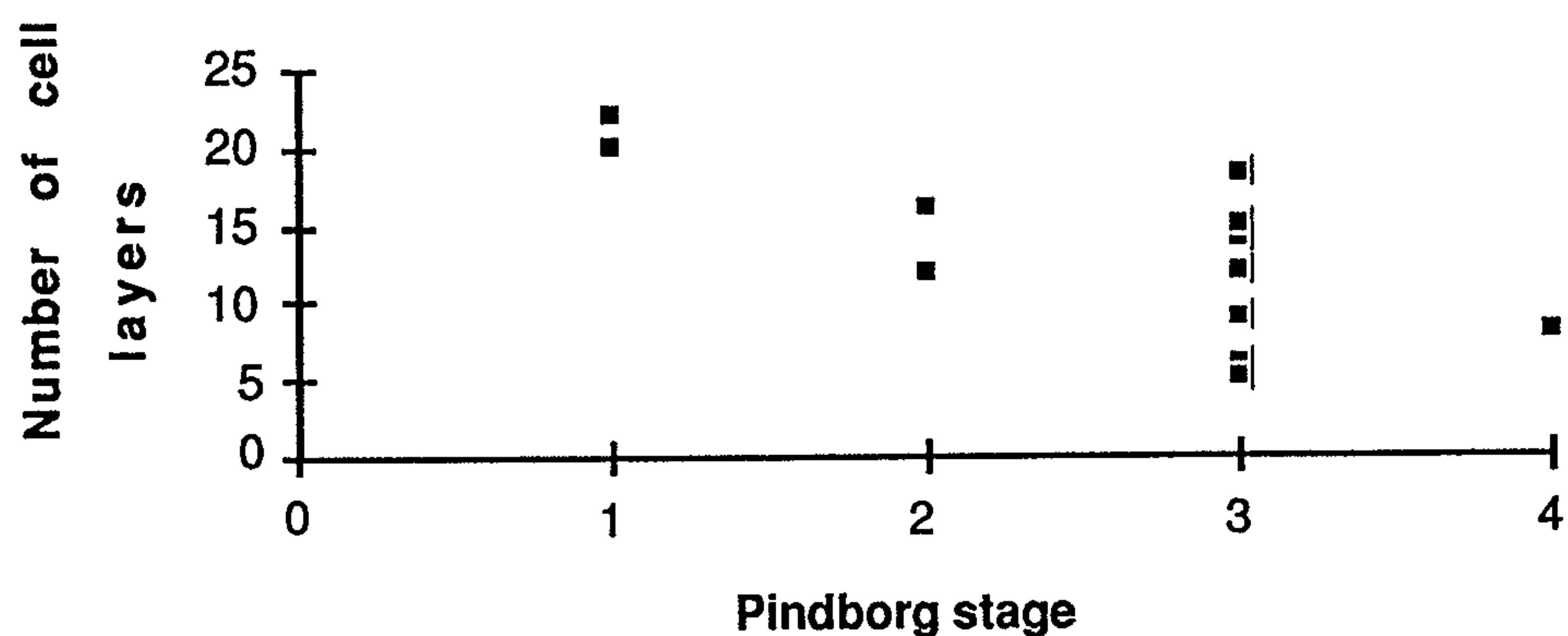
Regions that were not clinically affected by OSF, occasionally demonstrated areas positive for p53.

4.10.8.3. Analysis of the buccal mucosa of patients suffering from oral submucous fibrosis

Fig 4.5. demonstrates that if the grading system of Sirsat and Pindborg (1967) is used then there tends to be a reduction in epithelial thickness as the stage of the disease progresses. This suggests that the epithelial changes coincide with those changes taking place in the dermal region.

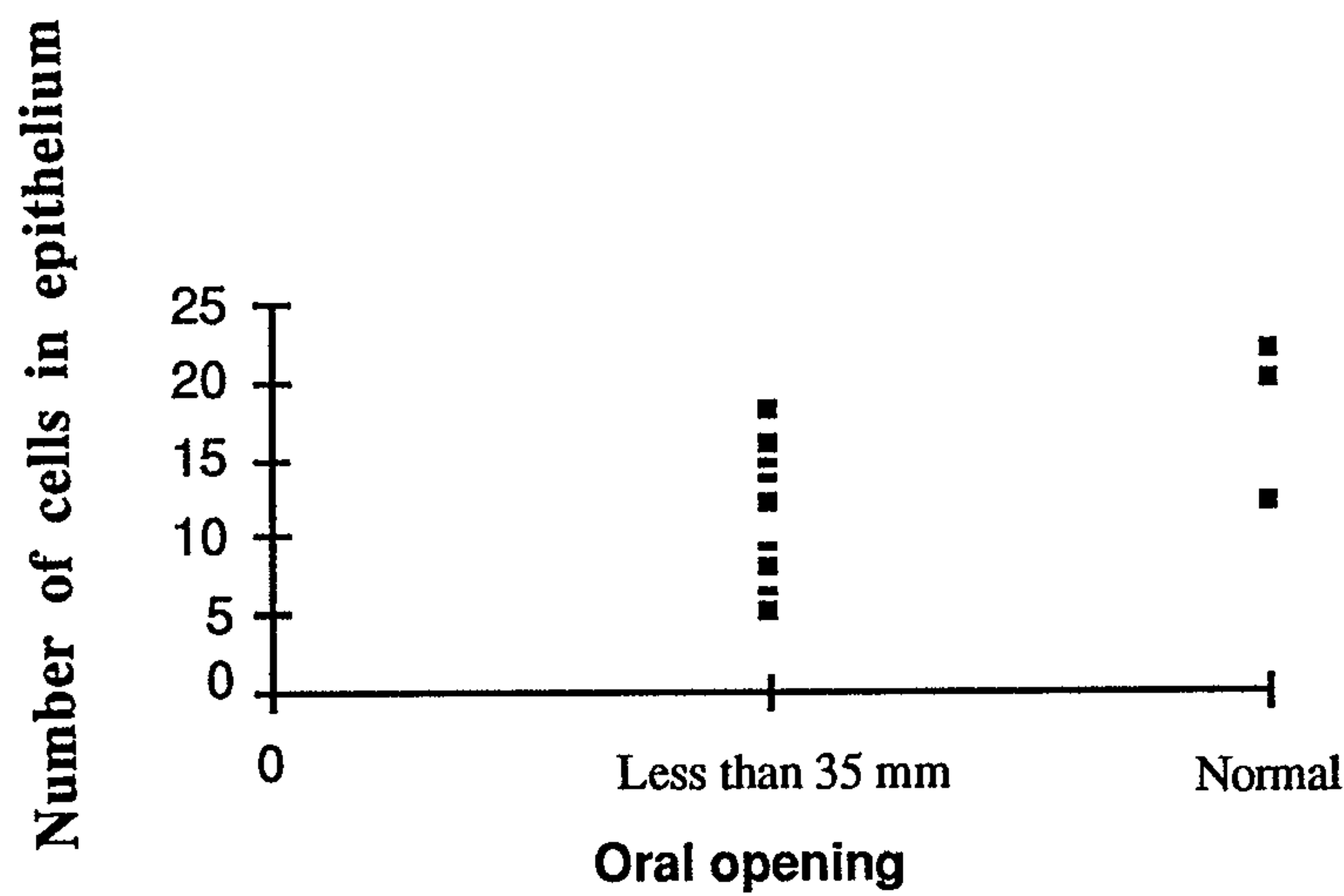
The clinically normal epithelial tissue had an average of 28 ± 6 cell layers. Although this tissue featured rete ridges, which was not present in the OSF material, an average was taken from a region of normal tissue.

Figure 4.5.. Correlation of Pindborg staging and number of cell layers in epithelium for mucosa affected by oral submucous fobrosis.



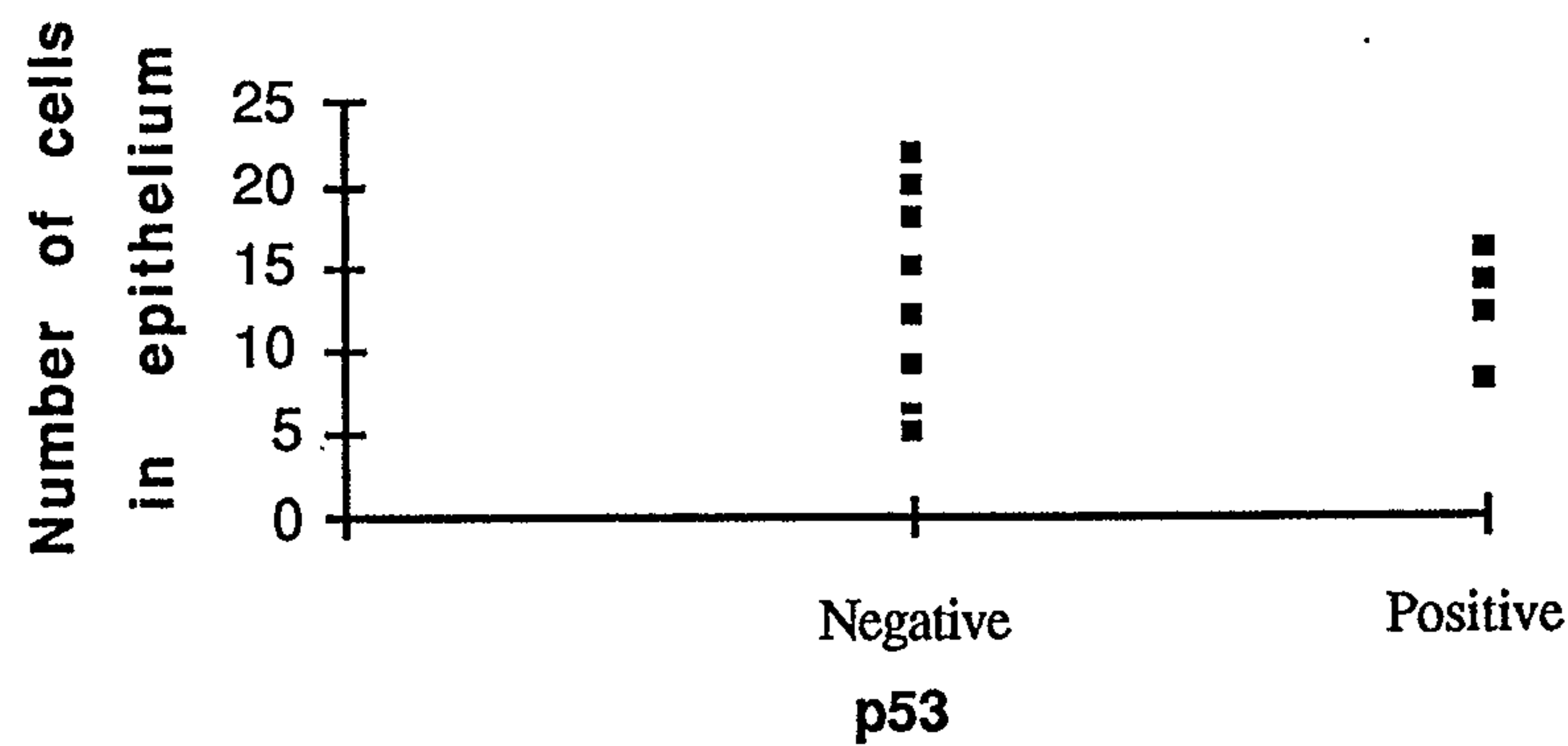
The same trend, for the epithelial cell layer to be reduced, is also seen if an alternative, and perhaps a more objective, parameter of 'oral opening' is used (Fig 4.6.)

Figure 4.6 Correlation of oral opening and the number of cells present in the epithelium



If the tissue is further examined for abnormal p53 protein then the more atrophic tissue tended to present with the abnormal protein. (Fig 4.7.). The small and unequal numbers in each subgroup did not permit a valid statistical analysis.

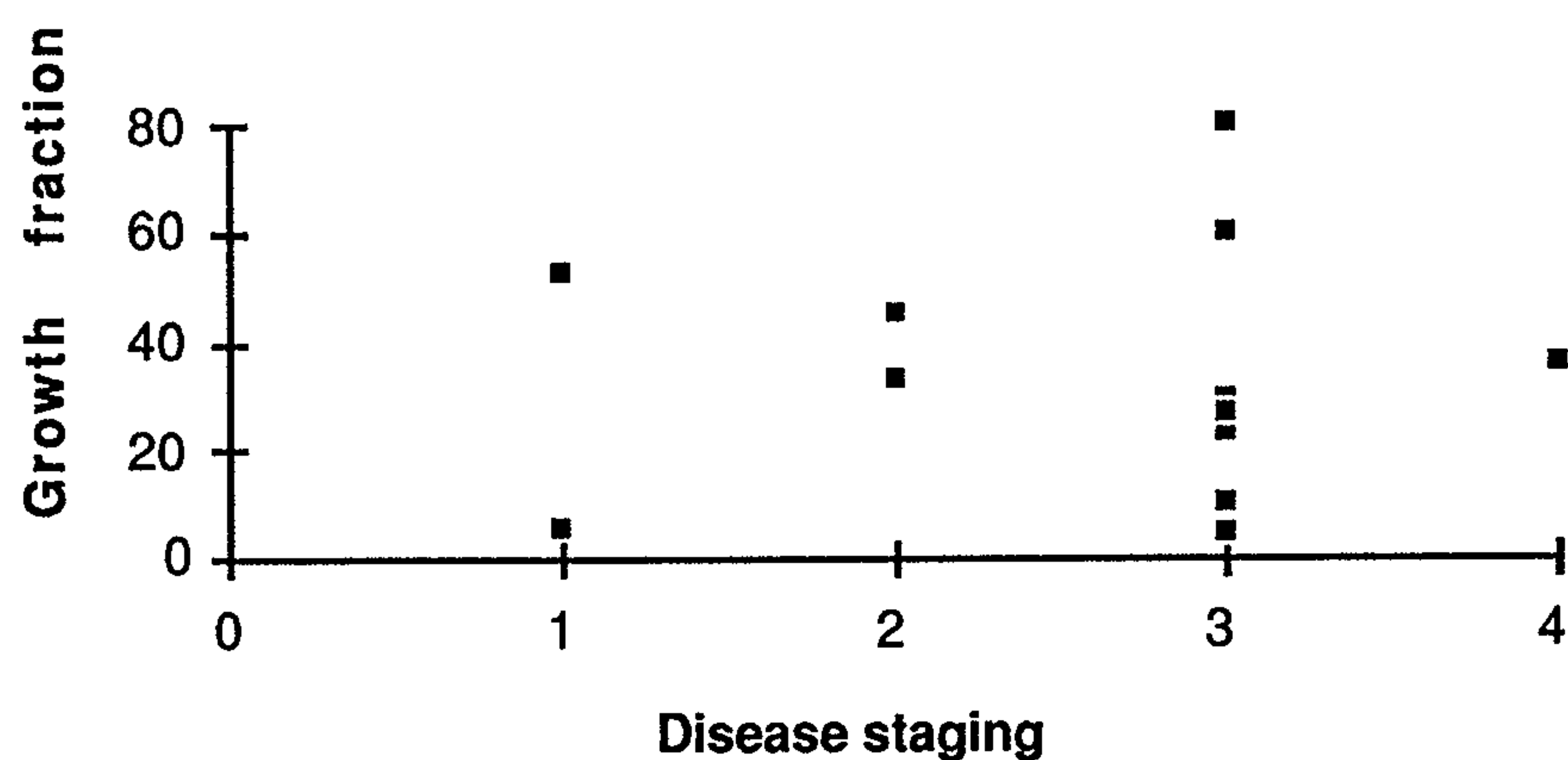
Figure 4.7 p53 positivity and number of cells in the mucosal epithelium



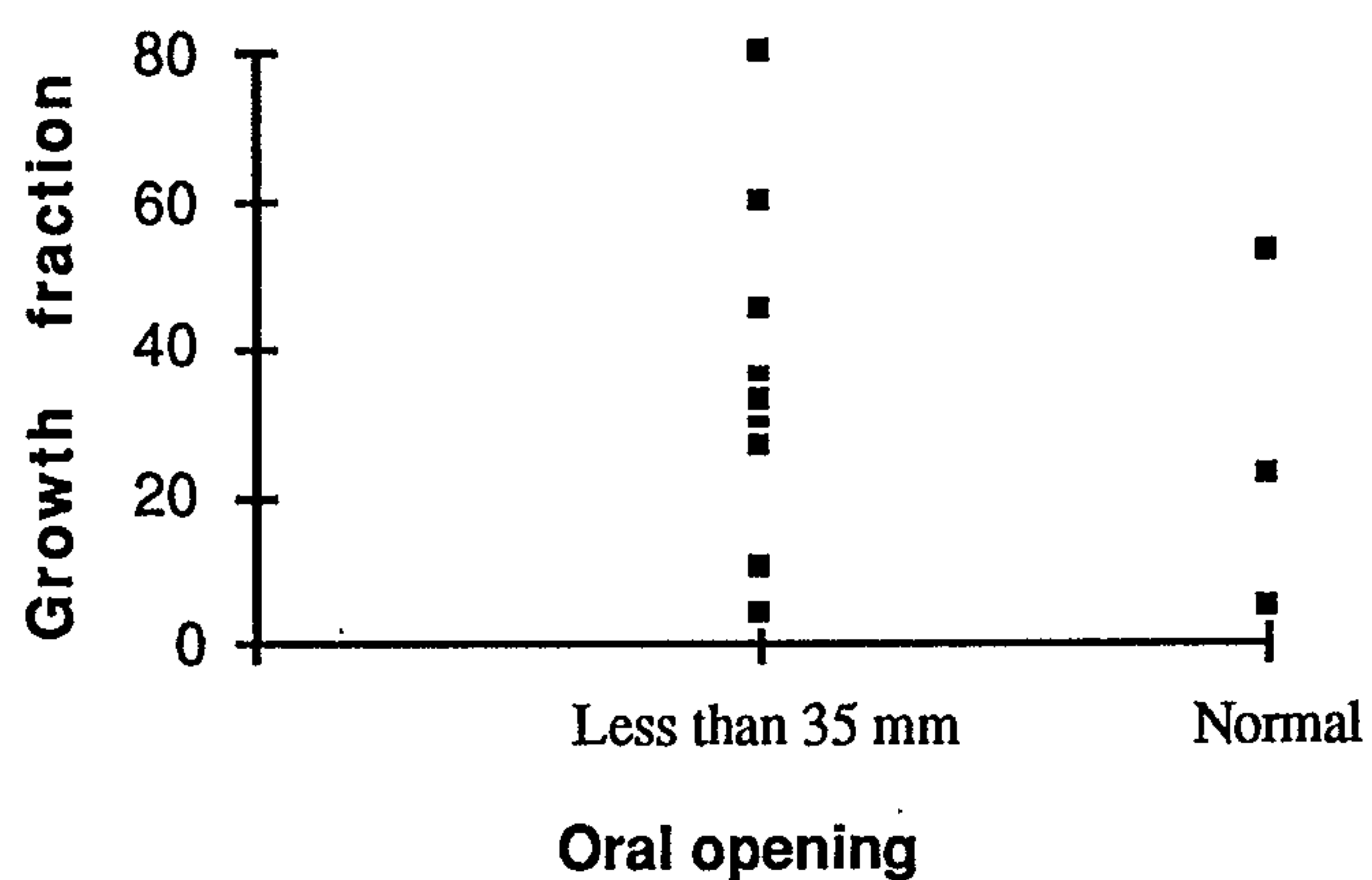
The growth fraction was also compared for the three variables:- disease staging, presence of abnormal p53, and oral opening (Fig.4.8.).

Figure 4.8. Epithelial growth fraction against the variables of disease staging, limited opening and p53 positive response in the tissue.

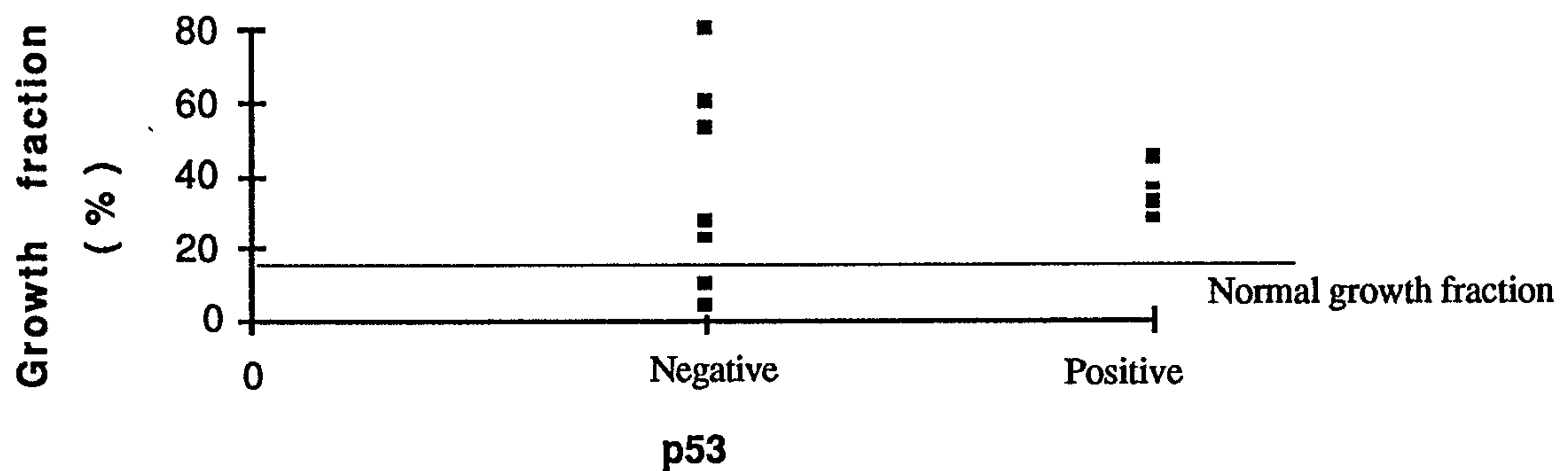
(A) **Growth fraction and disease staging in OSF**



(B) **Growth fraction and oral opening in OSF**



(C) Growth fraction and p53 positivity in OSF

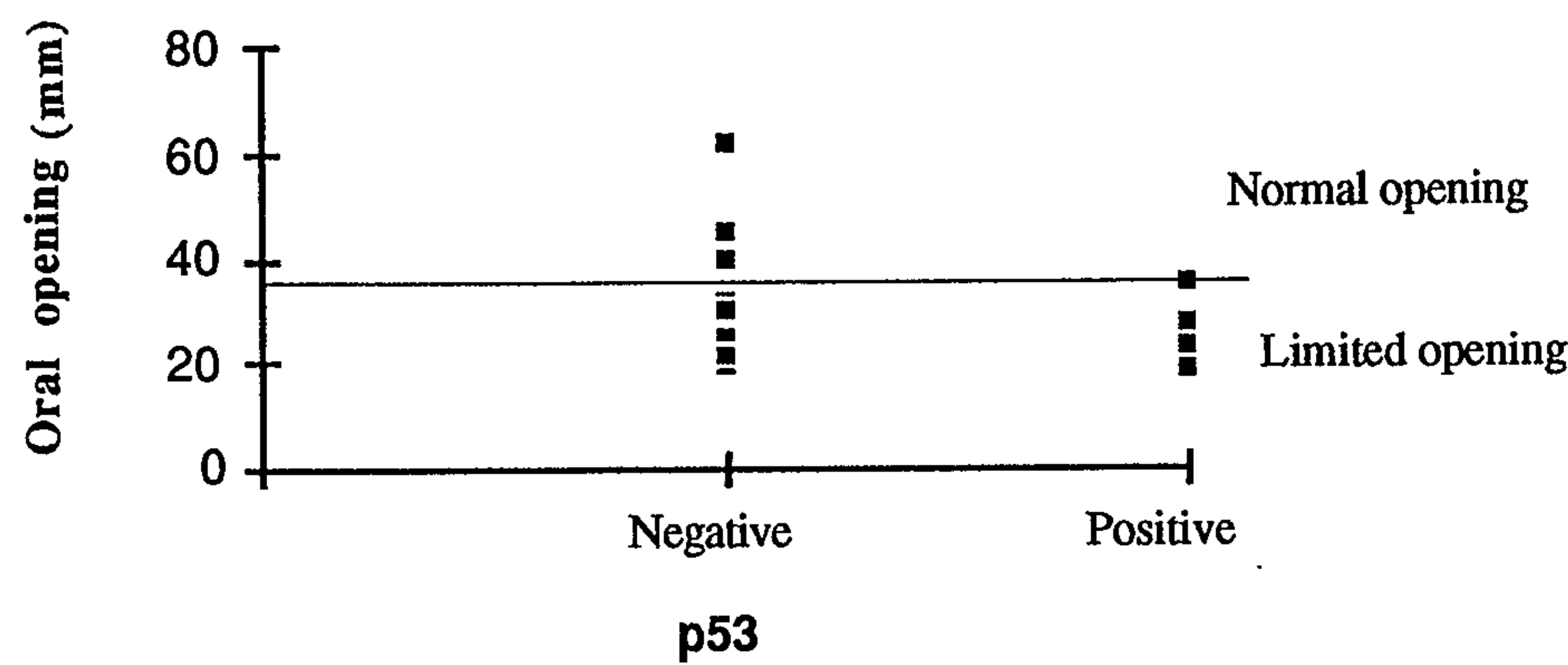


The present data in Fig 4.8. fails to demonstrate any significant trend for growth fraction against disease staging (A), whereas the detection of abnormal p53 protein via an immunohistochemical means was only demonstrated in subjects who had a high growth fraction (as determined by PAb PC-10) seen in (C). The presence of a limited oral opening (B) tended to also be associated with a raised epithelial growth fraction. These trends were not assessed for statistical significance in the small samples available.

Oral opening was taken as then used as the main variable and examined against the presence of abnormal p53 and the stage of the disease (Fig.4.9).

Figure 4.9. (following page) Analysis of the oral opening against the variables of p53, and disease stage.

(A) Oral opening and p53 in OSF



(B) Oral opening & Disease stage in OSF

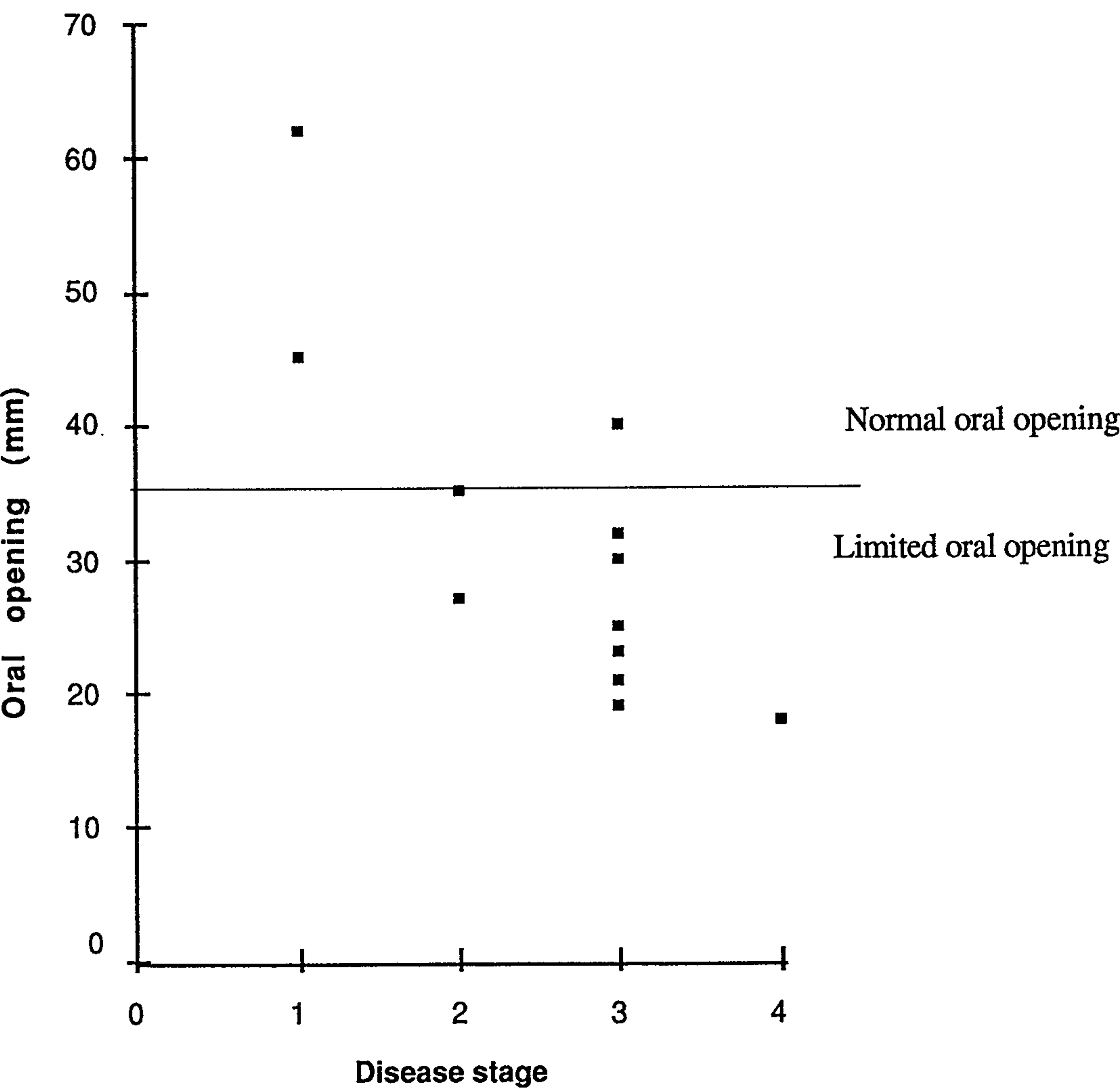
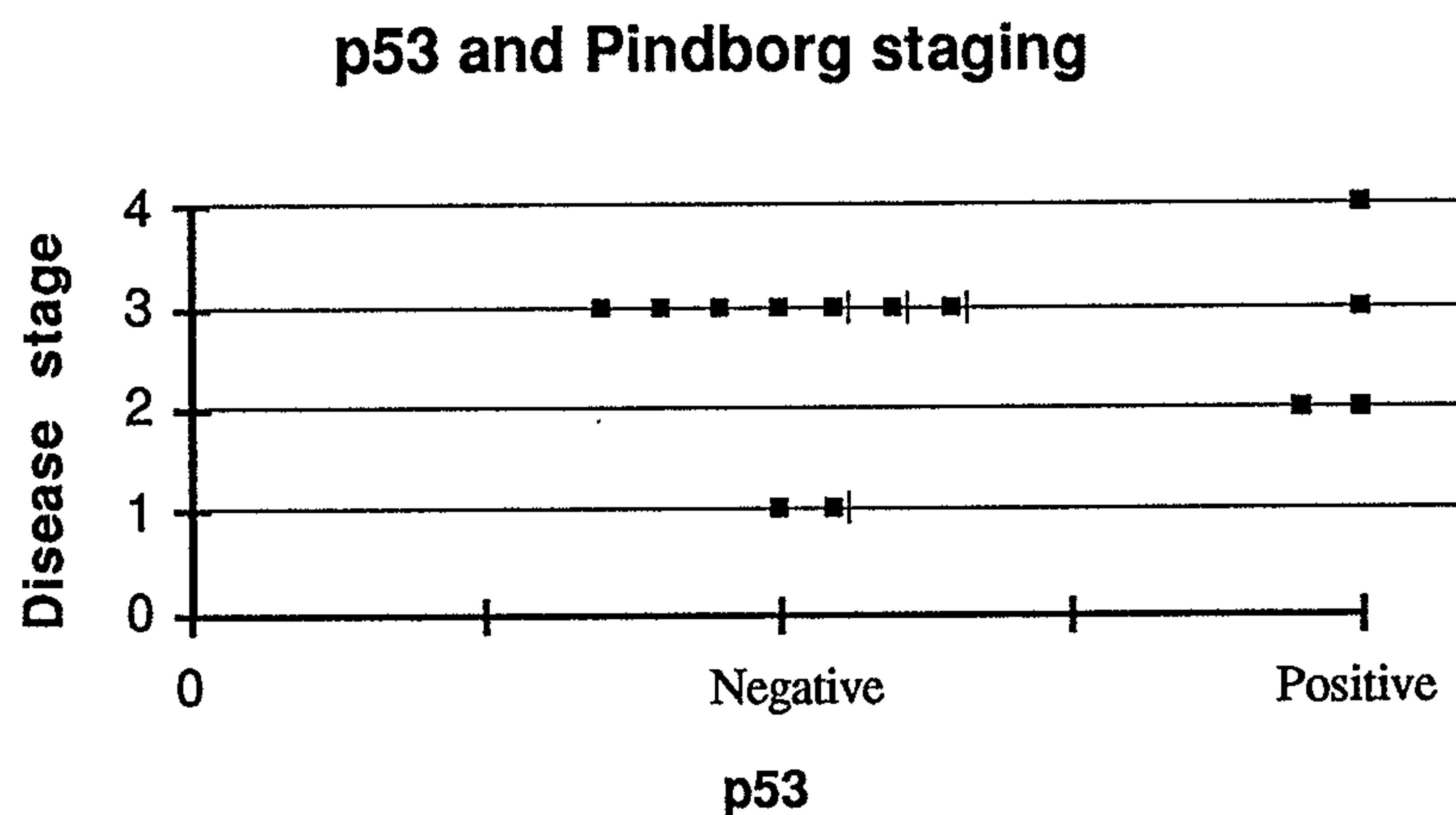


Figure 4.9 demonstrates a limited correlation between limited oral opening and abnormal p53 protein (A), in that tissue presenting abnormal p53 (with more than 10% of the cells affected) had been taken from patients with a limited oral opening. In addition the oral opening progressively decreasing as the stage of the disease progresses (B) -stage 4 being more severe than stage 1.

Figure 4.10 Abnormal p53 presentation and the stage of the disease.
(Presentation of <1% of cells excluded)



There does not appear to be any direct correlation between p53 and stage of the disease (Fig.4.11.). It may be that limited oral opening is a more reliable indicator than a grading system, to assess patients 'at risk'

4.10.9 p53 presentation - a comparative study

The profile presented in Table 4.21 attempts to draw out similarities, and differences, in all the sections presenting a positive product with the p53 antibodies. The main feature represented by Figure 4.12 which represents those sections which had groups or clusters of cells positive for p53 protein. The OSF with its linear presentation of p53 positive tissue demonstrates a higher proportion of positive biopsy specimens than the oral carcinomas.

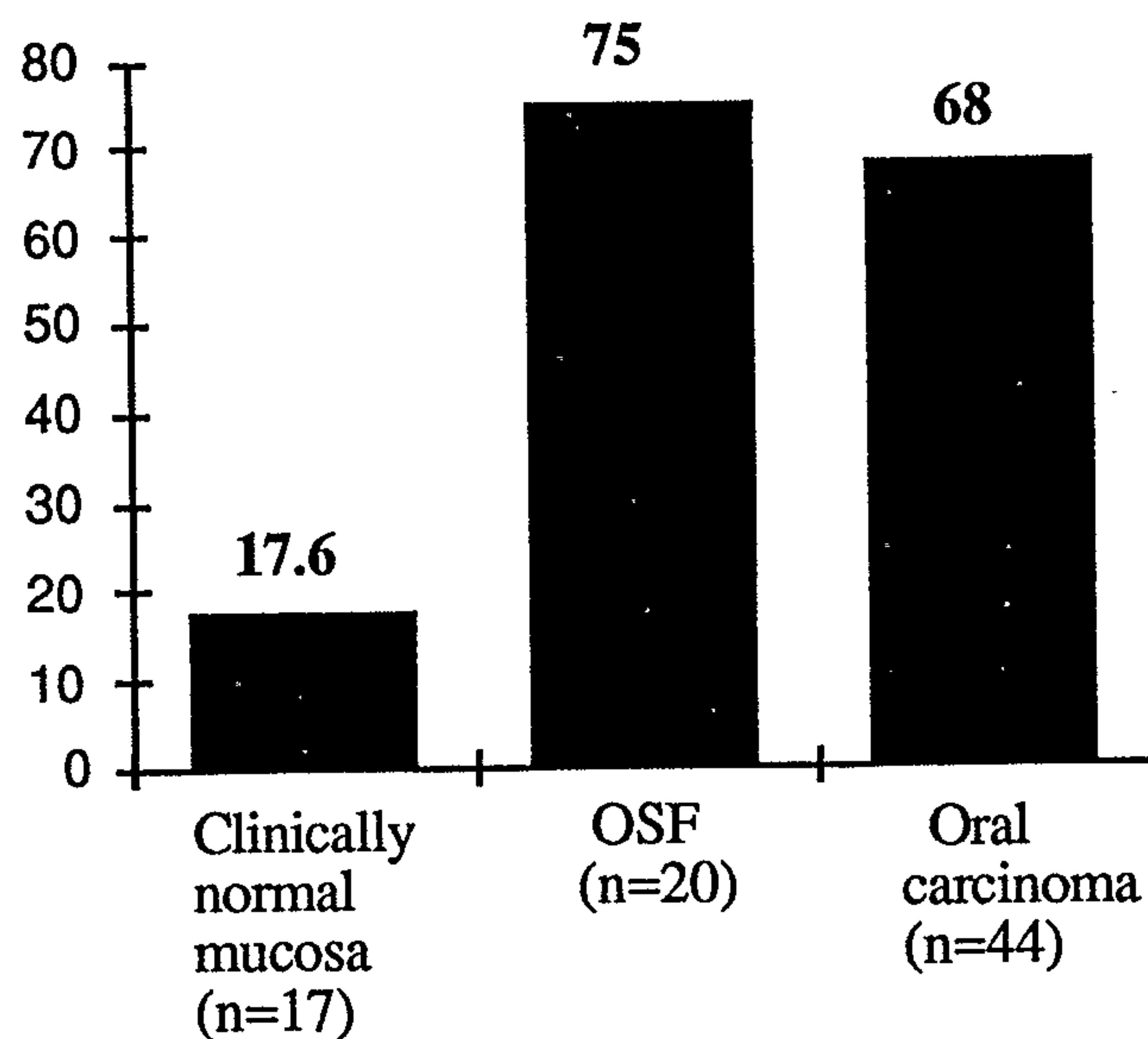
Table 4.21 Comparison of the distribution of p53 positive cells in oral epithelium of subjects who come from one of two groups : clinically normal mouths; oral submucous fibrosis; and oral carcinoma.

	Normal				OSF		Oral carcinoma	
	Australia		Nepal / India					
	No. of subjects	p53 +ve (%)	No. of subjects	p53 +ve (%)	No. of subjects	p53 +ve (%)	No. of subjects	p53 +ve (%)
Total	17	5 (29)	14	6 (43)	20	15 (75)	44	30 (68)
Basal	5	2 (40)	6	5 (83)	15	15 (100)	38	16 (42)
Regular	5	0	6	3 (50)	15	13 (72)	38	17 (45)
Single cells	5	4 (80)	6	3 (50)	15	6 (33)	38	0 (0)
Clusters	5	4 (80)	6	1 (17)	15	0	38	21 (55)
% of cells								
(1-10 %)	17	8 (100)	6	6 (100)	15	11 (61)	38	24 (63)
(11-50 %)	17	0	17	0	15	4 (22)	38	10 (26)
(51+ %)	17	0	17	0	15	3 (17)	38	4 (11)

basal = cells present in the basal layer and not in the supra-basal region of the epithelium; 'regular', 'single cells' and 'cluster' refer to the frequency that the positive cells present in the basal layer.

Note! - these terms were not used exclusively and any tissue section could be described by a number of these categories.

Figure 4.11 Immunohistochemical detection of p53 protein (%)



It is interesting to note that the growth fraction of the basal epithelial layer of the OSF tissue which was also positive for p53 was 53% (with a Standard deviation of 18.2) compared with that for p53 negative OSF basal epithelium which was $29.4 \% \pm 20.4$, and for control mucosa $6.1 \% \pm 6.0$. This did not prove to be statistically significant observation, possibly due to the low number involved.

4.10.9.1. Staining pattern of p53 in oral epithelium affected by OSF

Table 4.15 shows that there seems to be a characteristic pattern in the presentation of p53 product in this tissue with the positive product being confined to the basal layer, or immediate supra-basal region. and is regular, either being present in every cell or every 5th or every 10th. It is regular and not sporadic, or present in clusters. The same pattern is frequently repeated with all three antibodies. The p53 product is found in 75% (15/20) of cases.

4.10.10. Methodological problems with antibodies to p53 in immunohistochemical study

1. The 'blocking' serum (goat serum) developed problems - complexing with itself and with the primary antibody. This became more of a problem as the serum solution aged. At one stage sheep serum was substituted, and later smaller aliquots of the goat serum were used.
2. During the early stages in learning the technique, the chromogen diaminobenzidine (DAB) developed contaminants. It was initially thought that this was the cause of the speckled staining and the DAB was filtered through a micro filter, however, there was no change in the abnormal staining pattern. Later this was attributed to the 'old' goat serum.
3. The staining of supra-basal tissue with PAb PC-10 was originally thought to be related to a faulty technique. A number of trials were undertaken in an attempt to reduce the supra-basilar staining as much as possible. However, it was observed that the

concentration at which the "background" staining disappeared, also produced a negative result in the basilar region.

4. An unanticipated problem occurred with the use of the primary antibodies for p53. Initially the PAb CM-1 was used and a positive product was not obtained with the recommended positive control (carcinoma of the breast). The stromal tissue frequently stained positive, but nothing else - in particular the vascular smooth muscle and fibroblasts. At the suggestion of the manufacturer, the dilution of the primary antibody was reduced, but the result was a positive reaction in the cytoplasm of all keratinocytes of the oral mucosal epithelium[not glandular epithelium of the breast]. It appeared that the antibody was not reacting specifically in the tissues, and for the conditions employed, for the p53 protein. The manufacturer, Novacastra, was unable to provide a western blot for the batch of antibody supplied, and recommended instead the use of a second p53 antibody to be used in conjunction with PAb CM-1.

Professor Horne, of Novacastra (U.K.), reviewed the results and stated that the findings were normal for a polyclonal antibody, that the stromal staining was only "background" staining and that the dilution of the primary antibody should be increased to the point where the tumour cells were positive and the stromal staining was negligible.

The recommendation of Novacastra was that the two antibodies (PAb CM-1 and D07) should be used, a third antibody (PAb 1801) had been used by a number of researchers (Table 4.1.). Thus three primary antibodies (PAb CM-1, D07 and 1801) were assessed on oral carcinomas. A tongue carcinoma proved to be strongly and consistently positive with all three antibodies (all antibodies registering a positive product in the same region of the tumour with a similar proportion of reactive cells, and with insignificant stromal background staining). This was later used as the positive control. All three antibodies were used throughout the study, as there appeared to be a significant difference

between the antibodies in their ability to recognise mutant p53 protein in the different oral squamous cell carcinomas.

Similar problems were subsequently experienced by three separate research groups in the Sydney region. My experience has allowed me to successfully assist these groups, at the request of Novacastra (Sydney).

5. The effect of omitting the primary antibody was to always have an absence of any p53 product. However the finding of the occasional cluster of cells in clinically normal mucosa raised the possibility of false positives occurring.

p53 null allele cells were thought to be the only means of confirming the absence of false positives. A source of null allele cells was found in the Saos-2 cell line (Chen, Chen, 1990). Cells from this cell line were kindly donated by Dr Rodger Reddel, Children's Medical Research Institute, Sydney.

4.11. Discussion

4.11.1. P53 expression in oral mucosa

The panel of antibodies used in this study (PAb CM-1, D07 and 1801) react with human p53 antigen only, and identifies both the wild and mutant forms (Banks et al, 1986, Midgley et al, 1992, Vojtesek et al, 1992). However, wild-type p53 protein is present in normal cells only in minute concentrations, has a very short half life of approximately 5-20 minutes (Milner, 1991), and is not detected in tissue sections by standard immunohistochemical methods. The mutant forms however are stable (Milner, 1991; Jenkins et al, 1985) and can be visualised by relevant antibodies. While there are a number of studies which describe cytoplasmic staining of p53 protein, this has been ascribed to cross reaction with cytoskeletal proteins (Field et al, 1991). Although produced in the cytoplasm, p53 acts in, and is normally found only in the nucleus. Thus, although there was some cytoplasmic staining with PAb CM-1 and PAb 1801, there was no cytoplasmic staining with PAb D07 in cells with definite nuclear staining.

Bartek et al (1990) demonstrated the ability of several monoclonal antibodies specific for p53 proteins (PAb 1801, 240 and 421) to identify mutated p53 protein in a concordant fashion, irrespective of which exons of the gene were mutated. This is a consequence of active mutations in p53 producing a common conformational effect in the gene product (Gannon et al, 1990) although it is now known that different mutations may have different transforming potencies (Halevy et al 1990). Thus a gene which was originally a 'tumour suppressor gene' can undergo a mutation to become an oncogene.

One important, though perhaps theoretical drawback of immunohistochemical methods for identifying the p53 mutant protein is that there may be no immunoreactive protein present, due to allelic deletions or severe mutations. Both Bodner et al (1992) and Rodrigues et al (1990) confirm that immunohistochemical studies identify missense

mutations. In these situations complementary methods of analysis such as gene sequencing would provide additional information.

Although a number of genes are implicated in tumourigenesis the loss or deletion of the p53 suppressor gene is emerging as one of the most significant factors in the development of human malignancies (Hollstein et al, 1991).

The present study of primary oral squamous cell carcinomas, has demonstrated mutant p53 in 70% of cases. This result is comparable to the proportion reported by other investigators for epithelial malignancies, where a bank of antibodies has been used (ref to Table 4.1)

4.11.2. Oral Squamous cell carcinoma

The present study of primary oral carcinomas detected mutant p53 phosphoprotein in 68% (30/44) of the carcinomas (Table 4.8). This is comparable to that reported by investigators for other malignancies. The moderately well, and well differentiated squamous cell carcinomas were the categories in which the highest proportions of p53 positive cases were registered for each of the antibodies. The poorly differentiated squamous cell carcinomas consistently had low frequency of p53 antigen detectable with all 3 antibodies. This observation was supported by the results with the Bryne malignancy grading.

These tumour gradings in this study had been recorded by a number of different pathologists not adopting any single scheme for assessing tumour differentiation. This approach could be more susceptible to subjective error, and inter-observer variation than attempts at standardised numerical grading systems (also based on other facets of squamous cell carcinomas besides degree of differentiation) such as the Bryne index.

If the fraction of cells positive for p53 was arbitrarily taken as at least 10% of the total number of tumour cells before a carcinoma was considered positive then only

27% of the tumours would be considered as positive, with 7 of these being classified as moderately well differentiated..

There was no staining pattern characteristic of any of the cancer classifications. Great variation existed, from a negative or minimal result to one where the majority of the cells had a positive nuclear stain (70%), even within the same category of carcinoma

4.11.3. Oral Habits and p53

A great majority (88%) of patients with oral carcinoma had an associated oral habit (which was usually tobacco smoking, at least 20 cigarettes per day, or alcohol abuse (40 gm/day) or both. There were eight patients (18%) who were 'non-smokers'. This figure is comparable to those in other studies - Field et al (1991) 19% (7/37) were non-smokers. These patients had carcinomas in various sites (floor of mouth, buccal mucosa, tongue, and two on the alveolar ridge). Amongst the non smokers only 2 tumours were considered positive for p53 (out of a sample of 8 patients), whereas for the smokers 26 out of 33 carcinomas expressed positive for p53 ($p < 0.005$).

This study demonstrated that smokers had a higher risk of dying than non-smokers.(risk of 2.2 for smokers) though this failed to be significant ($p = 0.22$), which may be a reflection of the sample size. The group known as non-smokers included those who had ceased smoking for more than 10 years. However, in regards to aetiology, cessation of cigarette smoking only means that the patients risk remains at approximately the same level for one to two decades, but these individuals never have the same low risk factor as non-smokers (Doll et al, 1976).

4.11.4. Results from clinically normal oral mucosa

The results of the study on p53 in clinically normal oral mucosa would appear to challenge the findings in some of the previous reports. p53 has not been shown to be present in normal and infrequently in dysplastic tissue. It is most frequently identified in tissue which contains frank carcinoma (Harris, 1990, Bodner et al, 1992), though Gusterson et al (1991) have shown the presence of p53 positive cells at multiple sites well removed from the positive tumour. A correlation has also been demonstrated between smoking and positive p53 staining in patients with oral squamous cell carcinoma (Field et al, 1991). In addition, p53 expression has been previously described in dysplasias of the bronchial epithelium, and in gall bladder dysplasias (Kamel, Paakko, 1993). It seems therefore not impossible that patients with clinically normal mucosa who smoke tobacco and drink alcohol to excess, might have mutant p53 protein.

The present study also demonstrated that p53 positive cells can occur in clinically normal mucosa which may vary in the degree of dysplasia (all subjects who demonstrated positive p53 cells were tobacco smokers). This presented in approximately 5% of all patients (or 26% of smokers) with clinically healthy mouths. No particular intra-oral site was favoured and more than one site was affected in at least one patient, out of the minority, from whom multiple intra-oral sites had been sampled. The possibility that this could be a false positive finding tends to be discounted by: (1) negative reaction in the null-allele osteosarcoma cell line; (2) the restricted and anatomically consistent location of the p53 positive cells - within the epithelium only; (3) negative results when the primary antibody was omitted; (4) the appearance of these cells in the same location in 4-6 consecutive sections only; (5) reproducibility of positive reaction in a selected carcinoma with negative findings in adjacent epithelium in the same section; and (6) the fact that the positive reactions were restricted to tobacco smokers, some of whom also drank alcohol to excess.

Tobacco products and ethanol have been listed among the major risk factors for oral carcinoma. In a study of oral cancer in the developing world it has been estimated that 8% can be attributed to smoking alone (WHO 1984), and in the USA it is considered a major cause of oral cancer (US Public Health Service, 1982). The risk factor associated with alcohol consumption is not as clear because most heavy alcohol drinkers are also heavy smokers. In some countries in Asia alcohol drinking was found to be of minor importance (Hirayama, 1966), while in others (Wahi, 1968) it is found to have an important synergistic role. An Australian study (Rich and Raddon, 1984) demonstrated a greater percentage of heavy users of alcohol among patients with oral cancer, compared with the general population. It might be hypothesised therefore, that mutations or deletions in the p53 protein (tumour suppressor protein) genome, can be induced by tobacco and ethanol.

In support of this hypothesis this study has shown that mutant p53 protein was restricted to those patients who had used either alcohol or tobacco to excess. However, two patients with an oral squamous cell carcinoma, had detectable oral epithelial p53, but who had no history of tobacco smoking or alcohol consumption. The difference in prevalence of tobacco and or alcohol abuse between p53 positive and negative oral cancer sufferers was 23:9. Statistically the ethanol was not shown to be a significant factor in the present study (Table 4.13)

The survey also confirmed that oral habits are present in 89% of patients with oral squamous cell carcinoma (only 5 out of 46 patients are non-smokers).

4.11.5. Results from 'dysplastic' epithelium

Table 4.10 is also pertinent in considering whether p53 can be present in 'normal' tissue. Here abnormal p53 protein was demonstrated in the epithelium adjacent to invasive carcinoma in 46% of cases. Frequently this was in severely dysplastic

epithelium, but was also immunolocalised to histologically normal adjacent epithelium in three sections which were classified as carcinoma *in situ*.

Serial sections of the clinically normal mucosal biopsies were examined to estimate the three dimensional nature of the clusters. The single positive immunoreactive cells were not evident in more than two sections of tissue, whereas mucosa with a cluster of p53 positive cells demonstrated immunoreactivity in at least six tissue sections and sometimes up to ten sections.

An important feature was that when the p53 protein was detected in the dysplastic epithelium adjacent to a carcinoma, it most frequently presented in a 'sporadic' fashion. Positive cells occurred either individually, or as a 'cluster' comprising 6-10 cells, per section and were situated either in the basal and/or supra-basal regions. The relative frequency of these presentations varied and did not seem to be related to age, smoking habits, size or degree of differentiation of the tumour. (However, the number of oral carcinoma patients without an oral habit was very small (n=8).)

4.11.6. Lichen planus and mucosal keratosis

The results of the study of p53 expression in archival blocks from patients suffering from either lichen planus or a non-specific keratosis are presented in Table 4.5. and 13% of the blocks in the study demonstrated a sporadic distribution of p53 detected in individual cells rather than in clusters. These were biopsies of mucosal keratoses in patients over 40 years of age. In addition both these patients smoked. The proportion is probably larger than would be expected in a larger sample.

This investigation identified cells expressing p53 protein, probably mutant, in normal or dysplastic epithelium, although these cells always represented less than 10% of the total population in any particular biopsy. Some investigators, such as Bodner et al (1992), have apparently ignored the presence of p53 product when it had a frequency of less than 10%. This does not seem justified, as some of the squamous cell carcinomas also have frequencies of p53 antigen positive cells of less than 10%.

It seems that the likelihood of p53 protein being detected via immunohistochemical means is increased where there is a smoking habit.

Even considered in isolation the results of the studies on p53 product in 'normal' epithelium are significant on their own, but if the results of 'normal' tissue, dysplastic tissue, and tissue adjacent to invasive carcinoma, are taken together the evidence for the following conclusions is compelling:-

1. It confirms that mutant p53 protein can be found in normal tissue
2. **p53 mutations in oral mucosa may occur early in the multi step process of malignant transformation** and well before the malignant phenotype is manifest.

Both inferences will need to be confirmed by molecular biological methods using techniques such as the polymerase chain reaction.

It may even be possible to put forward the suggestion that what is being seen in the tissue accounts for the normal, accumulated effect of 'insult and injury' within the genetic framework of the cell. The majority of the cells in the epithelium are non-progenitor cells. They have only a transient existence and therefore any change in the genetic make-up of these cells is of little consequence, as the cells are consigned to a differentiation pathway and be ultimately shed.

The situation where this early mutation in the p53 gene occurs in progenitor (stem) cells is potentially of considerable significance. It may be predicted that if this were to take place then, histologically, a clone of cells expressing the mutant protein would result in the basal and suprabasal layer. If the mutation occurred in the daughter cells or in the suprabasal transient amplifying population, then this may explain the situation as presented in 4.5.3., where either basal or suprabasal cells were positive.

Clones of cells occupying half the population of a rete ridge representing the daughter cells of a stem cell situated at the base of the ridge have been demonstrated by enzyme

histochemistry by Seddon et al 1992. The p53 mutant cells were in much smaller clusters or as single cells presumably due to the limited half-life of the p53 protein in comparison with the total epithelial turnover time.

The findings in the 'normal' and 'dysplastic' epithelium are consistent with this prediction. A hypothesis may therefore be advanced stating that clusters of cells positive for the p53 protein are the result of a significant mutation in the p53 gene existing in a progenitor cell. These mutations may be similar to the early changes in the multi-step pathway of cancer development as suggested by Fearon et al (1990) in the model of colorectal carcinoma.

This hypothesis may be able to partially explain the phenomenon known as 'field cancerization'. Slaughter et al (1953) referred to the problem of multiple carcinomas occurring both synchronously and metachronously in the aerodigestive tract. Estimates of the proportion of patients who have or who will develop a second or even more multiple carcinoma in the mouth vary from 4-8% (Moetel and Foss, 1958, Shibuya, et al, 1986).

It remains to be determined whether patients with 'cluster presentations' of abnormal p53 in their oral epithelium are at an increased risk of developing a primary or recurrent oral carcinoma

Conclusion- These finding suggest exciting possibilities for the detection of early changes in the development of oral neoplasia. A longitudinal study of a larger series of biopsies of clinically healthy oral mucosa, and a wider spectrum of oral dysplastic lesions as well as frank carcinoma should be undertaken to determine the prognostic significance of the detection of p53 mutant protein. The genetic changes associated with these need to be investigated by the polymerase chain reaction and other molecular biology techniques.

It has been suggested that one of the functions of p53 is the regulation of cell proliferation, by the down-regulation of the gene responsible for producing

PCNA.(Mercer and Baserga, 1985, Mercer et al 1991, Milner, 1991). The work of Mercer et al (1991) shows that the increase in cell proliferation is associated with the detection of mutant p53. This has also been confirmed in the present study by the increase in PCNA detected in most of the cases where p53 is detected. However the studies of clinically normal mucosa show that the detection of p53 does not necessarily mark the presence of uncontrolled cell proliferation which is the hallmark of malignancy.

Table 4.8, correlates the detection of p53 with that of PCNA. Most tumours with a 25-50% growth fraction were p53 positive. Tumours with low histological grading (malignancy score of between 5-10 points) (Bryne et al, 1989) tended to be p53 negative. Tumours with a growth fraction of >50% as estimated by PCNA, tended to have a high malignancy grading demonstrated abnormal p53 protein. These features were not statistically significant however in the numbers investigated. It appears that abnormal p53 protein could present on either side of the normal growth fraction as presented by PCNA (ie <5% and > 18% -see Table 4.8). However, a negative p53 result occasionally occurred with carcinomas with a negative PCNA value or with an extremely high PCNA value. Both of these results could possibly be attributed to the absence of protein which could be recognised by the p53 antibody, though the negative result for PCNA is possibly due to denatured protein as a result of an extended period of fixation.

Bryne et al (1989) was able to demonstrate a worse 5 yr prognosis for patients with oral squamous cell carcinoma (all stages) with a malignancy score of >10, compared with those tumours with a score of 5-10. The results of the present study are consistent with the results of Bryne, with patients who had a high score having a risk of dying which was 2.3 times that of those who had a low Bryne score ($p = 0.066$), and a median life expectancy of 17 months, whereas those with a low Bryne score did not have a median life expectancy because of the fact that 50% of this population had not

died over the period from 1988 through to the end of 1992. These observations should be further tested in a larger study over a longer period of time.

During the follow-up period of this study, 26 patients died as a direct result of their carcinomas and 19 of these were positive for p53 product, though the significance is minimal as there were a large number of patients who did not die and yet were p53 positive ($p < 0.05$). Rodrigues et al (1990) and Bodner et al (1992) both state that the immunohistochemical technique seems to favour mutations of the 'missense' type. The results from this study would therefore suggest that the majority of oral carcinomas are associated with a missense mutation in the p53 gene. Further, the majority of deaths were also associated with carcinomas which had this form of mutation, as recognised by the immunohistochemical technique.

Mutation of the p53 gene therefore appears to be a very significant event in the development of squamous cell carcinoma of the oral cavity. It occurs in both the severely dysplastic (as defined by the biopsies demonstrating the carcinoma in situ) as well as the mildly dysplastic (epithelium adjacent to carcinomas), though not consistently in either. This situation could possibly be explained if p53 is a reasonably early, and frequent genetic mutation in the development of an oral carcinoma.

This study could be furthered by molecular biological studies into the type of mutation being detected by the immunohistochemical technique. This knowledge would also assist the understanding of the development of oral carcinomas, and how they compare with the studies undertaken in the colorectal region. (Fearon and Vogelstein, 1990)

4.11.7. p53 expression in oral submucous fibrosis

p53 was expressed in 75% of cases of OSF according to the immunohistochemical study. The pattern of the presentation of positive p53 in the epithelium was dissimilar from that obtained in clinically normal mucosa or in carcinoma. However, like the oral

carcinomas the presence of the mutant p53 was associated with an increased growth fraction.

A major concern for the patients with oral submucous fibrosis, which arose from the carcinoma study is that p53 mutations could be an early or very common event in the development of on oral malignancy.

The histology of the OSF sections suggests that there is increased apoptotic activity in this disease. However these regions and tissue sections frequently fail to stain positive for p53 protein, while the positive areas for p53 appear normal, or with a slightly enlarged nucleus. This presentation could perhaps suggest a mutated gene of the type described by Lowe et al (1993) and Clarke et al (1993).

However, the research undertaken by Yin et al (1992) has raised the possibility that p53 holds a cell in G₁ if there are insufficient nutrients for the cell to progress further in its cycle. Yin et al (1992) points out that under these circumstances normal p53 will be present in the cell for an extended period of time. Clarke et al (1993) and Lowe et al (1993) also show that where there is DNA damage then there is an increased level of p53 in normal tissue which leads to apoptosis.

However it is difficult to determine the chronological order in which the myriad of changes have taken place during the disease process. Certainly established OSF is noted for its reduction in the vascularity of the juxta-epithelial region, together with the appearance of hyalinised collagen. Whether this represents collagen formed as a result of external stimuli, present in the oral cavity, in the form of spices, betel nut or tobacco, is uncertain. Quite possibly the a state of general malnutrition in affected individuals predisposes the epithelial cells to abnormal patterns of repair. From a teleological standpoint, this fibrosis could be useful in limiting the transmucosal diffusion of irritants from the oral cavity.

The hyalinised collagen is always adjacent to the epithelium, in varying thicknesses. This would suggest that it may be the epithelium itself which "drives" this fibrotic

response. Cross-linking of the collagen or the accumulation of other materials such as the glycosaminoglycans or ground substance, or immunoglobulins could contribute to the hyalinised appearance.

One interpretation of a high growth fraction in OSF in the presence of an atrophic epithelium and a positive p53 product is that these cells are indeed being held in G₁ phase by normal p53 protein. The G₁ stage is the interval when PCNA would be usually expressed in a normal cell, and damaged cells would progress to apoptosis. The hypothesis of Yin et al (1992) could be therefore be applied here such that normal p53 is present in these cells for an extended period of time due to the relative nutritional deficiency in the lesional epithelium. This would allow adequate nucleosides to be accumulated for cell division to occur without mutations. Mention has already been made (1.5.2.) that deficiencies of vitamin and iron have been implicated as being of aetiological importance in OSF. However there is the additional factor that vitamin deficiency has an important role in the susceptibility to oral and pharyngeal carcinoma.

An alternative explanation is that the immunoreactive p53 protein represents the mutant form of the protein. The normal mitotic control over the cell has therefore been lost, and the increased PCNA demonstrates a high growth fraction. Large numbers of mitotic figures would not necessarily be present, as this is an inconsistent feature in carcinomas.

The study documented that a proportion of the biopsy specimens demonstrated epithelial atrophy while the remainder were either normal or hyperplastic. A number of the atrophic sections also demonstrated signs of oral lichen planus, and these were associated with the atrophic sections. Pindborg noted that erosive or atrophic lichen planus is frequently associated with OSF. Lichen planus was occasionally associated with OSF in the present study as well.

Chapter 5

General Discussion

The aim of the present study was to investigate the disease entity of oral submucous fibrosis (OSF), which is considered to be a premalignant condition, and to identify those patients particularly at risk of developing an oral squamous cell carcinoma.

The biopsy material from patients with OSF demonstrated an epithelial growth fraction, as determined by PCNA, which was significantly higher than that of the controls, and similar to that of the oral carcinomas. This characteristic was frequently associated with an abnormal immunohistochemical presentation of the tumour suppressor gene protein p53. However the frequency of this presentation (85%) was higher than the incidence of carcinomas developing in those with OSF estimated to be 7.6% by Murti et al (1985).

A separate investigation into the presence of abnormal p53 protein in oral squamous cell carcinoma, demonstrated that while it commonly presents when an immunohistochemical test is used, it is not a consistent feature. Surprisingly the abnormal protein occasionally presented in clinically normal oral mucosa, and seemed

to be associated with a smoking habit. This presentation was in the form of single cell or isolated clusters of cells. These features suggested that the mutation in the p53 gene is possibly an early event in the development of oral carcinoma. This is in agreement with the published work of others in this field.

The recent observation of the association between normal p53 and apoptosis could possibly present an alternative explanation for the apparent anomaly in OSF where an increased epithelial growth fraction is associated with an atrophic epithelium. In addition it was noted that there was an apparent association between the depletion of essential nucleosides available to the cell, and a raised presentation of p53, which arrests the cell in the G1 phase of mitosis, preventing progress to the S phase.

It is therefore possible to offer at least two explanations for the observations in this study, where there is a high frequency of presentation of p53, using the immunohistochemical technique.

1. The mutation of the p53 gene is a very early event in the development of an oral squamous cell carcinoma
2. The observed increase in p53 is of normal protein which is arresting the epithelial cells in the G1 phase, of the cell cycle, possibly due to the absence of essential nucleosides, which could be related to the underlying fibrotic changes occurring in the dermis in OSF.

Direction of future research

Doubt persists as to whether the immunohistochemical technique identified a true mutation, or merely a prolonged expression of the protein. One of the major

limitations of the present study was the inability to identify, the nature of the mutation of the p53 gene (or indeed if the expression of the protein was prolonged).

Therefore, the results presented in this study need to be validated using molecular biological techniques. This would also provide information regarding the nature of the mutation, if present.

Longterm followup of the patients presented in this study should be undertaken and this will greatly contribute to the overall understanding of the relationship between the presentation of p53 (immunohistochemically) and the subsequent outcome.

Extension of the study to include a greater number of patients with OSF into the study group would also make the results significant.

Management and control of the disease has not been feature of this study, however, it would seem appropriated in the light of our understanding of the important role for the carotenes and other vitamins in the susceptibility to oral cancer, to study the response of the epithelial tissue to vitamin supplementation. The response of the growth fraction and the presentation of p53 would be of particular interest.

Summary

1. Review of the material published on oral submucous fibrosis (OSF)

2. Presentation of clinical material obtained in Nepal, of patients suffering from OSF

i/ Use of oral opening as a means of screening a population, for the presence of the disease, was shown to have a weak sensitivity but good specificity.

3. An immunohistochemical technique was used to determine the 'growth fraction' in clinically normal mucosa. The results obtained using the proliferating cell nuclear antigen (PCNA), (primary antibody PC-10) as the marker for growth fraction, was found to have a good correlation with the results obtained by Ki-67. This correlation was only present in the region of the basal epithelial layer.

i/ Position '1' on the rete ridge was never observed to be positive for either of the antibodies used to probe for cycling cells. The cell position which corresponded to the greatest activity was position '21'

ii/ The growth fraction of the oral epithelium present in smokers was significantly higher than that in non smokers.

4. Comparison of the growth fraction of clinically normal epithelium with epithelium from OSF and oral carcinoma patients, demonstrated wide differences. The difference between OSF and clinically normal mucosa was significant ($p < 0.009$), however there was no significant difference between the other groups of tissue.

i/ The epithelium from OSF patients demonstrated a growth fraction which was consistently higher than that of the clinically normal mucosa.

ii/ Oral carcinomas failed to consistently present a high growth fraction, using the immunohistochemical technique.

5. The high growth found in the epithelium of OSF and oral cancer patients was frequently associated with an abnormal presentation of p53 protein (protein of the "tumour suppressor gene")

6. A univariate analysis of the data from the oral carcinoma patients showed the following:-

i/ Presence of palpable nodes in the area draining the oral carcinoma was a significant factor for the time of survival.

ii/ The 'Bryne index' had a weak level of significance.

iii/ Presentation of abnormal p53 was not significant in regards to survival time.

7. p53 presentation in patients with OSF occurred most frequently in the 'moderately advanced' and 'advanced' stages of the disease

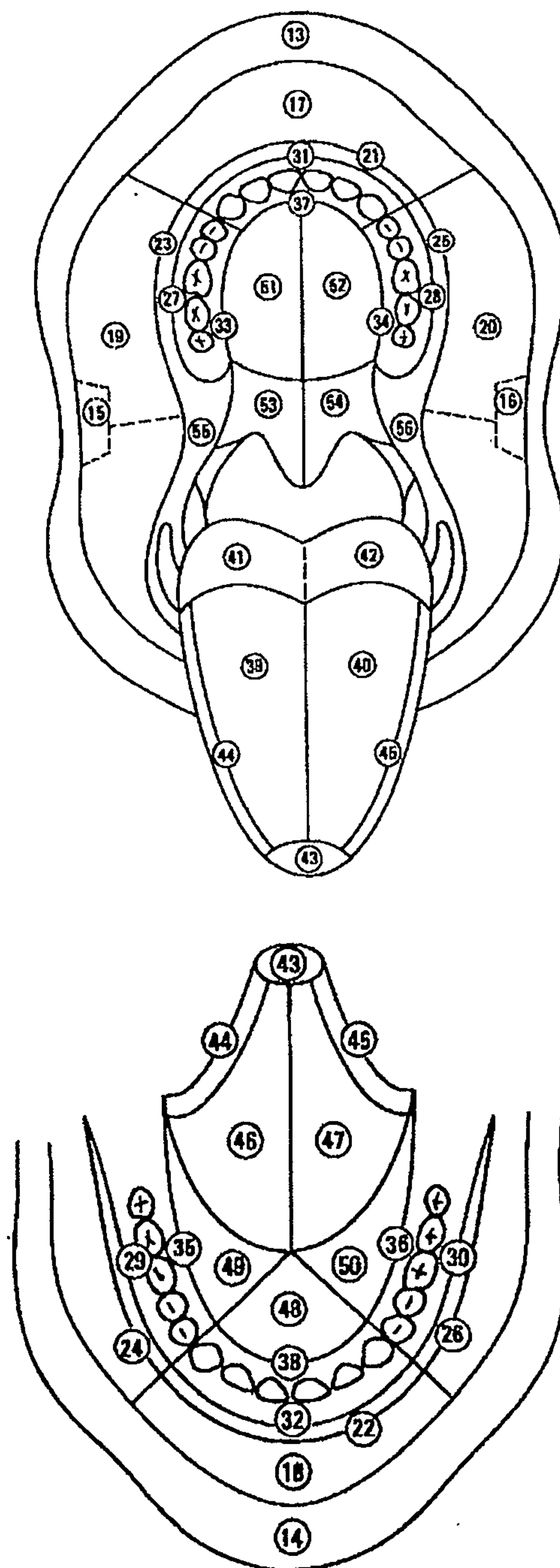
i/ Presentation of p53 was unlike that found in oral carcinoma. The 'regular' presentation of p53 was suggestive of changes consistent with cell damage. ("Stem cells" did not appear to be affected).

8. The latter stages of OSF were frequently associated with atrophic epithelium, a lower growth fraction (compared with the mean for OSF patients), a reduced oral opening, and p53 presentation.

Appendix A

Topographical record

The data pertaining to the oral mucosal lesions was recorded on a form with the following topographical division, modified after Roed-Pedersen et al [Roed-Petersen, 1969 #501]



Appendix B

A form for the recording of information on oral mucosal diseases and oral habits is reproduced in the following diagramme. The form consists of four parts, an identification section where personal and demographic information can be coded, a section for the recording of oral habits, a third for the recording the nature and location of lesions in the mouth and a section where various information on treatment provided and needed can be collected. This form was made available through the World Health Organisation - Oral Health Unit, Geneva, Switzerland

WHO ASSESSMENT FORM FOR ORAL MUCOSAL DISEASES

(1) T 1 (5)

Date 19 (6) (7)

Registration No. (8) (12)

PERSONAL AND DEMOGRAPHIC INFORMATION

Sex M = 1, F = 2 (13) (14)

Age in years (15)

Ethnic group (16)

Religion (17)

Occupation (18)

Diet (19)

Name family other

Address
.....
.....

Geographic location (20) (21)

Examiner (22)

SMOKING HABITS

If no habit present – leave box blank

1 = occasionally
2 = regularly

Number per day

Duration years

Cigarettes (23) (24) (26)

Cigars (28) (29) (31)

Others (specify) (33) (34) (36)

Grams per week

European pipe (38) (39) (41)

Water pipe (43) (44) (46)

Others (specify) (48) (49) (51)

CHEWING AND OTHER HABITS

1 = Occasionally
2 = Regularly

Location of quid
1 = left side
2 = right side
3 = both sides
4 = others (specify)

Areca nut, lime and leaf (53) (54) (56) (58)

(59) (60) (62) (64)

Tobacco chewing (65) (66) (68) (70)

grams per week

Other habits (specify) (71) (72) (74) (76)

(77) (78)

Card No. (80) 1

WHO ASSESSMENT FORM FOR ORAL MUCOSAL DISEASES

(1)

T

1

(5)

Date 19

(6)

(7)

Registration No.

(8)

(12)

CODES

If no disease or condition present — leave box blank.

		Location code	Carcinoma	Leuko-plakia	Erythro-plakia	Lichen planus	Submucous fibrosis	Candidiasis	
Carcinoma	1 = present								
Leukoplakia	1 = smooth & fissured 2 = ulcerated 3 = nodulo-speckled								
Erythroplakia	1 = present								
Lichen planus	1 = atrophic or ulcerative 2 = other types								
Submucous fibrosis	1 = present								
Candidiasis	1 = acute pseudo-membraneous 2 = other types								

Leukokeratosis nicotina palatini

(69)

Herpetic gingivostomatitis

(70)

Acute necrotizing gingivitis

(71)

Cancrum oris

(72)

Recurrent aphthae

(73)

Other specify

(74)

.....

(75)

PREVIOUS TREATMENT

0 = unknown 1 = yes

(76)

Biopsy 1 = punch, 2 = incisional

(77)

Photograph 1 = yes

(78)

TREATMENT REQUIREMENTS

0 = none

1 = oral hygiene instruction only

2 = recommended change of habit and follow-up

3 = treatment needed

4 = urgent treatment needed with referral

(79)

Card No.

(80)

2

i

HISTOLOGICAL GRADING
OF ORAL EPITHELIAL ATYPIA BY THE USE
OF PHOTOGRAPHIC STANDARDS

SCORE SHEET

OBSERVER		A.B.C.	
MASTER NUMBER		45988	
CLINICAL NUMBER		62	
SECTION NUMBER		36/69/8	
SITE		4	
AGE		37	
SEX	M	1	1
	F		2
CANDIDA PRESENT	YES		3
	NO	1	4

Characteristic to be observed	Magnification of obj. lens	Photograph no.	Grades	Score	
1. "DROP-SHAPED" RETE RIDGES	(up to) × 10			2	
			NONE (0)		5
		1	SLIGHT (2)		6
		2	MARKED (4)		7
2. IRREGULAR EPITHELIAL STRATIFICATION	× 10			0	
			NONE (0)		8
		3	SLIGHT (2)		9
		4	MARKED (5)		10
3. KERATINIZATION OF CELLS BELOW KERA- TINIZED LAYER	× 10			0	
			NONE (0)		11
		5	FEW/SHALLOW (1)		12
		6	MANY/DEEP (3)		13
4. BASAL CELL HYPER- PLASIA	× 10			1	
			NONE (0)		14
		7	SLIGHT (1)		15
		8	MARKED (4)		16
5. LOSS OF INTERCELLULAR ADHERENCE	× 10			0	
			NONE (0)		17
		9	SLIGHT (1)		18
		10	MARKED (5)		19
6. LOSS OF POLARITY	× 10			2	
			NONE (0)		20
		11	SLIGHT (2)		21
		12	MARKED (6)		22
7. HYPERCHROMATIC NUCLEI	× 10			5	
			NONE (0)		23
		13	SLIGHT (2)		24
		14	MARKED (5)		25
RUNNING TOTAL TO CARRY OVER				10	

RUNNING TOTAL BROUGHT FORWARD				10	
Characteristic to be observed	Magnification of obj. lens	Photograph no.	Grades	Score	
8. INCREASED NUCLEO- CYTOPLASMIC RATIO (INCREASED DENSITY) IN BASAL AND PRICKLE- CELL LAYERS	(up to)			2	
	× 40		NO INCREASE (0)		26
		15	SLIGHT INCREASE (2)		27
		16	MARKED INCREASE (6)		28
9. ANISOCYTOSIS AND ANISONUCLEOSIS	× 40		NONE (0)	0	29
		17	SLIGHT (2)		30
		18	MARKED (6)		31
	10. PLEOMORPHIC CELLS AND NUCLEI	× 40		NONE (0)	0
19			SLIGHT (2)	33	
20			MARKED (6)	34	
11. MITOTIC ACTIVITY		× 40		NORMAL (0)	1
	21		SLIGHT INCREASE (1)	36	
	21		MARKED INCREASE (5)	37	
	12. LEVEL OF MITOTIC ACTIVITY	× 40		NORMAL (0)	3
22			LOWER ½ ONLY (3)	39	
22			ALSO UPPER ½ (10)	40	
13. PRESENCE OF BIZARRE MITOSES		oil		NONE (0)	0
	23		SINGLE (6)	42	
	23		MULTIPLE (10)	43	
	EAI				16

Characteristic to be observed		Grades	Assessment	
14. HYPERPLASIA-ATROPHY				
		ALL NORMAL THICKNESS FOR SITE		45
		SLIGHT HYPERPLASIA		46
		SLIGHT ATROPHY		47
		MARKED HYPERPLASIA		48
		MARKED ATROPHY		49
15. SURFACE CHARAC-TERISTICS	(a) IN NORMALLY UNKERATINIZED SITES			
		NORMAL FOR SITE		50
		PARAKERATOSIS		51
		HYPERPARAKERATOSIS		52
		ORTHOKERATOSIS		53
		HYPERORTHOKERATOSIS		54
	(b) IN NORMALLY ORTHOKERA-TINIZED SITES	NORMAL FOR SITE		55
		HYPERORTHOKERATOSIS		56
		PARAKERATOSIS		57
		HYPERPARAKERATOSIS		58
		UNKERATINIZED		59

References

- Anneroth G, Hansen LS (1984)** A methodological study of histologic classification and grading of malignancy in oral squamous cell carcinoma. *Scand. J. Dent. Res.*;92:448-468.
- Adhvaryu SG, Bhatt RG, Dayal PK, et al. (1986)** SCE frequencies in lymphocytes of tobacco/betel nut chewers and patients with oral submucous fibrosis. *Br J Cancer*;53(1):141-3.
- Akbar M (1976)** Oral submucous fibrosis. *J. Indian Dent. Assoc.*;48:365-373.
- Allegranza A, Girlando S, Arrigoni GL, et al. (1991)** Proliferating cell nuclear antigen expression in central nervous system neoplasms. *Virchows Arch. A Path. Anat. Histo*;419:417-423.
- Allred D, Clark G, Elledge R, et al. (1993)** Association of p53 protein expression with tumour cell proliferation rate and clinical outcome in node-negative breast cancer. *J. Nat. Cancer Inst.*;85(3):200-206.
- Almendral J, Huebsch D, Blundell P, Macdonald-Bravo H, Bravo R (1987)** Cloning and sequence of the human nuclear protein cyclin: homology with DNA-binding proteins. *Proc. Natl. Acad. Sci. USA*;84:1575-1579.
- Baker SJ, Fearon ER, Nigro J, et al. (1989)** Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science*;244:217-221.
- Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B (1990)** Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science*;249:912-915.

- Banks L, Matlashewski G, Crawford L (1986)** Isolation of human-p53-specific monoclonal antibodies and their use in the studies of human p53 expression. *Eur J Biochem*;159(3):529-34.
- Banoczy J, Csiba A (1976)** Occurrence of epithelial dysplasia in oral leukoplakia. *Oral Surg*;42:223-231.
- Barnard N, Hall P, Lemoine N, Kadar N (1987)** Proliferative index in breast carcinoma determined in situ by Ki-67 immunostaining and its relationship to clinical and pathological variables. *J. Pathol.*;152:287-295.
- Bartek J, Bartkova J, Vojtesek B, et al. (1991)** Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. *Oncogene*;6:1698-1703.
- Bartek J, Bartkova J, Vojtesek B, et al. (1990)** Patterns of expression of the p53 tumour suppressor in human breast tissues and tumours in situ and in vitro. *Int J Cancer*;46(5):839-44.
- Bartek J, Iggo R, Gannon J, Lane DP (1990)** Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. *Oncogene*;5(6):893-9.
- Bartkova J, Bartek J, Lukas J, et al. (1991)** p53 protein alterations in human testicular cancer including pre-invasive intratubular germ-cell neoplasia. *Int J Cancer*;49(2):196-202.
- Barton CM, Staddon SL, Hughes CM, et al. (1991)** Abnormalities of the p53 tumour suppressor gene in human pancreatic cancer. *Br J Cancer*;64(6):1076-82.
- Bataini JP, Bernier J, Brugere J, Jaulerry C, Picco C, Brunin F (1985)** Natural history of neck disease in patients with squamous cell carcinoma of oropharynx and pharyngolarynx. *Radiother Oncol*;3(3):245-55.
- Behl P (1962)** Practice of dermatology. Bombay: Allied Publishers Private Limited,.
- Bennett WP, Hollstein MC, He A, et al. (1991)** Archival analysis of p53 genetic and protein alterations in Chinese esophageal cancer. *Oncogene*;6(10):1779-84.

- Bhonsle R, Murti P, Gupta P, Mehta F (1976)** Reverse dhumti smoking in Goa: an epidemiological study of 5449 villagers for oral precancerous lesions. *Indian J Cancer*;13:301-305.
- Bhonsle RB, Murti PR, Daftary DK, et al. (1987)** Regional variations in oral submucous fibrosis in India. *Community Dent Oral Epidemiol*;15(4): 225-9.
- Bhonsle RB, Murti PR, Pindborg JJ, Daftary DK, Mehta FS (1981)** Focal vascular dilatations and petechiae in oral submucous fibrosis. *Scand J Dent Res*;89(3):270-4.
- Bishop J (1987)** The molecular genetics of cancer. *Science*;235:305-311.
- Bodner SM, Minna JD, Jensen SM, et al. (1992)** Expression of mutant p53 proteins in lung cancer correlates with the class of p53 gene mutation. *Oncogene*;7(4):743-9.
- Borle RM, Jagtap MW (1987)** Estimation of complement C3 in oral submucous fibrosis. *Int J Oral Maxillofac Surg*;16(6):753-6.
- Bradfield JS, Scruggs RP (1983)** Carcinoma of the mobile tongue: incidence of cervical metastases in early lesions related to method of primary treatment. *Laryngoscope*;93(10):1332-6.
- Braithwaite AW (1987)** Mouse p53 inhibits SV40 origin-dependent DNA replication. *Nature*;329:458-460.
- Braithwaite AW, Blair GE, Nelson CC, McGovern J, Bellett AJ (1991)** Adenovirus E1b-58 kD antigen binds to p53 during infection of rodent cells: evidence for an N-terminal binding site on p53. *Oncogene*;6(5):781-787.
- Branch P, Aquilina G, Bignami M, Karran P (1993)** *Nature*;362:652-654.
- Bravo R (1984)** Coordinated synthesis of the nuclear protein cyclin and DNA in serum-stimulated quiescent 3T3 cells. *FEBS Lett*;169:185-188.

- Bravo R, Fey S, Bellatin J, Larsen P, Arevalo J, Celis J (1981)**
Identification of a nuclear and of a cytoplasmic polypeptide whose relative proportions are sensitive to changes in the rate of cell proliferation. *Exp. Cell Res.*;136:311-319.
- Bravo R, Frank R, Blundell PA, Macdonald-Bravo H (1987)**
Cyclin/PCNA is the auxilliary protein of DNA polymerase- δ . *Nature*;326:515-517.
- Bravo R, Macdonald-Bravo H (1984)** Induction of the nuclear protein cyclin in quiescent mouse 3T3 cells stimulated by serum and growth factors: correlation with DNA synthesis. *EMBO* ;3(13):3177-3181.
- Bravo R, Macdonald-Bravo H (1987)** Existence of two populations of cyclin/PCNA during the cell cycle: Association with DNA replication sites. *J. Cell Biol.*;105:1549-1554.
- Brown DC, Gatter KC (1990)** Monoclonal antibody Ki-67: its use in histopathology. *Histopathol*;17:489-503.
- Bryne M, Koppang HS, Lilleng R, Stene T, Bang G, Dabelsteen E (1989)** New malignancy grading is a better prognostic indicator than Broder's grading in oral squamous cell carcinomas. *J. Oral. Path.*;18:432-437.
- Canniff JP, Batchelor JR, Dodi IA, Harvey W (1985)** HLA-typing in oral submucous fibrosis. *Tissue Antigens*;26(2):138-42.
- Canniff JP, Harvey W (1981)** The aetiology of oral submucous fibrosis: the stimulation of collagen synthesis by extracts of areca nut. *Int J Oral Surg*;10(S 1):163-167.
- Canniff JP, Harvey W, Harris M (1986)** Oral submucous fibrosis: its pathogenesis and management. *Br Dent J*;160(12):429-34.
- Carter R (1992)** Pathology of squamous carcinomas of the head and neck. *Current Opinion in Oncology*;4:485-490.

- Casson AG, Mukhopadhyay T, Cleary KR, Ro JY, Levin B, Roth JA (1991) Oncogene activation in esophageal cancer. *J Thorac Cardiovasc Surg*;102(5):707-9.
- Cattoretti G, Rilke F, Andreola S, D'Amato L, Delia D (1988) P53 expression in breast cancer. *Int.J.Cancer*;41:178-183.
- Celis JE, Madsen P (1986) Increased nuclear cyclin/PCNA antigen staining of non-S-phase transformed human amnion cells engaged in nucleotide excision DNA repair. *FEBS*;209(2):277-283.
- Chan G, Little J (1981) The influence of cellular proliferative history on the susceptibility to oncogenic transformation. *J.Cell.Physiol.*;109:317-.
- Chauvel P, Courdi A, Gioanni J, Vallicioni J, Santint J, Demard F (1989) The labelling index: a prognostic factor in head and neck carcinoma. *Radiother. Oncol*;14:231-237.
- Chen P-L, Chen Y, Bookstein R, Lee W-H (1990) Genetic mechanisms of tumour suppression by the human p53 gene. *Science*;250:1576-1580.
- Cibull M, Heryet A, Gatter K, Mason D (1989) The utility of Ki67 immunostaining, nuclear organizer region counting, and morphology in the assessment of follicular lymphomas. *J. Pathol.*;158:189-193.
- Clarke A, Purdie C, Harrison D, et al. (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*;362:849-852.
- Cleaver JE (1989) DNA repair in man. *Birth Defects*;25:61-82.
- Coltrera MC, Gown AM (1991) PCNA/cyclin expression and BrdU uptake define different subpopulations in different cell lines. *J. Histochem. Cytochem.*;39(1):23-30.
- Coltrera MD, Zarbo RJ, Sakr WA, Gown AM (1992) Markers for dysplasia of the upper aerodigestive tract. Suprabasal expression of PCNA, p53, and CK19 in alcohol-fixed, embedded tissue. *Am J Pathol*;141(4):817-25.

- Costa A, Banadonna G, Villa E, Valagussa P, Silvestrini R (1981)**
Labelling index as aprognostic marker in non-Hodgkin's lymphoma.
JNCI;66:1-5.
- Costa J, Wesley R, Glastein E, Rosenberg S (1984)** The grading of soft tissue sarcomas: Results of a clinicohistopathologic correlation in a series of 163 cases. *Cancer*;53:530-541.
- Cox D (1972)** Regression models and life tables. *J Roy Statist Soc*;34:187-220.
- Cox S** Oral submucous fibrosis - some clinical observations. *J Nepal Med Ass*;(in press).
- Crook T, Wrede D, Tidy J, Scholefield J, Crawford L, Vousden K (1991)** Status of c-myc, p53 and retinoblastoma genes in human papillomavirus positive and negative squamous cell carcinoma of the anus. *Oncogene*;6:1251-1257.
- Daftary D (1992)** Oral precancerous lesions and conditions of tropical interest. In: Prabhu S, Wilson D, Daftary D, Johnson N, ed. *Oral diseases in the tropics*. Oxford: Oxford University Press, : 419.
- Daftary D, Murti P, Bhonsle R, Gupta P, Mehta F, Pindborg J (1991)** Risk factors and risk markers for oral cancer in high incidence areas of the world. In: Johnson N, ed. *Risk markers for Oral Diseases*. Cambridge: Cambridge University Press, : 29-62. vol 2 Oral Cancer).
- Davidoff A, Kerns B, Iglehart J, Marks J (1991)** Maintenance of p53 alterations throughout breast cancer progression. *Cancer Res*.;51:2605-2610.
- Deppert W, Haug M, Steinmayer T (1987)** Modulation of p53 protein expression during cellular transformation with simian virus 40. *Mol Cell Biol*;7(12):4453-63.
- Desa J (1957)** Submucous fibrosis of the palate and cheek. *Annals of Otology, Rhinology and Laryngology*;66:1143-1159.

- Dierendock J, Wijsman R, Keizer R, Velde C, Cornelisse C (1991)** Cell cycle related staining patterns of anti-proliferating cell nuclear antigen monoclonal antibodies: comparison with BrdU labeling and Ki-67 staining. *Am.J.Pathol.*;138:1165-1172.
- Dockrat I, Shear M (1969)** Oral submucous fibrosis in Natal. *Proc Int Acad Oral Pathol*;1969(57):57-63.
- Doll R, Peto R (1976)** Mortality in relation to smoking: 20 years observation on male British doctors. *Br Med J*;2:1525-1536.
- Donehower LA, Harvey M, Slagle BL, et al. (1992)** Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*;356:215-221.
- Dover R, Watt F (1987)** Measurement of the reate of epidermal terminal differentiation: expression of involucrin by S-phase keratinocytes in culture and in psoriatic plaques. *Journal of Investigative Dermatology*;89:349-52.
- Dunphy W, Newport J (1988)** Unravelling of mitotic control mechanisms. *Cell*;55:925-928.
- Ebling F, Eadym R, Leigh I (1992)** Anatomy and organisation of human skin. In Champion R.H., Burton J.L. Ebling F.J.G. *Textbook of Dermatology*. Oxford, Blackwell Publications;:65.
- El-Labban N, Canniff JP (1985)** Ultrastructural findings of muscle degeneration in oral submucous fibrosis. *J Oral Pathol*;14(9):709-17.
- Eliyahu D, Michalovitz D, Eliyahu S, Pinhasi KO, Oren M (1989)** Wild-type p53 can inhibit oncogene-mediated focus formation. *Proc Natl Acad Sci U S A*;86(22):8763-7.
- Ervin TJ, Clark JR, Weichselbaum RR (1985)** Multidisciplinary treatment of advanced squamous carcinoma of the head and neck. *Semin Oncol*;
- Fearon E, Vogelstein B (1990)** A genetic model for colorectal tumorigenesis: Review. *Cell*;61:759-767.

- Field JK, Spandidos DA, Malliri A, Gosney JR, Yiagnisis M, Stell PM (1991)** Elevated P53 expression correlates with a history of heavy smoking in squamous cell carcinoma of the head and neck. *Br J Cancer*;64(3):573-7.
- Finlay C, Hinds P, Levine A (1989)** The p53 proto-oncogene can act as a suppressor of transformation. *J. Cell Biol.*;57:1083-1093.
- Finlay CA, Hinds PW, Tan T-H, Eliyahu D, Oren M, Levine AJ (1988)** Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. *Mol. Cell Biol.*;8:531-539.
- Foulds L (1958)** The natural history of cancer. *J. Chronic Disease*;8: 2-37.
- Galand P, Degraf C (1989)** Cyclin/PCNA immunostaining as an alternative to tritiated thymidine pulse labelling for marking S-phase cells in paraffin sections from animal and human tissues. *Cell Tissue Kinet*;22:383-392.
- Gannon J, Lane D (1991)** Protein synthesis required to anchor a mutant p53 protein which is temperature-sensitive for nuclear transport. *Nature*;349:802-806.
- Gannon JV, Lane DP (1987)** p53 and DNA polymerase α compete for binding to SV40 T antigen. *Nature*;329:456-458.
- Gannon JV, Greaves R, Iggo R, Lane DP (1990)** Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *Embo J*;9(5):1595-602.
- Garcia R, Coltera M, Gown A (1989)** Analysis of proliferative grade using anti-PCNA/cyclin monoclonal antibodies in fixed embedded tissues. *Am.J.Pathol.*;134:733-739.
- Gerdes J, Lemke H, Baish H, Wacker H, Schwab U, Stein H (1984)** Cell cycle analysis of a cell proliferation associated human nuclear antigen defined by the monoclonal antibody Ki67. *J Immunol*;133:1710-1716.

- Gerdes J, Schwab U, Lemke H, Stein H (1983)** Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int.J.Cancer*;31:13-20.
- Giglio A, O'Brien S, Ford R, et al. (1992)** Prognostic value of proliferating cell nuclear antigen expression in chronic lymphoid leukemia. *Blood*;79(10):2717-2720.
- Greene J, Vermillion J (1964)** The simplified oral hygiene index. *JADA*;68:7-13.
- Gupta P.(1975)** Estimation of relative risk and attributable risk from epidemiological studies. [DSc thesis.]. Department of Epidemiology, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, USA.
- Gupta PC, Mehta FS, Daftary DK, et al. (1980)** Incidence rates of oral cancer and natural history of oral precancerous lesions in a 10-year follow-up study of Indian villagers. *Community Dent Oral Epidemiol*;8(6):283-333.
- Gusterson BA, Anbazhagan R, Warren W, et al. (1991)** Expression of p53 in premalignant and malignant squamous epithelium. *Oncogene*;6:1785-1789.
- Hahn P, Kapp L, Morgan W, Painter R (1986)** Chromosomal changes without DNA over-production in Hydroxyurea-treated mammalian cells: implications for gene amplification. *Cancer Res.*;46:4607-4612.
- Hahn SS, Spaulding CA, Kim JA, Constable WC (1987)** The prognostic significance of lymph node involvement in pyriform sinus and supraglottic cancers. *Int J Radiat Oncol Biol Phys*;13(8):1143-7.
- Halevy O, Michalovitz D, Oren M (1990)** Different tumor-derived p53 mutants exhibit distinct biological activities. *Science*;250(4977):113-6.
- Hall P, Richards M, Gregory W, d'Ardenne A, Lister T, Stansfeld A (1988)** The prognostic value of Ki67 immunostaining in non-Hodgkin's lymphoma. *J. Pathol.*;154:223-235.
- Hall P, Watt F (1989)** Stem cells:the generation and maintenance of cellular diversity. *Development*;106:619-633.

- Hall PA, Levison DA (1990)** Review: Assessment of cell proliferation in histological material. *Journal of Clinical Pathology*;43:184-192.
- Hall PA, Levison DA, Woods AL, et al. (1990)** Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. *J Pathol*;162:285-294.
- Hamner J, Looney PD, Chused TM (1974)** Submucous fibrosis. *Oral Surg Oral Med Oral Pathol*;37(3):412-21.
- Harris AL (1990)** Mutant p53 - the commonest genetic abnormality in human cancer? *J. Pathol.*;162:5-6.
- Harris M (1991)** Clinical signs and symptoms of the high risk lesion. In: Johnson NW, ed. *Risk markers of Oral Diseases*. Cambridge: Cambridge University Press, : 157-164. vol Vol. 2 Oral Cancer).
- Harvey W, Scutt A, Meghji S, Canniff JP (1986)** Stimulation of human buccal mucosa fibroblasts in vitro by betel-nut alkaloids. *Arch Oral Biol*;31(1):45-9.
- Hinds P, Finlay C, Levine A (1989)** Mutation is Required to activate the p53 gene for cooperation with the *ras* oncogene and transformation. *J.Virol.*;63(2):739-746.
- Hirayama T (1966)** An epidemiological study of oral and pharyngeal cancer in Central and South-east Asia. *Bulletin of the World Health Organization*;34:41-69.
- Hollstein M, Sidransky D, Vogelstein B, Harris CC (1991)** p53 mutations in human cancers. *Science*;253(5015):49-53.
- Howard A, Pelc S (1951)** Nuclear incorporation of ^{32}P as demonstrated by autoradiographs. *Exp.Cell Res.*;2:178-187.
- Hume W (1985)** Keratinocyte proliferative hierarchies confer protective mechanisms in surface epithelia. *Brit. J. Dermatol.*;112:493-502.

- Iggo R, Gatter K, Bartek J, Lane D, Harris AL (1990)** Increased expression of mutant forms of p53 oncogene in primary lung cancer. *Lancet*;335(8691):675-9.
- Jaskulski D, Gattu C, Travali S, Calabretta B, Baserga R (1988)** Regulation of the proliferating cell nuclear antigen cyclin and thymidine kinase mRNA levels by growth factors. *J.Biol.Chem.*;263(21):10175-10179.
- Jenkins JR, Rudge K, Chumakov P, Currie GA (1985)** The cellular oncogene p53 can be activated by mutagenesis. *Nature*;317(6040):816-8.
- Joshi S (1953)** Submucous fibrosis of the palate and pillars. *Indian J Otolaryngol*;3:1-4.
- Jüptner H (1968)** cited in *Trop Dis Bull*;65:1176.
- Kamel D, Paakko P, Nuorva K, Vahakangas K, Soini Y (1993)** p53 and *c-erb B-2* protein expression in adenocarcinomas and epithelial dysplasias of the gall bladder. *J. Pathol.*;170:67-72.
- Kamel O, Franklin W, Ringus J, Meyer J (1989)** Thymidine labeling and Ki-67 growth fraction in lesions of the breast. *Am.J.Pathol.*;134:107-113.
- Kamel OW, LeBrun DL, Davis RE, Berry GJ, Warnke RA (1991)** Growth fraction estimation of malignant lymphomas in formalin-fixed paraffin-embedded tissue using anti-PCNA/cyclin 19A2. *Am.J.Pathol.*;138(6):1471-1477.
- Kaplan E, Meier P (1958)** Nonparametric estimation for incomplete observations. *J Am Statist Assoc*;53:457-481.
- Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, Craig RW (1991)** Participation of p53 protein in the cellular response to DNA damage. *Cancer Res*;51 (23 pt 1):6304-11.
- Kastan MB, Zhan Q, el DW, et al. (1992)** A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*;71(4):587-97.

- Kavarana NM, Bhathena HM (1987)** Surgery for severe trismus in submucous fibrosis. *Br J Plast Surg*;40(4):407-9.
- Kellett M, Hume W, Potten C (1989)** A topographical study of the circadian rhythm in labelling index of mouse gingival and floor of mouth epithelium, including changes in labelling activity with individual cell position on epithelial ridges. *Archives of Oral Biology*;34:321-328.
- Kern S, Fearon E, Tersmette K, et al. (1989)** Allelic loss in colorectal carcinoma. *JAMA*;261(21):3099-3103.
- Kern S, Pietenpol J, Thiagalingam S, Seymour A, Kinzler K, Vogelstein B (1992)** Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science*;256:827-830.
- Kern SE, Kinzler KW, Baker SJ, et al. (1991)** Mutant p53 proteins bind DNA abnormally in vitro. *Oncogene*;6(1):131-6.
- Kerns BJ, Jordan PA, Moore MB, et al. (1992)** p53 overexpression in formalin-fixed, paraffin-embedded tissue detected by immunohistochemistry. *J Histochem Cytochem*;40(7):1047-51.
- Kerr JF, Wyllie AH, Currie AR (1972)** Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*;26(4):239-57.
- Knudson AGJ (1985)** Hereditary Cancer, Oncogenes, and Antioncogenes. *Cancer Res.*;45:1437-1443.
- Knudson AJ (1983 a)** Cancer genes in man. *Curr Probl Cancer*;7(12):4-14.
- Knudson AJ (1983 b)** Model hereditary cancers of man. *Prog Nucleic Acid Res Mol Biol*;29(17):17-25.
- Kramer R, Lucas R, Pindborg J, Sobin L (1978)** Definition of leukoplakia and related lseions: An aid to studies on oral precancer. *Oral Surgery*;46(4):518-539.

- Kramer S, Marcial VA, Pajak TF, MacLean CJ, Davis LW (1986)**
Prognostic factors for loco/regional control and metastasis and the impact on survival. *Int J Radiat Oncol Biol Phys*;12(4):573-8.
- Kurki P, Ogata K, Tan EM (1988)** Monoclonal antibodies to proliferating cell nuclear antigen (PCNA) cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. *Journal of Immunological Methods*;109:49-59.
- Lajtha L (1979)** Stem cell concepts. *Differentiation*;14:23-43.
- Lal D (1953)** Diffuse oral submucous fibrosis. *J All India Dent Assoc*;26:1-3.
- Landberg G, Roos G (1991)** Antibodies to proliferating cell nuclear antigen as S-phase probes in flow cytometric cell cycle analysis. *Cancer Res.*;51:4570-4574.
- Landberg G, Tan EM, Roos G (1990)** Flow cytometric multiparameter analysis of proliferating cell nuclear antigen/cyclin and Ki-67 antigen: a new view of the cell cycle. *Exp.Cell Res.*;187:111-118.
- Landtwing K (1978)** Evaluation of the normal range of vertical mandibular opening in children and adolescents with special reference to age and stature. *Journal of Maxillofacial Surgery*;6:157.
- Lane D, Benchimol S (1990)** p53: oncogene or anti-oncogene? *Genes Dev.*;4:1-8.

- Lane D, Crawford LV (1979)** T antigen is bound to a host protein in SV40-transformed cells. *Nature*;278:261-263.
- Lane DP (1992)** Cancer. p53, guardian of the genome [news; comment]. *Nature*;358(6381):15-6.
- Langdon J, Rapidis A, Harvey P, Patel M (1977)** STNMP - A new classification for oral cancer. *British Journal of Oral Surgery*;15:49-54.
- Lavigne A, Bernstein A (1991)** p53 transgenic mice: accelerated erythroleukemia induction by Friend virus. *Oncogene*;6:2197-2201.
- Lavker R, Sun T (1983)** Rapid modulation of keratinocyte differentiation by the external environment. *Journal of Investigative Dermatology*;80:228-237.
- Leblond C, Greulich R, Pereira J (1964)** Relationship of cell formation and cell migration in the renewal of stratified squamous epithelia. In: Montagna W, Billingham R, ed. *Advances in Biology of Skin*. New York: Macmillan, : 39-67. vol 5).
- Lee E (1980)** Statistical Methods for Survival Data Analysis. Nonparametric Survival Distribution. Belmont, California: Life Learning Publication,:
- Lee M, Nurse P (1988)** Cell cycle control genes in fission yeast and mammalian cells. *Trends in Genetics*;4:287-290.
- Lee NK (1992)** Tumor suppressor genes. *Head Neck*;14(5):407-14.
- Leonardi E, Girlando S, Serio G, et al. (1992)** PCNA and Ki67 expression in breast carcinoma: correlations with clinical and biological variables. *J Clin Pathol*;45(5):416-9.
- Levine AJ, Momand J, Finlay CA (1991)** The p53 tumour suppressor gene. *Nature*;351:453-456.
- Loe H, Karring T, Harra K (1972)** The site of mitotic activity in rat and human oral epithelium. *Scandinavian Journal of Dental Research*;80:111-119.

- Löe H, Silness J (1963)** Periodontal disease in pregnancy 1. Prevalance and severity. *Acta Odontol Scan*;21(533-551).
- Lowe S, Schmitt E, Smith S, Osborne B, Jacks T (1993)** p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*;362:847-849.
- Lozano G, Levine AJ (1991)** Tissue-specific expression of p53 in transgenic mice is regulated by intron sequences. *Mol Carcinog*;4(1):3-9.
- Mani N, Singh B (1968)** Studies on oral submucous fibrosis.1. Clinical aspects. *J All India Acad Dent*.;9:27-36.
- Marshall J, Graham S, Mettlin C, Shedd D, Swanson M (1982)** Diet in the epidemiology of oral cancer. *Nutr Cancer*;3(3):145-9.
- Martinez J, Georgoff I, Martinez J, Levine A (1991)** Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. *Genes Dev*.;5:151-159.
- Masuda H, Muller H, Koeffler H, Battifora H, Kline M (1987)** Rearrangement of the p53 gene in human osteogenic sarcoma. *Proc Natl Acad Sci USA*;84:7716.
- Mathews MB, Bernstein RM, Franza BR, Garrels JI (1984)** The identity of the proliferating cell nuclear antigen and cyclin. *Nature*;309:374-376.
- Matsumoto K, Moriuchi T, Koji T, Nakane P (1987)** Molecular cloning of cDNA coding for rat proliferating cell nuclear antigen (PCNA)/cyclin. *European Molecular and Biological Organ Journal*;6:637-642.
- McFarlane J, Quirke P, Bird C (1986)** Flow cytometric analysis of DNA heterogeneity in non-Hodgkin's lymphoma. *J. Pathol*.;149:236a.
- McLaughlin JK, Gridley G, Block G, et al. (1988)** Dietary factors in oral and pharyngeal cancer. *J Natl Cancer Inst*;80(15):1237-43.

- Mehta FS, Pindborg JJ, Gupta PC, Daftary DK (1969)** Epidemiologic and histologic study of oral cancer and leukoplakia among 50,915 villagers in India. *Cancer*;24(4):832-49.
- Mercer WE, Baserga R (1985)** Expression of the p53 protein during the cell cycle of human peripheral blood lymphocytes. *Exp Cell Res*;160(1):31-46.
- Mercer WE, Shields MT, Amin M, et al. (1990)** Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. *Proc. Natl. Acad. Sci. USA*;87:6166-6170.
- Mercer WE, Shields MT, Lin D, Appella E (1991)** Growth suppression induced by wild-type p53 protein is accompanied by selective down-regulation of proliferating-cell nuclear antigen expression. *Proc. Natl. Acad. Sci. USA*;88:1958-1962.
- Meyer J, Friedman E, McCrate M, Bauer W (1983)** Prediction of early course in breast carcinoma by thymidine labelling. *Cancer*;51:1879-1886.
- Mezitis M, Rallis G, Zachariades N (1989)** The normal range of mouth opening. *Journal of Oral & Maxillofacial Surgery*;47:1028-1029.
- Midgley CA, Fisher CJ, Bartek J, Vojtesek B, Lane D, Barnes DM (1992)** Analysis of p53 expression in human tumours: an antibody raised against human p53 expressed in *Escherichia coli*. *J Cell Sci*;101:183-9.
- Milner J (1991)** The role of p53 in the normal control of cell proliferation. *Curr Opin Cell Biol*;3(2):282-6.
- Miyachi K, Fritzler MJ, Tan EM (1978)** An autoantibody to a nuclear antigen in proliferating cells. *J Immunol*;121:2228-2234.
- Moetel C, Foss E (1958)** Multicentric carcinomas of the oral cavity. *Surgery, Gynecology and Obstetrics*;106:652-659.
- Moore K, Sullivan K, Tan E, Prystowsky M (1987)** Proliferating cell nuclear antigen/cyclin is an interleukin 2-responsive gene. *J. Biol. Chem.*;262(18):8447-8450.

- Morgan W, Bodycore J, Fero J, et al. (1986)** A cytogenetic investigation of DNA rereplication after Hydroxyurea treatment: implications for gene amplification. *Chromosoma*;93:191-196.
- Morris GF, Mathews MB (1989)** Regulation of proliferating cell nuclear antigen during the cell cycle. *J.Biol.Chem.*;264:13856-13864.
- Mowat MA, Cheng A, Kimura N, Bernstein A, Benchimol S (1985)** Rearrangements of the cellular p53 gene in erythroleukaemic cells transformed by Friend virus. *Nature*;314:633-636.
- Mukherjee A, Biswas S (1972)** Oral submucous fibrosis-A search for aetiology. *Indian Journal of Otolaryngology*;24:11-15.
- Murti PR, Bhonsle RB, Pindborg JJ, Daftary DK, Gupta PC, Mehta FS (1985)** Malignant transformation rate in oral submucous fibrosis over a 17-year period. *Community Dent Oral Epidemiol*;13(6):340-1.
- Nigro J, Baker S, Preisinger S, et al. (1989)** Mutations in the p53 gene occur in diverse human tumour types. *Nature*;342:705.
- Nishida C, Reinhardt P, Linn S (1988)** DNA repair synthesis in human fibroblasts requires DNA polymerase δ . *J.Biol.Chem.*;263(1):501-510.
- Nowell PC (1982)** Genetic instability in cancer cells: relationship to tumor cell heterogeneity. In: Owens AH, ed. *Tumor Cell Heterogeneity*. New York: Academic Press, Inc. : 351-365.
- Oliner JD, Kinzler KW, Meltzer PS, George DL, Vogelstein B (1992)** Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature*;358(6381):80-3.
- Ostrowski JL, Sawan A, Henry L, et al. (1991)** p53 expression in human breast cancer related to survival and prognostic factors: an immunohistochemical study. *J Pathol*;164(1):75-81.

- Patsouris E, Stocker U, Kallmeyer V, Keiditsch E, Mehraein P, Stavrou D (1988)** Relationship between Ki-67 positive cells, growth rate and histological type of human intracranial tumours. *Anticancer Res.*;8:537-544.
- Pavelic ZP, Gluckman JL, Gapany M, et al. (1992)** Improved immunohistochemical detection of p53 protein in paraffin--embedded tissues reveals elevated levels in most head and neck and lung carcinomas: correlation with clinicopathological parameters. *Anticancer Res*;12(5):1389-94.
- Paymaster J (1956)** Cancer of the buccal mucosa: a clinical study of 650 cases in Indian patients. *Cancer*;9:431-435.
- Penneys N, Fulton J Jr, Weinstein G, al e (1970)** Location of proliferating cells in human epidermis. *Brit. J. Dermatol.*;49:323-327.
- Phatak AG (1984)** Hypercoagulation and fibrinolysis in oral sub-mucous fibrosis. *Am J Clin Pathol*;81(5):623-8.
- Pindborg J, Bhatt M, Devanath K, Narayana H, Ramachandra S (1966)** Frequency of oral white lesions among 10,000 individuals in Bangalore, South India. Preliminary report. *Indian J Med Sci*;2(349-352).
- Pindborg J, Chawla T, Misra R, Nagpaul R, Gupta V (1965)** Frequency of oral carcinoma, leukoplakia, leukokeratosis, leukoedema, submucous fibrosis, and lichen planus in 10,000 Indians in Lucknow, Uttar Pradesh, India. Preliminary report. *J. Dent. Res.*;44:615.
- Pindborg J, Kalapesi H, Kale S, Singh B, Taleyarkhan B (1965)** Frequency of oral leukoplakia and related conditions among 10,000 Bombayites. Preliminary report. *J All India Dent Assoc*;37:228-229.
- Pindborg J, Mehta F, Gupta P, Daftary D (1968)** Prevalence of oral submucous fibrosis among 50915 Indian villagers. *Brit J Cancer*;22:646-654.
- Pindborg J, Singh B (1964)** Formation of vesicles in oral submucous fibrosis. *Acta Pathologica et Microbiologica Scandinavia*;62:562-566.

- Pindborg JJ (1965)** Frequency of oral submucous fibrosis in North India. *Bull World Health Organ*;32(5):748-50.
- Pindborg JJ (1972)** Is submucous fibrosis a precancerous condition in the oral cavity? *Int Dent J*;22(4):474-80.
- Pindborg JJ (1989)** Oral submucous fibrosis: a review. *Ann Acad Med Singapore*;18(5):603-7.
- Pindborg JJ, Bhonsle RB, Murti PR, Gupta PC, Daftary DK, Mehta FS (1980)** Incidence and early forms of oral submucous fibrosis. *Oral Surg Oral Med Oral Pathol*;50(1):40-4.
- Pindborg JJ, Mehta FS, Daftary DK (1970)** Occurrence of epithelial atypia in 51 Indian villagers with oral submucous fibrosis. *Br J Cancer*;24(2):253-7.
- Pindborg JJ, Mehta FS, Gupta PC, Daftary DK (1968)** Prevalence of oral submucous fibrosis among 50,915 Indian villagers. *Br J Cancer*;22(4):646-54.
- Pindborg JJ, Murti PR, Bhonsle RB, Gupta PC, Daftary DK, Mehta FS (1984)** Oral submucous fibrosis as a precancerous condition. *Scand J Dent Res*;92(3):224-9.
- Pindborg JJ, Sirsat SM (1966)** Oral submucous fibrosis. *Oral Surg Oral Med Oral Pathol*;22(6):764-79.
- Pindborg JJ, Zachariah J (1965)** Frequency of oral submucous fibrosis among 100 South Indians with oral cancer. *Bull World Health Organ*;32(5):750-3.
- Potten C (1974)** The epidermal proliferative unit: the possible role of the central basal cell. *Cell Tissue Kinetics*;7:77-88.
- Potten C (1992)** Cell lineages. In: McGee J, Isaacson P, Wright N, ed. *Oxford Textbook of Pathology, Principles of Pathology*. Oxford: Oxford University Press, : 43-52. vol 1).
- Potten C, Loeffler M (1990)** Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development*;110:1001-1020.

- Potten C, Morris R (1988)** Epithelial stem cells in vivo. *J Cell Sci;Suppl.* 10:45-62.
- Prelich G, Stillman B (1988)** Coordinated leading and lagging strand synthesis during SV40 DNA replication in vitro requires PCNA. *Cell*;53:117-126.
- Purdie C, O'Grady J, Piris J, Wyllie A, Bird C (1991)** p53 expression in colorectal tumours. *Am.J.Pathol.*;138(807-813).
- Quinn C, Wright N (1990)** The clinical assessment of proliferation and growth in human tumours: evaluation of methods and application as prognostic variables. *J. Pathol.*;160:93-102.
- Rajendran R, Sugathan CK, Remani P, Ankathil R, Vijayakumar T (1986)** Cell mediated and humoral immune responses in oral submucous fibrosis. *Cancer*;58(12):2628-31.
- Ramanathan K (1981)** Oral submucous fibrosis--an alternative hypothesis as to its causes. *Med J Malaysia*;36(4):243-5.
- Rao A (1962)** Idiopathic palatal fibrosis. *British Journal of Surgery*;50:23-25.
- Reich NC, Levine AJ (1984)** Growth regulation of a cellular tumour antigen, p53, in nontransformed cells. *Nature*;308(5955):199-201.
- Reich NC, Oren M, Levine AJ (1983)** Two distinct mechanisms regulate the levels of a cellular tumor antigen, p53. *Mol Cell Biol*;3(12):2143-50.
- Reihsaus E, Kkohler M, Kraiss S, Oren M, Montenarh M (1990)** Regulation of the level of the oncoprotein p53 in non-transformed and transformed cells. *Oncogene*;5:137-145.
- Rich A, Raddon B (1984)** Squamous cell carcinoma of the oral mucosa: a review of 244 cases in Australia. *Journal of Oral Pathology*;13:459-471.
- Rodrigues NR, Rowan A, Smith ME, et al. (1990)** p53 mutations in colorectal cancer. *Proc Natl Acad Sci U S A*;87(19):7555-9.

- Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM (1990)** The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell*;63(6):1129-36.
- Schuller DE, McGuirt WF, McCabe BF, Young D (1980)** The prognostic significance of metastatic cervical lymph nodes. *Laryngoscope*;90(4):557-70.
- Schwarting R, Gerdes J, Niehus J, Jaeschke, Stein H (1986)** Determination of the growth fraction in cell suspensions by flow cytometry using monoclonal antibody Ki-67. *Journal of Immunological Methods*;90:65-70.
- Schwartz J.** Atrophica idiopathica (tropica) mucosa oris. In: Presented at the Eleventh International Dental Congress, London., 1952:
- Scully C (1992)** The oral cavity. In Champion R.H., Burton J.L. Ebling F.J.G. *Textbook of Dermatology*. Oxford, Blackwell Publications;:2689-2760.
- Seddon S, Walker D, Williams D, Williams E (1992)** The clonal organisation of the squamous epithelium of the tongue. *Cell Proliferation*;25:115-124.
- Seedat HA, van Wyk C (1988)** Betel-nut chewing and submucous fibrosis in Durban. *S Afr Med J*;74(11):568-71.
- Sharan J (1959)** Histopathological observations on cases of submucous fibrosis of the oral cavity. *Indian Journal of Pathology and Bacteriology*;2:150-152.
- Shaulsky G, Ben-Ze'ev A, Rotter V (1990)** Subcellular distribution of the p53 protein during the cell cycle of Balb/c 3T3 cells. *Oncogene*;5:1707-1711.
- Shear M, Lemmer J, Dockrat I (1967)** Oral submucous fibrosis in South African Indians: an epidemiological study. *S Afr J Med Sci*;32(1):41-6.
- Shiau YY, Kwan HW (1979)** Submucous fibrosis in Taiwan. *Oral Surg Oral Med Oral Pathol*;47(5):453-7.

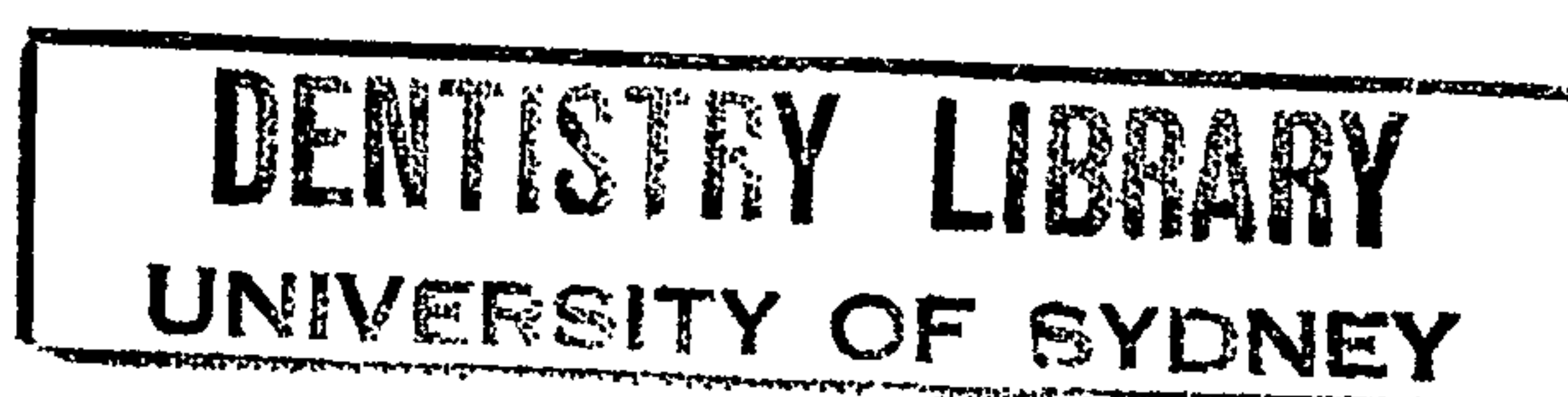
- Shibuya H, Amagasa T, Seto K, Horiuchi J, Suzuki S (1986)**
Leukoplakia-associated multiple carcinomas in patients with tongue carcinoma.
Cancer;6:843-846.
- Silness J, Loe H (1964)** Periodontal disease in pregnancy. II. Correlation
between oral hygiene and periodontal condition. *Acta Odontol Scan*;22:121-
135.
- Silvestrini R, Costa A, Veneroni S, Del Bino G, Persici P (1988)**
Comparative analysis of different approaches to investigate cell kinetics. *Cell
Tissue Kinet*;21:123-131.
- Sirsat S, Khanolkar V (1957)** A histochemical and electron-microscope study of
submucous fibrosis of the palate. *Indian Journal of Pathology and
Bacteriology*;73:439-442.
- Sirsat S, Khanolkar V (1960)** Submucous fibrosis of the palate in diet
preconditioned Wistar rats: induction by local painting of capsaicin-an optical
and electron microscopic study. *Archives of Pathology*;70:171-179.
- Sirsat SM, Pindborg JJ (1967)** Subepithelial changes in oral submucous
fibrosis. *Acta Pathol Microbiol Scand*;70(2):161-73.
- Slaughter H, Smejkal W, Southwick D (1953)** Field cancerization in oral
stratified squamous epithelium: Clinical implications of multicentric origin.
Cancer;57:843-846.
- Smith CJ, Pindborg JJ (1969)** Histological Grading of Oral Epithelial Atypia by
the use of Photographic Standards. Copenhagen: C. H. Munksgaard
Bogtrykkeri,;
- Soussi T, Caron DE, Fromental C, May P (1990)** Structural aspects of the
p53 protein in relation to gene evolution. *Oncogene*;5(7):945-52.
- Steel G (1977)** Growth kinetics of tumours. Oxford: Clarendon Press. .

- Steinmeyer K, Macke H, Deppert W (1990)** Cell cycle control by p53 in normal (3T3) and chemically transformed (Meth A) mouse cells. 1. Regulation of p53 expression. *Oncogene*;5:1691-1699.
- Stell P, McCormic M (1985)** Cancer of the head and neck: are we doing any better? *Lancet*;2:1127.
- Strang P, Eklund G, Stendahl U, Frankendal B (1987)** S-phase rate as a predictor of early recurrences in carcinoma of the uterine cervix. *Anticancer Res*;7:807-810.
- Sturzbecher HW, Addison C, Jenkins JR (1988)** Characterization of mutant p53-hsp72/73 protein-protein complexes by transient expression in monkey COS cells. *Mol Cell Biol*;8(9):3740-7.
- Su I (1954)** Idiopathic scleroderma of the mouth. Report of three cases. *Archives of Otolaryngology*;59:330-332.
- Suzuki H, Takahashi T, Kuroishi T, et al. (1992)** p53 mutations in non-small cell lung cancer in Japan: association between mutations and smoking. *Cancer Res*;52(3):734-6.
- Takahashi H, Strutton G, Parsons P (1991)** Determination of proliferating fractions in malignant melanomas b anti-PCNA/cyclin monoclonal antibody. *Histopathology*;18:221-227.
- Takahashi T, D'Amico D, Chiba I, Buchhagen DL, Minna JD (1990)** Identification of intronic point mutations as an alternative mechanism for p53 inactivation in lung cancer. *J Clin Invest*;86(1):363-9.
- Takahashi T, Nau MM, Chiba I, et al. (1989)** p53: a frequent target for genetic abnormalities in lung cancer. *Science*;246(4929):491-4.
- Tan TH, Wallis J, Levine AJ (1986)** Identification of the p53 protein domain involved in formation of the simian virus 40 large T-antigen-p53 protein complex. *J Virol*;59(3):574-83.

- Thor A, Yandell D (1993)** Editorial: Prognostic significance of p53 overexpression in node-negative breast carcinoma: preliminary studies support cautious optimism. *Journal of the National Cancer Institute*;85(3):176-177.
- Tlsty TD (1990)** Normal diploid human and rodent cells lack a detectable frequency of gene amplification. *Proc. Natl. Acad. Sci. USA*;87:3132-3136.
- Toschi L, Bravo R (1988)** Changes in cyclin/PCNA distribution during DNA repair synthesis. *J. Cell Biol.*;107:1623-1628.
- Tsuji T, Shrestha P, Yamada K, et al. (1992)** Proliferating cell nuclear antigen in malignant and pre-malignant lesions of epithelial origin in the oral cavity and the skin: an immunohistochemical study. *Virchows Archiv A Pathological Anatomy and Histopathology*;420:377-383.
- Tubiana M, Courdi A (1989)** Cell proliferation kinetics in human solid tumours: relation to probability of metastatic dissemination and long-term survival. *Radiother. Oncol*;15:1-18.
- Uchino S, Tsuda H, Noguchi M, et al. (1992)** Frequent loss of heterozygosity at the DCC locus in gastric cancer. *Cancer Res*;52(11):3099-3102.
- Underwood J (1987)** Introduction to biopsy interpretation and surgical pathology. (2nd ed.) Springer-Verlag:
- US Public Health Service.** The Health Consequences of Smoking. In: Washington DC: US Department of Health and Human Services, 1982:
- van Nest D, Staquet M, Viac J, et al. (1983)** A new way to evaluate the germinative compartment in human epidermis using [3H] thymidine incorporation and immunoperoxidase staining of 67k polypeptide. *Brit. J. Dermatol.*;108:433.

- van den Bogaert W, Ostyn F, van der Schueren E (1983) The different clinical presentation, behaviour and prognosis of carcinomas originating in the epilarynx and the lower supraglottis. *Radiother Oncol*;1(2):117-31.
- van Wyk C, Seedat HA, Phillips VM (1990) Collagen in submucous fibrosis: an electron-microscopic study. *J Oral Pathol Med*;19(4):182-7.
- van Wyk C, Staz J, Farman A (1977) The prevalence of oral mucosal lesions among a random sample of Asian residents in Cape Town. *J Dent Ass S Afr*;32:589-592.
- Verheijen R, Kuipers HJH, Schlingemann RO, et al. (1989) Ki-67 detects a nuclear matrix-associated proliferation-related antigen. II. Localization in mitotic cells and association with chromosomes. *J Cell Sci*;92:531-540.
- Vogelstein B, Fearon E, Hamilton S, et al. (1988) Genetic alterations during colorectal-tumor development. *New England Journal of Medicine*;319:525-532.
- Vojtesek B, Bartek J, Midgley CA, Lane DP (1992) An immunochemical analysis of the human nuclear phosphoprotein p53. New monoclonal antibodies and epitope mapping using recombinant p53. *J Immunol Methods*;151(1-2):237-44.
- Wahi P (1968) The eidemiology of oral and oropharyngeal cancer. A report of the study in Mainpuri district, Uttar Pradesh, India. *Bulletin of the World Health Organization*;38:495-521.
- Wahi P, Kapur V, Luthra U, Srivastava M (1966) Submucous fibrosis of the oral cavity. 2. Studies on epidemiology. *Bulletin of the World Health Organization*;35:793-799.
- Wahi P, Mital V, Lahiri B, Luthra U, Seth R, Arora G (1970) Epidemiological study of precancerous lesions of the oral cavity:A preliminary report. *Indian Journal of Medical Research*;58:1361-1391.
- Weinberg R (1989) Oncogenes, antioncogenes and the molecular basis of multistep carcinogenesis. *Cancer Res*;49:3713-3721.

- Windle B, Wahl G (1992)** Molecular dissection of mammalian gene amplification: new mechanistic insights revealed by analyses of very early events. *Mutation Research*;276:199-224.
- Wood G, Branco J (1979)** A comparison of three methods of measuring maximal opening of the mouth. *Journal of Oral Surgery*;37:175.
- World Health Organization (1984)** Control of oral cancer in developing countries. A World Health Organization meeting. *Bulletin of the World Health Organization*;62:817-830.
- World Health Organization.** International Classification of Diseases. Geneva: WHO., 1977:
- Wright N (1984)** Cell proliferation on health and disease. In Anthony P.P., MacSween RNM eds. *Recent advances in Histopathology 12*. Edinburgh: Churchill Livingstone.;17-33.
- Yewdell J, Gannon J, Lane D (1986)** Monoclonal antibody analysis of p53 expression in normal and transformed cells. *J.Virol.*;59:444-452.
- Yin Y, Tainsky MA, Bischoff FZ, Strong LC, Wahl GM (1992)** Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell*;70(6):937-48.



- Yonish RE, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M (1991)**
Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature*;353(6333):345-7.
- Zachariah J, Mathew B, Varma N, Iqbal A, Pindborg J (1966)** Frequency of oral mucosal lesions among 5,000 individuals in Trivandrum, South India. *J All India Dent Assoc*;38:290-294.
- Zantema A, Schrier P, Davies-Olivier A, Van Laar T, Vaessen R, Van Der Eb A (1985)** Adenovirus serotype determines association and localisation of the large E1b tumor antigen with cellular tumor antigen p53 in transformed cells. *Mol.Cell Biol.*;5:3084-3091.