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The Suitability of Paraffin-embedded Material to Predict Metastatic Potential of Oral Squamous Cell Carcinoma

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Abstract. *Background: The presence of regional metastasis in oral squamous cell carcinoma (OSCC) is an important prognostic factor. This study was undertaken to identify histological features and biological markers from paraffin-embedded primary OSCC that may predict the presence of regional metastases. Materials and Methods: Fifty-three en-bloc primary OSCC resections were divided into two groups, 26 with lymph node metastases and 27 without metastases. The pattern of infiltration, presence of vascular or perineural infiltration and tumour necrosis were evaluated while expression of p53, p21 and Rb were assessed in the two groups. DNA ploidy status was also determined with a flow cytometer. Results: The presence of DNA aneuploidy was found to be the only statistically significant predictor of regional metastases. Seventy-seven per cent of the primary OSCC with lymph node metastases showed DNA aneuploidy. Conclusion: DNA flow cytometry obtained from archival material could be used as a parameter to predict regional metastases.*

The TNM staging system for patients with oral squamous cell carcinoma (OSCC) is widely used to facilitate treatment planning and to predict behaviour and prognosis. Patients with advanced disease have the worst prognosis while the presence of regional nodal metastasis is considered to be the most important prognostic factor (1, 2). Although T3-4 OSCC's of the tongue and floor of mouth have a higher incidence of nodal metastasis than do T1-2 carcinomas (2, 3), the ability of OSCC to metastasise is not always related to clinical staging (4). Evaluation of the primary tumour for features to predict metastatic potential is important because regional metastases are only detected with an accuracy of around 70% using clinical examination (5). Although modern imaging techniques may improve the detection of overt metastases, even the most sophisticated methods may not disclose occult metastases (6).

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Conflicting results have been reported when using histological grading and expression of a variety of biological markers to predict lymph node metastasis of head and neck squamous cell carcinomas (4, 7-9). The significance of flow cytometric DNA content in OSCC is well documented. Aneuploidy has been correlated with metastatic spread (10, 11) and shown to be an independent prognostic factor (12, 13). Significant differences are found between flow cytometric data obtained from analyses of paraffin-embedded material *versus* fresh tissue (14) and therefore the source of the material should always be taken into account when comparing results.

The concept of tumour cell heterogeneity with regard to the metastatic potential is widely accepted (15). It is at present not possible to divide the cells in a primary tumour into those with metastatic potential and those without this potential, although completion of the human genome sequence would facilitate studies on possible genetic alterations (16). This retrospective study was undertaken to identify biological markers in paraffin-embedded primary OSCC that might predict the presence of regional metastases.

Materials and Methods

En-bloc tumour and lymph node resections of primary OSCC from the tongue or floor of mouth were retrieved from the files of the departments of Anatomical Pathology and Oral Pathology, University of Pretoria. Fifty-three cases were selected and divided into two groups based on the presence or absence of metastatic tumour in the regional lymph nodes. Twenty-six cases had metastatic tumour in one or more lymph nodes while no metastases could histologically be demonstrated in 27 cases.

All the H&E sections of the primary tumour were reviewed and certain grading features were assessed. These included: the presence of vascular invasion, presence of neural or perineural infiltration, presence of eosinophils in the surrounding inflammatory infiltrate, presence of tumour necrosis and presence of individual tumour cell infiltration in the deep invading margins.

Tumour sections and positive lymph nodes were also evaluated for immunohistochemical expression of p53 (DO-7, DAKO Corporation, Carpinteria, CA 93013 USA; prediluted), $p21^{WAF1/Cip1}$ (Clone SX118, DAKO Corporation, Produktionsvey 42, DK-2600 Glostrup, Denmark; 1:50 dilution) and retinoblastoma tumour-suppressor gene (Rb) (DAKO Corporation, Produktionsvey 42, DK-2600 Glostrup, Denmark; 1:25 dilution). The staining techniques were performed according to the manufacturer's instructions. Antigen retrieval was performed with a microwave pressure cooker in citric acid buffer (pH 7.6) in all cases. For

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Table I. *Histomorphological features used to access metastatic potential of OSCC.*

Grading features	Tumours with metastases (n=26)	Tumours without metastases (n=27)
individual cell infiltration	24	21
presence of eosinophils	17	16
tumour necrosis	7	7
vascular invasion	2	1
neural/perineural spread	7	6

Table II. *Immunohistochemical detection of protein expression in OSCC.*

Antibody		Tumours with metastases (n=26)		Tumours without metastases (n=27)	
p53	-	6		9	
	(+)	3	38%	1	44%
	+	1		2	
	++	5	62%	7	56%
	+++	11		8	
Rb	-	2		2	
	(+)	1	19%	2	18%
	+	2		1	
	++	12	81%	17	82%
	+++	9		5	
p21	-	2		7	
	(+)	6	77%	3	77%
	+	12		11	
	++	6	23%	6	23%
	+++	0		0	

all antibodies, only nuclear staining was regarded as positive. The following scoring system was used for the immunohistochemistry staining: negative - no positive cells; (+) - scattered positive cells; + - less than 10% positive cells; ++ - between 10 and 50% positive cells; +++ - more than 50% positive cells.

Sections from the same tumour block used for immunohistochemical analyses were used for DNA ploidy analysis. The sections were prepared according to the modified Hedley method (17). In short, four to six 40 μm sections were cut, wrapped in 50 μm nylon mesh, placed in a histocasssette and manually dewaxed and hydrated to distilled water. The sections were left in distilled water overnight where after they were digested in subtilisin Carlsberg solution at 37°C for 120 minutes. The cell suspension was then stained with DAPI (4'6 diamidino 2 phenylindole) (Research Organics, Cleveland, OH, USA). Flow cytometry was carried out using a PAS II flow cytometer equipped with a high-pressure 100W mercury lamp (Partec, Münster, Germany). DNA histograms of at least 10,000 cells were plotted. The diploid cell population was used as an internal reference standard for the identification of aneuploid clones.

All the primary tumours were evaluated without knowing the metastatic status of the tumours. The factors evaluated were related to the nodal status in 2X2 contingency tables. The Chi square with Yates correction and Fischer's exact tests were used for the analysis of the categorical data. Correlations with a p <0.05 were considered statistically significant.

Table III. *Flow cytometric analysis of DNA ploidy status of OSCC.*

Ploidy status	Tumours with metastases (n=26)	Tumours without metastases (n=27)
Aneuploid	20 (77%)	12 (44%)
Diploid	6 (23%)	15 (56%)

Results

The grading features of the primary tumours are reflected in Table I. These differences were not statistically significant. No correlation was found between the expression or lack of expression of p53 (p=0.87), Rb (p= 1) and p21 (p=0.80) in the primary tumour and the presence of lymph node metastases. Neither was a correlation apparent for any of these markers if the cases were divided in two groups: one group with more than 10% positive staining cells (++ and +++) and the other group with less than 10% positive staining cells. The immunohistochemical results are summarised in Table II. Virtually the same expression of the three antibodies used was also found when the primary tumours cells were compared to its metastatic tumour cells.

Twenty of the primary tumours with metastases were aneuploid while the majority of the group without metastases were diploid (Table III). These differences were highly significant (p=0.033). The coefficient of variation (CV) ranged between 2.8% and 6% with a mean of 4.4% (SD = 0.7).

Discussion

Grading features are often considered to be unreliable due to interobserver and intraobserver variability although this can be minimised when using well-defined criteria (18, 19). A relation between growth pattern and regional metastases has been described (20, 21) but could not be confirmed in the present study or by others (7). Contradictory results regarding the prognostic value of an eosinophilic infiltrate adjacent to the infiltrating tumour islands have also been reported.

Although a few studies link a favourable prognosis to an eosinophilic infiltrate (22, 23), a correlation between lymph node metastases and stromal eosinophylia could not be demonstrated in previous studies (23, 24) and this study. Vascular and perineural infiltration are well-documented to be important predictors of metastases in OSCC (25-27) but this could not be confirmed in our study.

The overexpression of p53 in head and neck carcinomas is well-established. p53 protein alteration is considered an early event that is maintained during tumour progression and metastases (28). p53 has been widely used as a marker to predict metastatic potential with conflicting results. Expression of p53 has been reported to be associated with lymph node metastases (29) while others, including the present study, found no association (30).

p21 arrest the cell cycle in G1-or S-phase by forming a complex with cyclin A (or B or D or E), cdk2 (cyclin-dependent kinase) and PCNA (31). This complex inhibits the kinase activity of the cdk's and for that reason plays a role in keeping cells in a non-mitotic state. There is evidence that p53 regulates p21 expression directly, although recent studies have suggested that p53 independent pathways may also lead to p21 expression (32). Although no difference in p21 expression could be detected in the two groups in the present study, the expression also appears to be lower than reported in the literature (32-34) Comparison with published reports was difficult due the different criteria used to define positivity. No significant relationship could be demonstrated in our study between p53 and p21 expression supporting the co-existence of p53-independent pathways.

The meaning of expressed Rb is unclear. It could present functional protein, mutated protein or a combination of both. Phosphorylation of pRb during the G1-phase of the cell cycle allows for the transition from G1-to S-phase (35). pRb also has a possible role in the regulation of apoptosis. Using a panel of immunohistochemical markers in laryngeal carcinomas to predict nodal metastases, Rb was the only nuclear factor that showed a positive correlation with nodal metastases (7), although this could not be confirmed by others (36) or the current study.

Inconsistency in results based on immunohistochemistry exists. The scoring categories for immunohistochemical analysis were chosen subjectively without any biological criteria. It is well-known that most tumours express heterogeneity in markers or chromosomal aberrations in different areas due to clonal variation (37). A small subclone may therefore develop alterations reflected in the immunohistochemical profile that lead to metastasis without affecting the scoring of the primary tumour as a whole. In this case, it would be expected that metastatic tumour cells would show a similar staining pattern with the subclone responsible for the metastasis. The fact that expression of the three antibodies in the primary tumour and its metastases were similar, suggested that these alterations were early events in carcinogenesis. Reliable and reproducible results and interpretations of immunohistochemical markers are essential before it can be used on a large scale as a prognostic marker. The need expressed for collaboration to standardize the use of immunohistochemical markers for specific applications (38) are supported.

DNA aneuploidy in the primary tumour was found to be the only statistically significant marker of regional metastases in this study. Although the occasional presence of metastatic disease in diploid OSCC cannot be excluded and should be accepted, it is likely that the majority of the diploid tumours in the positive nodal group were false diploid due to the inability to detect tumour cell populations with only small deviations of their DNA content from normal diploid cells (39). This inability to detect tumour cell populations with small DNA abnormalities is further compromised because of the use of formalin-fixed paraffin-embedded blocks (14). This is most likely a major contributing factor in the conflicting findings reported about the prognostic value of DNA flow cytometry in OSCC. Numerous studies on DNA flow cytometry in OSCC do not reveal the CVs of the measurements and were predominantly done on paraffin-embedded material (40-42). The quality of DNA flow cytometric analyses is measured by its CV. A mean CV of 4.4% was obtained in the present study while most studies described CVs higher than 5% when using archival material (11, 43). The ability to use the more sensitive DAPI staining procedure (12) had contributed towards the relatively lower CV obtained for paraffin-embedded material.

It is well-documented that the development of aneuploid clones contributes to the metastatic development of OSCC (10). It was possible that aneuploid clones in the 12 aneuploid tumours without metastases had not yet disseminated to the regional lymph nodes at the time of surgery or that the metastatic lesions were too small to be detected by routine histological examination (6).

Tumour metastases is a highly intricate and dynamic process consisting of a number of chronological steps based on several interactions between tumour cells and host tissue (44). High-resolution flow cytometry using fresh tumour tissue has been shown to be an independent prognostic factor in OSCC (12). The results of this study indicated that the metastatic potential of OSCC could largely be predicted with the use of DNA flow cytometry, even with the shortcomings of using paraffin-embedded blocks instead of fresh tissue.

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RESEARCH ARTICLE

Detection of p53 Gene Mutations in Oral Squamous Cell Carcinomas of a Black African Population Sample

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Mutations in the p53 gene have been reported in head and neck carcinomas. We determined the p53 mutation profile in 55 oral squamous cell carcinomas (OSCCs) from a black African population sample. DNA from all the patients were investigated using PCR amplification of the p53 gene (exons 5–9), followed by heteroduplex single-stranded conformational polymorphism (HEX-SSCP) analysis on the PCR products. Direct sequencing was performed on cases where mutations were identified. The results showed mutations in 13 of 55 (23.6%) tumours. Eleven of 13 (85%) were single base pair substitutions (9 transitions and 2 transversions), and 2 were deletions. Two novel mutations were identified: a large 63-base pair deletion, and a single base pair substitution. The mutations in our study occurred outside the head and neck tumour hot spot region (codons 238–248). Hum Mutat 11:39–44, 1998.

KEY WORDS: p53; mutations; oral carcinoma; African

INTRODUCTION: DETERMINING P53 MUTATION PROFILE

Approximately 3% of all malignancies arise in the oral cavity (Silverman and Grosky, 1990). Recent studies suggest a global increase in the incidence of oral squamous cell carcinoma (OSCC), especially in developing countries where it is the third most common malignant disease among men (Johnson, 1991). The age standardised incidence rate of oral and pharyngeal cancer in the black population of South Africa is 14.2 for males and 2.6 for females, as obtained from the National Cancer Registry of 1990/1 (Sitas et al., 1996). Historically, there was a strong predilection for the occurrence of OSCC in men, but today, in most parts of the world, the male: female ratio is 2 : 1 (Silverman and Grosky, 1990). OSCC in South Africa is the fourth most prevalent malignancy among black males and the eleventh for females.

OSCC occurs over a wide age range, with a peak incidence in the sixth and seventh decades. A significant different age distribution was found between black and white male patients, where 33.4% of black male patients were below the age of 50 years compared to 15.6% of white males (Flemming et al., 1982). This difference may be the result of exposure to carcinogenic agents at a young age. Conflicting findings regarding tobacco use, a well-documented carcinogenic agent, have been reported in young OSCC patients. Lower tobacco use among young cancer patients was present to site-matched and stage-matched older patients (Cusamo and Persky, 1988; Schantz et al., 1988), whereas Lipkin et al., (1985) found a high exposure to tobacco and alcohol in their young head and neck cancer patients.

The p53 protein was first discovered in association with the SV40 large T-antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). The p53 suppressor gene, located on the short arm of chromosome 17, is 20 kilobases long and encodes

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for a 53-kd phosphoprotein consisting of 393 amino acids (Lamb and Crawford, 1986; Malkin, 1993). The gene consists of 11 exons, interrupted by 10 introns, of which the first comprises approximately half the gene in the human genome (Levine and Momand, 1990; Soussi et al., 1990). It was initially thought to be an oncogene, but later evidence demonstrated that p53 is a tumour-suppressor gene. About 60% of cancers in humans have mutations in the p53 gene, which takes the form of a missense mutation plus a selection for a reduction to homozygosity and a complete loss of the wild-type alleles (Levine et al., 1991).

Epidermal cells with mutations in one copy of the p53 gene are slightly resistant to death by apoptosis (Ziegler et al., 1994). In addition to an inability to suppress cell division, many mutants express a gain of function that actively promotes the tumorgenic potential of cells lacking endogenous p53 protein. Missense mutations can generate mutant p53 protein, which is more stable than wild-type p53 protein and can sequester normal protein into inactive oligomeric complexes rendering the normal p53 inactive (Vogelstein and Kinzler, 1992; Zambetti and Levine, 1993). Mutant p53 protein can act as an oncogene and can transform rodent cells in cooperation with the *ras* gene (Eliyahu et al., 1984; Jenkins et al., 1985).

Mutations in the p53 gene, or increased expression of the p53 protein have also been reported in head and neck carcinomas (Hollstein et al., 1990; Sakai et al., 1992; Somers et al., 1992; Boyle et al., 1993; Caamano et al., 1993; Shin et al., 1994). The objective of our study was to determine the p53 mutation profile in 55 OSCCs from a black African population sample from the North Western Transvaal.

MATERIALS AND METHODS

Patients

Formalin-fixed, paraffin-embedded blocks of OSCCs were retrieved from the files of the Department of Oral Pathology, Medical University of Southern Africa (MEDUNSA). Fifty-five blocks containing OSCC were selected. The mean age of the group was 50.8 ± 15.9 years; 45 were male and 10 were female.

Preparation of Cell Lysates

Cell lysates were prepared according to the method described by van Rensburg et al. (1996). All specimens were subjected to amplification using human β-globin primers (Saiki et al., 1986) to evaluate their suitability for DNA amplification.

Nested PCR Amplification of the p53 Gene (exons 5–9)

The genomic region containing p53 coding exon 5 to exon 9, together with flanking intron sequences, were amplified in three separate fragments, adapted from manufacturers' instructions (p53 genomic DNA screening module Catalog # 1610, Ambion, Austin, TX): fragment # 1, exons 5–6; fragment # 2, exon 7, and fragment # 3, exons 8–9. A list of the nucleotide sequences of each primer and the sizes of the amplified PCR products are shown in Table 1. Briefly, thermal cycling was carried out using a Perkin-Elmer (Norwalk, CT) GeneAmp® PCR system 9600 cycler. Forty amplification cycles were used: denaturing of DNA (94°C; 1 min); annealing of primers (55°C; 1 min), and extension of the annealed primers (72°C; 1.5 min). PCR was performed using the following reagents: 5 µl of cell lysate; 200 µM of each nucleotide; 250 nM of each primer (Ambion); 1 U of *Taq* DNA polymerase (Promega, Madison, WI); 5 µl of Promega 10x buffer and 1.5 mM $MgCl_2$ made up in a final volume of 50 µl.

Template DNA for the nested amplification round of each exon consisted of 3–7 µl of reaction product from the first round. Each exon 5–9 was amplified separately using primers previously described (Eeles et al., 1993). Briefly, thermal cycling was carried out using a Perkin-Elmer GeneAmp® PCR system 9600 cycler. Forty amplification cycles were used: denaturing of DNA (94°C; 1 min); annealing of primers (55°C; 1 min), and extension of the annealed primers (72°C, 1.5 min). PCR was performed using the following reagents: either 5 µl of first round PCR

TABLE 1. Sequences of p53 Genomic Primers With Expected Sizes of the PCR Products

Primer	p53 sequence	Size of PRC product
#1-S	CCTGAGGTGTAGACGCCAACTCTCT[a]	659 bp
#1-AS	ACTTTGCACATCTCATGGGGTTAT[a]	
#2-S	GGCCTCCCCTGCTTGCCA[a]	405 bp
#2-AS	CTCCAGCTCCAGGAGGTG[a]	
#3-S	AAGGGTGGTTGGGAGTAGA[a]	464 bp
#3-AS	ACGGCATTTTGAGTGTTAGAC[a]	
exon 5a	ATCTGTTCACTTGTGCCCTG[b]	308 bp
exon 5b	ATCAGTGAGGAATCAGAGGG[b]	
exon 6a	GCCTCTGATTCCTCACTGAT[b]	202 bp
exon 6b	GGAGGGCCACTGACAACCA[b]	
exon 7a	CTTGCCACAGGTCTCCCCAA[b]	236 bp
exon 7b	AGGGGTCAGCGGCAAGCAGA[b]	
exon 8a	TTCCTTACTGCCTCTTGCTT[b]	238 bp
exon 8b	TGAATCTGAGGCATAACTGC[b]	
exon 9a	GCAGTTATGCCTCAGATTCA[b]	161 bp
exon 9b	ACTTTCCACTTGATAAGAGG[b]	

[a]Ambion, Austin, TX
[b]Eeles et al., 1993.

products (fragment #1 to amplify exons 5 and 6), or 3 µl of first round PCR products (fragment #2, to amplify exon 7), or 7 µl of first round PCR products (fragment # 3, to amplify exons 8 and 9); 200 µM of each nucleotide; 0.4 µM of each primer; 1 U of *Taq* DNA polymerase (Promega); 10 µl of Promega 10x buffer and 1.5 mM $MgCl_2$ made up in a final volume of 100 µl. Two units of *Taq* polymerase were used in reactions to amplify exons 8 and 9. Reagent controls contained sterile water instead of sample DNA.

Aliquots of 8 µl of the amplification reaction products were analysed by electrophoresis through 3% LE agarose gels (FMC Products, Rockland ME), stained with ethidium bromide and evaluated by UV transillumination as described above.

Heteroduplex Single-stranded Conformational Polymorphism (SSCP) Analysis

DNA fragments of exons 5–9 were amplified as described above. Heteroduplex-SSCP analysis of these fragments were done to observe mutations, essentially as described with a few modifications (Kotze et al., 1995). Briefly, 10 µl of each PCR product was mixed with an equal volume of gel loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) containing 0.5 X TBE or 1.5 X TBE for either glycerol or urea containing gels, denatured at 95°C for 2–5 min and immediately placed on ice. The samples were loaded on a 24 cm vertical 1.5 mm thick (Hoefer Scientific Instruments, San Francisco, CA) 10% polyacrylamide gels with 1% cross-linking and run overnight at both room temperature and 12°C at 180 to 250 V (Bio-Rad Laboratories, Richmond, CA) depending on the size of the DNA fragments. Gels were supplemented with 7.5% and 5% urea and glycerol, respectively, stained for 10 min in a solution of 0.6 X TBE containing 1 µg/ml of ethidium bromide, and destained for 10 min in water before being evaluated by UV transillumination as described before.

Direct Sequencing

Sequencing of PCR products was performed for exons 5–9 to confirm the presence of mutations that had been detected by heteroduplex-SSCP. The primers used for sequencing was the same as for the nested PCR (Eeles et al., 1993), and the Sequenase PCR product sequencing kit (USB Catalog US70170, supplied by Amersham, Buckinghamshire, UK, life science) was used. The enzymes shrimp alkaline phosphatase and exonuclease 1 were used to remove unwanted materials from the PCR products and inactivated each at 80°C for 15 min after a 15-min incubation step at 37°C. The template was denatured and annealed to the sequencing primer using a heating step (99°C for 3 min) and a snap-cooling procedure on ice at 0°C. This is followed by using normal sequencing protocols including a labelling and termination step. Chain-termination sequencing is achieved with this kit using Sequenase™ Version 2.0 DNA polymerase. Manganese is added to improve the band uniformity and the ability to read sequences close to the priming site. Standard denaturing gel electrophoresis was done (Sambrook et al., 1989) using a glycerol tolerant gel buffer containing taurine (Amersham).

Sequence Analysis

Sequences were visualised by autoradiography, read from both directions, and analysis was done with the Genepro V5.0 software program (Riverside Scientific Enterprises, Bainbridge Island, WA).

RESULTS

Using a combination of SSCP and direct genomic sequencing, we examined exons 5–9 of the p53 gene in 55 OSCCs. DNA fragments showing an electrophoretic mobility shift were identified as positive for p53 gene mutations. They were subsequently selected for DNA sequencing to confirm the SSCP results and to characterise the types and locations of mutations. We scanned the GenBank (Bilofsky and Burks, 1988) data bank for sequence similarities, using GenBank accession number X54156. Mutations of the gene were identified in 13 of 55 (23.6%) tumours (Table 2). One of the 13 mutations was a two nucleotide deletion, the second was a large, novel deletion of 63 base pairs (Fig. 1), and the other 11 (85%) were single base pair substitutions (9 transitions and 2 transversions). One of the single base pair substitutions was

TABLE 2. p53 mutation summary in 13 OSCCs

Patient no.	Exon	Nucleotide change	Codon change
B14	5	C454→T	Pro152→Ser
A70	5	C459→T	Silent (153)
C2	5	G507→A	Met169→Ile
C33	5	G527→T	Cys176→Phe
C34	6	T584→C	Ile195→Thr
A67	6	del625-626	Frameshift
A48	6	A659→G	Tyr220→Cys
B43	7	C689→T	Thr230→Ile
C28	8	C817→T	Arg273→Cys
B34	8	C832→T	Pro278→Ser
C59	8	A874→T	Lys292→Stop
B5	8	C916→T	Arg306→Stop
B4	8	del814-876	del272-292

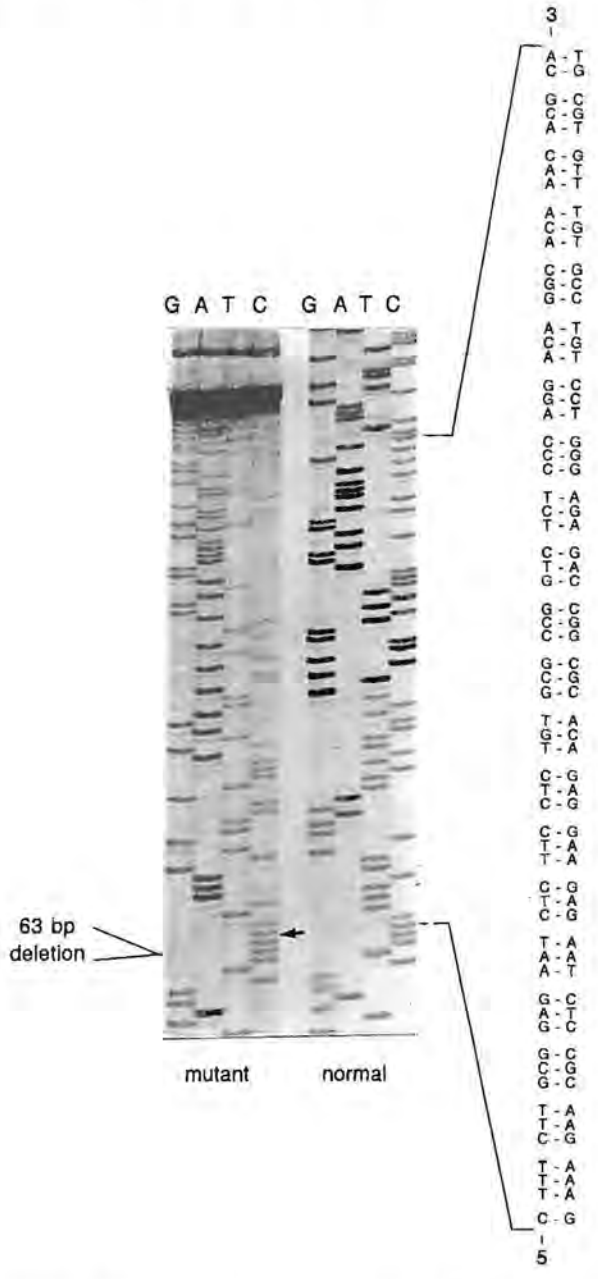

FIGURE 1. **Sequence analysis of a part of the p53 exon 8 gene from patient B4, showing the 63bp deletion.**

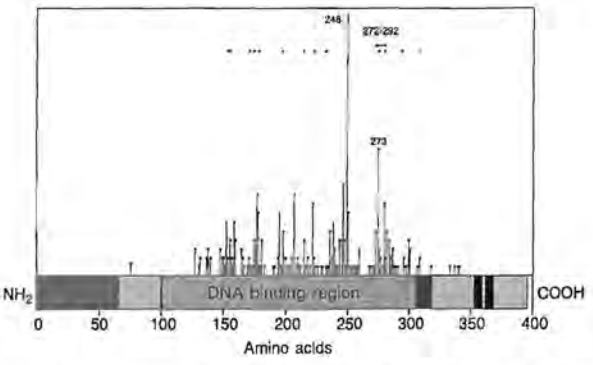

FIGURE 2. **Frequency of p53 mutations found in head and neck tumours (bottom) compared to mutations found in our study (top). The p53 molecule is schematically represented showing the DNA binding region and relative positions of the amino acid codons. Hot spot areas are evident at amino acid sites 248 and 273.**

also a novel mutation when compared to the p53 Database (De Vries et al., 1996; Hollstein et al., 1996). It was an A to T transversion at nucleotide 874. Of the previously described single base pair mutations, one was a silent mutation, two resulted in premature stop codon formation, and the other 8 in amino acid changes. Six of the 9 transitions were C to T changes, one was a G to A change, and one each was T to C, and A to G, respectively. The transversions were G to T, and A to T in one tumour each, respectively. All the mutations in this study occurred in different codons, except in the region of the large deletion, where three other mutations occurred as well. In total, four mutations occurred in exon 5, three in exon 6, one in exon 7, five in exon 8, and none in exon 9. In Figure 2, the mutations found in our study are compared to the frequency of p53 mutations in other head and neck tumours (Hollstein et al., 1996).

DISCUSSION

Approximately 45% of invasive head and neck squamous cell carcinomas have a mutated p53 gene (Koch et al., 1994). Most of the mutations in the p53 gene in general are missense mutations, causing a change in an amino acid, and a probable increase in stability of the protein (Harris, 1993). In a recent review, where 115 head and neck cancer samples were studied, 47% of the mutations were found to be transitions, 37% transversions, and 16% frameshifts, the majority of these being small insertions or deletions of 1–20 base pairs (Brachman, 1994). In our study, 69% were transitions, 15% were transversions, and 15% frameshifts. Two were novel mutations.

Two mutations, at codons 292 and 306, were nonsense mutations, and one mutation at codon 153 was a silent mutation. Of the missense mutations, seven were transitions, and one was a transversion. The most common transition was C to T in six of the nine (67%) cases. There seems to be distinct differences in the mutations reported from different geographical regions, as 50% of p53 mutations in Japan had G to T transitions (Sakai and Tsuchida, 1992), whereas 63% of head and neck cancers in the United States showed G to T transversions (Somers et al., 1992). Two of our tumours (14%) had deletions; one of them was also a frame shift mutation. This is in keeping with other studies, where frame shift mutations are

less commonly seen (Cotran et al., 1989). The novel deletion occurred at codons 272 to 292, which constitutes a 21 amino acid (63 base pair) deletion in the DNA binding region of the p53 protein, which is situated between amino acid 91 and 309 (Bargonetti et al., 1993). The large deletion in this area may cause the mutant p53 protein to loose its DNA binding capacity, with the cells expressing the mutation showing a selective growth advantage.

The p53 mutations in head and neck tumours seem to have a hot spot region at codons 238–248 as summarized by Hollstein et al. (1996). This region was not prominent in our study, but rather the region between 272 to 292 where the large deletion occurred and where three other point mutations occurred, i.e., condons 273, 278, and 292. There also appear to be a geographic distribution in the exons affected. In our study, as well as in a Japanese study (Sakai and Tsuchida, 1992), the majority of mutations were found in exons 5 and 8; exon 4 was the most frequent location for mutations in the United Kingdom (Field et al., 1993) and exon 7 in the United States (Somers et al., 1992).

To conclude, the number of OSCCs in our study with p53 mutations confirmed the importance of this gene in oral carcinogenesis. The unique distribution of the mutatons in the population sample studied suggested a different response to etiological agents. This may be linked to genetic factors that alter the molecular steps leading to cancer.

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Correlation Between p53 Gene Mutation, p53 Protein Labeling and PCNA Expression in Oral Squamous Cell Carcinomas

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Abstract. *Background: The prevalence of oral squamous cell carcinoma (OSCC) among the Black community in South Africa is unacceptably high. The association between p53 protein, and PCNA overexpression and the presence of p53 gene mutations was evaluated. Materials and Methods: One hundred and ten formalin-fixed, paraffin-embedded blocks of OSCC were selected for immunohistochemical studies for p53 protein and PCNA expression using the DO-7 and PC10 monoclonal antibodies, respectively. DNA was extracted from fifty-five blocks and exons 5 to 9 of the p53 gene were amplified with nested primers, thereafter sequencing was performed to confirm the presence of mutations detected by single stranded conformational polymorphism. Results: Fifty-six cases (51%) showed p53 expression, while fourteen mutations (25%) were detected. A significant difference was found between the PCNA index in p53 positive and p53 negative tumors while the mean PCNA index for the tumors with p53 mutations was not significantly different from the tumors without mutations. Conclusions: No association between p53 protein overexpression and p53 gene mutations could be demonstrated.*

Squamous cell carcinoma is the most common malignancy of the oral cavity. Despite the relatively easy accessibility of the oral mucosa to detect premalignant lesions, recent studies suggest a global increase in the incidence of oral squamous cell carcinoma. This increase is especially evident in developing countries (1). The prevalence of oral squamous cell carcinoma (OSCC) in the Black community in South Africa is high. In the period 1990-1991, an average of 1363 new intraoral cancers per year were reported in South Africa, resulting in an average incidence of 3.4% of all diagnosed cancers, excluding basal cell carcinomas and squamous cell carcinomas of the skin (2). In combination with cancers of the pharynx, these malignancies were the 4th most common in South African males and the 2nd most common in South African Black males.

In an attempt to contribute towards the understanding of OSCC in the Black community, p53 gene mutations and their correlation with p53 protein and PCNA expression were evaluated.

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Key Words: p53, PCNA, oral cancer.

Materials and Methods

Patients. Formalin fixed paraffin embedded blocks of Black patients diagnosed with OSCC were retrieved from the files of the Department of Oral Pathology, Medical University of Southern Africa, MEDUNSA. These consisted of material from resection specimens as well as biopsies. The sections were screened to confirm the diagnosis.

Immunohistochemistry. One hundred and ten cases were selected for immunohistochemical analyses. The mean age of the patients was 50.1 ± 16.6 years. Seventy-three patients were male and 27 female. A 4 µm section of each block was cut and picked up using amino-alkyl saline-coated glass slides. Sections were deparaffinised, hydrated with graded alcohol and brought to distilled water. The sections were placed in a jar filled with 10 mM citrate buffer, pH 6.0. For p53 antibody labeling, the samples were treated in a microwave oven for 2 × 5 minutes. The PCNA slides were immediately incubated at 37°C with 3% H_2O_2 and washed with PBS for 5 minutes as were the p53 slides following microwave antigen retrieval. The sections were then incubated at 37°C for 15 minutes with a p53 protein-specific DO-7 monoclonal antibody (Dako, Southern Cross, Cape Town, South Africa) and a PCNA protein-specific PC10 antibody (Dako, Southern Cross, Cape Town, South Africa). A working dilution of 1:100 with PBS was used for both antibodies. The sections were incubated in the Link antibody from the Steptavidin ABC Complex kit (Dako, Southern Cross, Cape Town, South Africa) for 10 minutes and washed in PBS for 5 minutes. The sections were incubated in Streptavidin for 10 minutes, washed again in PBS for 5 minutes and were then incubated for 20 minutes in a substrate solution at 37°C. The slides were counterstained in Mayers haemotoxylin for 1 minute, and blued for 5 minutes in running water.

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Negative controls consisted of the replacement of the primary antibody with normal mouse serum while positive controls consisted of known positive tumors.

Only nuclear p53 and PCNA staining was regarded as positive. Tumors were defined as p53 positive when more than 30% of the nuclei showed DO-7 labeling. When in doubt, 1000 tumor cells were evaluated at 400× magnification with the aid of an eyepiece graticule.

PCNA activity was quantified by assessing the staining in 1000 cells in areas within each section showing maximum reactivity for PCNA. All nuclei found to exhibit fine to coarse granular staining at ×400 magnification were rated as positive for PCNA. The number of positive cells was expressed as a percentage of the total number counted to give a PCNA index. An eyepiece graticule was used to ensure that all cells were evaluated once only. The Student's t-Test for uncorrelated data was used to compare the means of the PCNA index between the p53 positive and negative tumors

p53 gene analysis. Only fifty-five blocks of the cases used for immunohistochemistry were selected for analysis of exons 5 to 9 of the p53 gene. This was done to reduce costs. Cases were included for mutation analysis if all the exons could be amplified. Mutations were identified by heteroduplex single-stranded conformational polymorphism and confirmed by direct sequencing. The detailed methodology followed has been reported previously (3). The correlation between the presence of DNA mutations in any of the exons and the presence of p53 overexpression was determined using the χ^2-Test with Yates correction, while the significance of the mean PCNA counts between the tumors with mutations and those without was determined using the studentfs t-Test.

Results

The p53 protein expression was observed as a granular nuclear stain. Some tumor cells also expressed cytoplasmic p53 protein labeling. Cytoplasmic staining in this study was present in the minority of cases, and then in all instances associated with nuclear staining. The pattern of p53-positive nuclei in tumor sections varied from diffuse positive, to focal positive areas, to individual cell positivity. PCNA expression was also confined to the nuclei. The PCNA labeling index of p53-positive tumors was significantly higher (Student's t-Test, $p=0.0008$) than in the p53-negative group (Table I).

Mutations of the p53 gene were identified in 14 of 55 (25,5%) tumors (Table II). Two of the mutations resulted in premature stop codon formation. One of these showed no protein overexpression while the other was positive for p53 protein. Details of the mutation profile recorded in this study have been reported elsewhere (3). Only nine of 31 tumors exhibiting p53 protein overexpression showed mutations of the p53 gene. Five of 24 p53-negative tumors had mutations of the corresponding p53 gene. There was no statistically significant correlation between p53 protein expression and p53 mutation ($\chi^2=0.45$, $p=0.50$).

Discussion

Only nuclear staining was considered as a positive immunoreaction for p53 in this study. The cytoplasmic staining found has several explanations. Mutated p53 protein may have accumulated in the cytoplasm because transport to the nucleus was defective due to the altered amino acid sequences. Mutated p53 may also have formed stabilized complexes with wild-type p53 which kept it in the cytoplasm (4). Cross reactivity between the p53 antibodies and cytoplasmic cytokeratins has also been described (5)

Table I. *p53 protein expression and PCNA labeling indices for p53-positive and -negative OSCC patients.*

	n	PCNA index
p53+	56 (51%)	65.8 ± 14
p53-	54 (49%)	55.8 ± 16
Total		61.1 ± 16

The p53-negative tumors presumably contained wild-type p53 at levels too low to detect immunohistochemically. The negativity might also reflect the loss of both p53 alleles or the presence of non sense mutations leading to termination of protein synthesis.

PCNA immunoreactivity does not always correlate with other indices of proliferation (6). This is due to the long half-life of PCNA resulting in persistent staining in cells that have recently left the cell cycle (7). PCNA may also be expressed in association with DNA repair rather than proliferation (8). Quantitative studies of cell proliferation in solid tumors have disadvantages in that only a static moment in the cycle of cellular kinetics is evaluated (9). Techniques to determine cellular kinetics are available, such as the assessment of S-phase fractions using DNA flow cytometry (10). This was modified by estimating bromodeoxyuridine incorporation in DNA synthesis, assessed simultaneously with the S-phase fraction through bivarate flow cytometry (11).

The PCNA count was statistically higher in the tumors expressing p53 protein compared to the p53-negative tumors. Immunohistochemical detection of p53 protein may be due to a mutated p53 gene with intervention in its growth-inhibitory capacity resulting in increased tumor cell proliferation. The altered form of the mutated protein can interact directly or indirectly with growth related genes activating their expression (12). It is postulated that the conformational change associated with the mutated p53 protein can affect its binding to the PCNA promoter, releasing its repressor effect and inducing PCNA transcription (12). It may also be due to accumulation of wild-type p53 induced by DNA damage or specific viral protein binding. These observations are compatible with the functions of p53 as an inducer of DNA repair and of PCNA as a DNA repair protein. Tumors without detectable p53, with a high PCNA index, can also contain cells with a non sense mutation in the p53 gene. No definite conclusions can therefore be drawn on p53 and tumor cell proliferation based the on immunohistochemical data only.

Numerous studies on the relationship between p53 and

Table II. *p53 mutations (3) with p53 protein overexpression profile of the OSCC.*

Exon	Base change	Nucleotide position	Type of mutation	Codon position	Amino acid change	p53 protein expression
5	C>T	459	transition	153	silent	positive
5	C>T	454	transition	152	Pro>Ser	negative
5	G>A	507	transition	169	Met>Ile	negative
5	G>A	514	transition	172	Val>Ile	negative
5	G>T	527	transversion	176	Cys>Phe	positive
6	A>G	659	transition	220	Tyr>Cys	positive
6	del 2	625-6	frameshift	212	Phe del	negative
6	T>C	584	transition	195	Ile>Phe	positive
7	C>T	689	transition	230	Thr>Ile	positive
8	C>T	916	transition	306	Arg>stop	positive
8	del 63	814-876	frameshift	272-292	del	positive
8	C>T	832	transition	278	Pro>Ser	positive
8	C>T	817	trarfsition	273	Arg>Cys	positive
8	A>T	874	transversion	292	Lys>stop	negative

PCNA expression have been conducted. Most of the authors found a positive correlation between p53 and PCNA expression (13, 14, 15). A major disadvantage of immunohistochemical techniques is that comparisons between different proteins cannot be drawn on a cell to cell basis. Double staining techniques are a possibility, but not when a different technique, *e.g.* antigen retrieval, is necessary for one of the antibodies used. Direct comparisons between cells expressing p53 and PCNA were therefore not possible.

Only 9 (64%) of the 14 tumors with p53 mutations showed p53 protein overexpression. Two of the mutations resulted in premature stop codon formation, one of which showed no protein overexpression with immunohistochemistry. The other tumor with a nonsense mutation was positive for p53 protein. The overexpression seen in this patient might have been because only one allele was affected by the mutation and that the wild-type protein was stabilized by protein to protein or protein to gene interaction (16). The other tumors with overexpressed p53 without detectable mutations were probably the result of stabilized protein. It may, however, also be possible that mutations were present in other exons not examined or in the introns.

Tumors with nonsense mutations can be expected to be p53-negative with immunohistochemistry, if p53 protein complex formation is not considered. Only two of the detected mutations in this study were nonsense in nature. The tumors with missense mutations without p53 overexpression could be samples where the mutation resulted in an ephemeral protein or changes in the epitope recognized by the antibody.

The PCNA gene is regulated in a complex manner and its overexpression may be a result of increased PCNA mRNA stability induced by growth factors, regardless of the p53 status (17). This study suggested a possible association between p53 and PCNA based on the immunohistochemical findings. The difference between the PCNA index in tumors with p53 mutations and those without was statistically not significant. This would suggest that overexpressed p53 may influence the PCNA status and not the mutation of the p53 gene alone. The fact that p53 overexpression is not necessarily the result of a mutated gene has been discussed previously.

In conclusion, no association between p53 protein overexpression and p53 gene mutation could be demonstrated in this study. Overexpressed p53 can therefore not be described as a mutated protein, and no conclusions can be made on the presence of p53 gene mutations based on immunohistochemical evaluation of the p53 protein alone. Although an association between p53 protein overexpression and the PCNA expression was demonstrated, it could not be demonstrated with p53 gene analysis. As a result of the

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limitations of immunohistochemistry techniques, the unknown factors involved made the results regarding an association between the two protein expressions inconclusive.

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Rapid Acetone Tissue Processing: An Economical Alternative

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Abstract
This report describes the use of acetone for the rapid processing of small biopsy specimens. Two tissue blocks of approximately equal size and thickness were prepared from a palatine tonsil, submandibular salivary gland and squamous cell carcinoma (SCC) of the tongue. Three blocks, one from each tissue, were routinely processed while the other three blocks were acetone processed and impregnated with paraffin wax. Four (m sections were subjected to different histochemical and immunohistochemical stains. All the stains were performed according to prescribed procedures and evaluated light microscopically by two oral pathologists who were blinded to the labelling of the sections from the rapid processed tissue as well as the staining intensity of all the routine and immunohistochemical stains applied compared equal or superior to the sections of the routine processed tissue.

Introduction

Special circumstances necessitate the availability of a rapid processing technique in a histopathology laboratory. Large throughput diagnostic laboratories are concerned with the speed of fixation and processing as well as the costs. Various rapid processing techniques, such as frozen sections and heat and vacuum processing are available that yield histotechnical results that are comparable to routinely processed tissue. An increasingly important aspect of fixation and processing is the preservation of tissue antigens.

In the past few years there have been several advances in rapid fixation and processing methods by microwave (MW) irradiation[1-4]. MW irradiation however, yields artifacts such as lysis of red blood cells due to irradiation of fresh, unfixed tissue[1]. Furthermore, overexposure to MW's results in smudging and pyknosis of nuclei and intense cytoplasmic eosinophilia, whereas underexposed tissues fail to take up stains adequately[1]. Most of the published results of MW procedures and current usage relate to adapted domestic ovens, probably due to the high cost of laboratory dedicated MW's.

Acetone is a known dehydrate in processing techniques, but prolonged exposure causes hardening of tissue[5]. It has, however been shown that fixation in cold-acetone may be preferable to formalin fixation for immunohistochemical detection of certain antigens[6].

This study evaluated rapid acetone processing pertaining to histotechnical quality of sections, preservation of cellular and nuclear morphology, staining intensity and reliability of immunohistochemical analyses.

Material and Methods

Two tissue blocks of approximately equal size and thickness were prepared from each of a palatine tonsil, submandibular salivary gland and squamous cell carcinoma (SCC) of the tongue. Three blocks, one from each tissue, were routinely processed and the other three blocks were acetone processed by dehydration in two changes of fresh acetone for 30 minutes each. As over-dehydration with acetone leads to tissue brittleness, tissue processing was monitored by gentle squeezing with a pair of forceps in order to detect tissue firmness that indicates adequate dehydration. The blocks were then directly impregnated with paraffin wax for 30 minutes and 45 minutes respectively. Impregnation in the second paraffin wax bath was aided with a vacuum unit of the Shandon Citadel processor (Labotec, Midrand, SA). No clearing agent was used with the rapid acetone processing technique.

The routine overnight processing consisted of dehydration with 50%, 70%, 90%, 96% and absolute alcohol. Clearing was done in two changes of xylene followed by impregnation with two changes of paraffin wax. Four (m sections from the six blocks were secretly labelled in order to differentiate the two processing techniques, and subjected to the histological stains listed (Table 1). Sections for immunohistochemical staining were mounted on 3-aminopropyltriethoxysilane coated slides. A standard Haematoxylin & Eosin (H&E) stain, a variety of special staining and immunohistochemical techniques were performed on the sections that were incubated at 54°C. All the investigations were done on both the routine and rapid processed tissue for comparison.

The special stains included a Periodic Acid Schiff (PAS), PAS-diastase and Southgate's mucicarmine on sections from the submandibular salivary gland, while the Masson trichrome technique of Bancroft & Stevens' was performed on the tongue SCC.

TABLE 1
Histological stains used with staining intensity and tissue brittleness evaluation

Stain	Staining intensity		Tissue brittleness	
	Routine processing	Acetone processing	Routine processing	Acetone processing
Palatine tonsil				
H & E	strong	strong	absent	mild
Anti-LCA	strong	strong	absent	absent
Anti-B-cell (CD20)	strong	strong	absent	absent
Anti-Ki-67	weak	strong	absent	absent
Salivary gland				
H & E	strong	strong	absent	absent
PAS	strong	strong	absent	absent
PAS-d	strong	strong	absent	absent
Southgate's mucicarmine	strong	strong	absent	absent
Anti-SMA antigen	moderate	strong	absent	absent
Anti-S100 antigen	moderate	strong	absent	absent
Tongue with SCC				
H & E	moderate	strong	absent	absent
Masson-trichrome	strong	strong	absent	mild
Anti-Cytokeratin antigen	strong	strong	absent	mild

The immunohistochemical stains evaluated were an anti-cytokeratin antigen (HMW, 34BE12) on the tongue SCC, anti-leukocyte common antigen (LCA) (2B11 & PD7/26), the proliferation marker Ki-67 and a B-cell marker (CD20) on the palatine tonsil, and anti-alpha smooth muscle actin (SMA)(1A4) and anti-S100 alpha specific protein on the submandibular salivary gland sections. Dako (DAKO CORPORATION, Carpenteria CA) prediluted antibodies were used for the investigations.

In all the immunohistochemistry procedures, except with SMA antigens, unmasking of the antigens were done in a domestic microwave oven using a pressure cooker. All the sections, including the SMA were treated with 5% hydrogen peroxide at 37°C for 5 min. to quench endogenous peroxidase activity. Slides were incubated with the primary antibodies for 20 min at 37°C. DAKO LSAB 2 Peroxidase kit (DAKO CORPORATION, Carpenteria CA) was used to detect the antibodies. S100 antibodies were detected using the Immunotech Universal immunostaining kit (Coulter Co. BP177-13276 Marseille Cedex 9-France). AEC substrate was the chromogen of choice. Sections were counterstained with Haematoxylin and mounted with Dako Faramount aqueous mounting media.

The tissue sections were evaluated light microscopically for intensity of staining and tissue brittleness. Staining intensity was subjectively recorded as weak, moderate and strong. Tissue brittleness was subjectively recorded as absent, mild, moderate or severe. Two oral pathologists who were blinded to the labelling of the sections performed all evaluations independently and on consensus basis.

Results

Routine processing was completed after 12 hours compared to 2½ hours of acetone processing. Minimal shrinkage of the tissue was observed with both the routine and acetone processed tissue blocks. Four (m sections of good histochemical quality were easily obtained from the acetone processed tissue blocks and the cellular and nuclear morphology were well preserved (Figs 1, 2). The tissue antigens were also well preserved with rapid acetone processing and heat induced epitope retrieval (HIER) was successfully carried out on these tissue sections. No false positive or false negative staining was observed. Acetone processing yielded improved (stronger) anti-Ki-67 staining of the tonsil, anti-SMA and anti-S100 staining of the salivary gland and H&E of the tongue with SCC. The other stains yielded staining

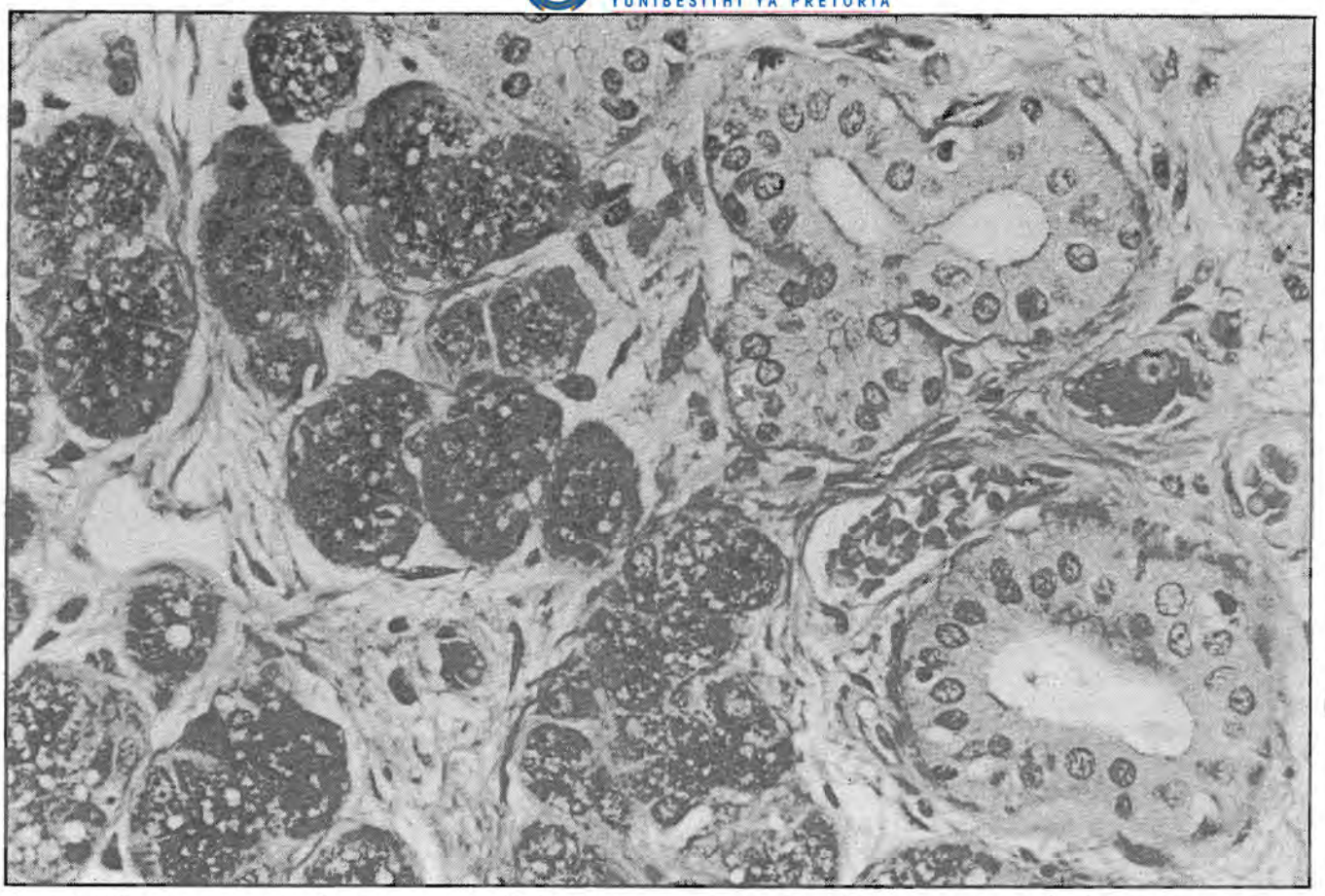

Figure 1: H&E staining of a routinely processed submandibular gland section showing striated ducts, serious acini and small vascular vessels (original magnification × 125).

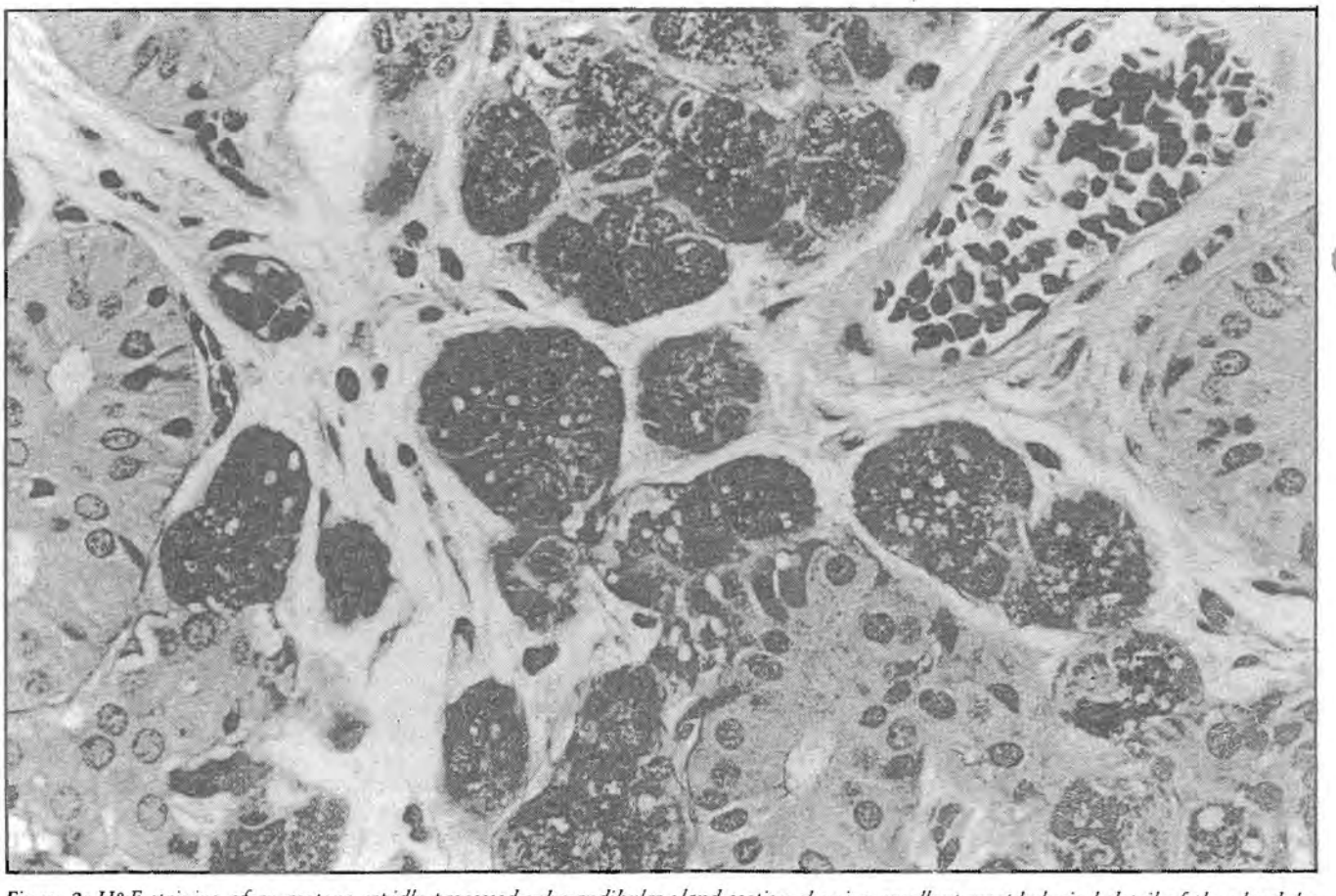

Figure 2: H&E staining of an acetone rapidly processed submandibular gland section showing excellent morphological detail of the glandular structures (original magnification × 125).

intensity similar to the routine processed tissue. Mild tissue brittleness was observed in the H&E section of the palatine tonsil, the Masson trichrome and cytokeratin stained sections of the SCC (Table 1).

Discussion

The use of MW ovens for rapid fixation and tissue processing has increased over the past few years. The standard domestic MW oven is not ideal for this purpose and certain adjustments need to be incorporated in the oven to render it more suitable. Leong suggests that a "stirrer" in the roof of a domestic MW oven should be present to prevent uneven heating and that a large output MW oven (1000 W) is preferable in order to provide a more even field of irradiation[4] This however has financial implications. When a standard domestic MW oven is the only available option in a laboratory, rapid tissue processing with acetone is an economical alternative to adjusting the oven.

No clearing agent was used with the rapid processing technique because the paraffin wax could impregnate the tissue blocks completely without having to remove the acetone with a clearing agent. This saved considerable time.

Tissue processing with acetone has to be monitored manually as processing time may vary according to tissue thickness and tissue type. This requires some practice and is somewhat labour intensive, especially if large numbers of blocks are processed simultaneously. As one becomes more familiar with the technique and tissue types this problem will be eliminated. When single cases are rapid processed the abovementioned yields no problem as the blocks are easily monitored.

The mild tissue brittleness that was observed in three of the twelve rapid processed sections could be due to mild over-dehydration as these sections were probably prepared from the surfaces of the tissue blocks which had been exposed to acetone for a longer period, compared to sections from deeper in the blocks. Minor reductions in the dehydration time of acetone rapid processing should therefore eliminate tissue brittleness. Further investigation is required to prove these statements.

Biopsy specimens are ideal to rapid process because of their size. Routine-size tissue blocks of 10 × 10 × 2mm can also be successfully rapid processed with acetone, as long as the tissue block thickness does not exceed 2mm. Incomplete processing of tissue blocks resulted in the dispersion of sections on the water bath. This can be rectified in the same way as incomplete routinely processed blocks. Acetone processed sections are more likely to wash off the slides when antigen retrieval for immunohistochemistry is performed in a MW. When ample tissue is available this problem can be overcome by rapid processing of half of the tissue and routine processing of the other half. This will ensure that one can obtain sections from the routine processed block that will not wash off the slides during the HIER step. In this study, however, no problem was encountered with washing off of sections.

In our laboratory, acetone processing has been successfully employed as the only rapid processing method for urgent biopsy specimens since 1998. This technique yields thin sections with good cellular and nuclear morphology. There is no difference in the shrinkage of tissue blocks compared to the routine processed tissue blocks. The artifacts encountered with MW irradiation as previously discussed were absent. No signs of smudging or pyknosis of cell nuclei were observed. The staining intensity of the sections was similar or superior to that of the routine processed sections, but as it is a labour intestive method, it is not suitable for a routine procedure. Rapid acetone processing is a simple, rapid and economical technique that can be used with confidence in histopathology laboratories.

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DISCREPANCIES IN HUMAN SERUM ALBUMIN CONCENTRATIONS AS DETERMINED BY COLORIMETRIC, ELECTROPHORETIC AND NEPHELOMETRIC METHODS

(Continued from page 319)

Materials and Methods

1. Colorimetric methods — Albumin concentrations in serum samples utilising BCG and BCP dye were estimated with a Beckman Synchron CX7 Clinical System. (Beckman Coulter Inc., Brea, California).

2. Electrophoretic methods — Cellulose Acetate electrophoresis was performed on a Helena Laboratories (HE) System. (Helena Laboratories, Beaumont, Texas). CZE was performed using a Paragon CZE 2000 System. (Beckman Coulter Inc.). The albumin concentrations in the serum samples were calculated as a percentage of the total protein concentration.

3. Nephelometric methods — Serum albumin concentrations were determined with a Beckman Array 360 System (ARRAY). (Beckman Coulter Inc.) and a Behring Nephelometer-Analyzer (BNA) (Behring Werke AG Diagnostica, Marburg).

Results

Serum albumin concentrations in ten patients, as determined by the different methods, are listed in Table 1. (A/G — Albumin: Globulin ratio for the two electrophoretic methods respectively).

Discussion

From this study it is clear that the BCG dye binding method is not specific for albumin but also binds globulins although with less intensity compared to albumin. The cellulose acetate electrophoretic method in which the proteins are stained (Helena Laboratories) also overestimates albumin. The BCP dye binding method is in line with the CZE electrophoretic and the nephelometric methods.

Conclusion

Although significant differences in human serum albumin concentrations between these methods may exist, the colorimetric method utilising bromcresol purple (BCP) dye is today the most suitable and practical method for routine applications. The capillary zone (CZE) electrophoretic method is superior to cellulose acetate electrophoresis which requires staining of proteins to quantitate albumin. In general, nephelometry is the most specific method for human serum albumin quantitation and is suitable for quantification of albumin in solutions with a low protein concentration. However, nephelometry is expensive to use and not ideal for routine applications. It is therefore not surprising that the BCG reagent has become obsolete as the BCP reagent has now become the method of choice for routine applications.

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Immunohistochemical evaluation of Fhit protein expression in oral squamous cell carcinomas

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van Heerden WFP, Swart TJP, van Heerden MB, van Rensburg EJ, Engelbrecht S, Dreyer L, Huebner K: Immunohistochemical evaluation of Fhit protein expression in oral squamous cell carcinomas. J Oral Pathol Med 1999; 28: 433–7.

The expression of Fhit (fragile histidine triad) protein in oral squamous cell carcinoma (OSCC) and adjacent oral epithelium was evaluated by immunohistochemistry on formalin-fixed paraffin-embedded blocks of 32 cases of OSCC. Rabbit polyclonal anti-GST-Fhit antiserum at 1:600 was used, after antigen enhancement in a microwave pressure cooker, in a saturated lead thiocyanate solution. This antiserum has been shown specifically to detect human Fhit by immunohistochemistry at dilutions up to 1:10,000. The Fhit protein expression was evaluated using both the intensity and extent of staining. Normal stratified squamous epithelium showed strong positivity, especially in the stratum spinosum and areas of keratinisation. Basal and parabasal cells were negative or expressed low levels of Fhit relative to the squamous epithelium. Mild and moderate epithelial dysplasia showed Fhit expression in the superficial layers, while Fhit expression was absent from severely dysplastic lesions. A reduction or loss of Fhit expression was found in 21 (66%) of the OSCC. The alterations in Fhit protein expression in OSCC, and not in normal tissues, are consistent with the proposal that Fhit inactivation plays a role in oral carcinogenesis.

Key words: chromosome 3; Fhit; immunohistochemistry; oral cancer; tumour suppressor genes

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The incidence of oral squamous cell carcinoma (OSCC) appears to be on the increase in developing countries (1). During the period 1990–1992, an average of 1336 new intraoral cancers per year was reported in South Africa, resulting in a frequency of 3.4% of all diagnosed cancers, excluding basal cell carcinomas and squamous cell carcinomas of the skin (2, 3). Oral cancer ranked fifth behind cancer of the prostate, oesophagus, lung and colon in males in South Africa. In combination with cancers of the pharynx, these malignancies were the fourth most common in South African males and the second most common in Black males (2, 3).

The *FHIT* (fragile histidine triad) gene was isolated from chromosome band 3p14.2 (4). The gene is encoded by 10 exons in a 1.1-kb transcript encompassing at least 1 Mb of genomic DNA (5). The protein has 147 amino acids and shows homology to diadenosine 5′,5‴-P¹,P⁴-tetraphosphate (Ap4A) hydrolase from the yeast *Schizosaccharomyces pombe* (6). The *FHIT* gene contains the FRA3B fragile site and is the target of homozygous deletions in many human cancer cell lines (4); *FRA3B* is also the most common fragile site in humans (7). The 3p region is one of several regions of frequent loss of heterozygosity (LOH) that have been identified in head and neck squamous cell carcinomas (HNSCC) (8). The others include 9p, 11q, 13q and 17p (9).

FHIT allele alterations were detected in 55% of HNSCC cell lines using a reverse transcription-polymerase chain reaction (RT-PCR), Southern blot analysis and interphase fluorescence *in situ* hybridisation (FISH) (10). Abnormal transcription of the *FHIT* gene in HNSCC was assessed by RT-PCR amplification by Mao *et al.* (11), who suggested that loss of *FHIT* function might be important in the development of HNSCC and proposed it as a potential tumour suppressor gene for HNSCC (11).

In a more comprehensive study of nine HNSCC cell lines and 32 primary HNSCCs, Kisielewski *et al.* (12) examined the *FHIT* gene for alterations at the DNA, RNA and protein levels. The HNSCC cell lines were examined for *FHIT* transcription by Northern blot analysis and *FHIT* transcript was not detected in the seven HNSCC cell lines, suggesting little or no *FHIT* transcript. RT-PCR amplification of *FHIT* RNA from these cell lines showed complete absence of a normal-sized *FHIT* transcript in two of the cell lines; the others showed a full-size RT-PCR product,

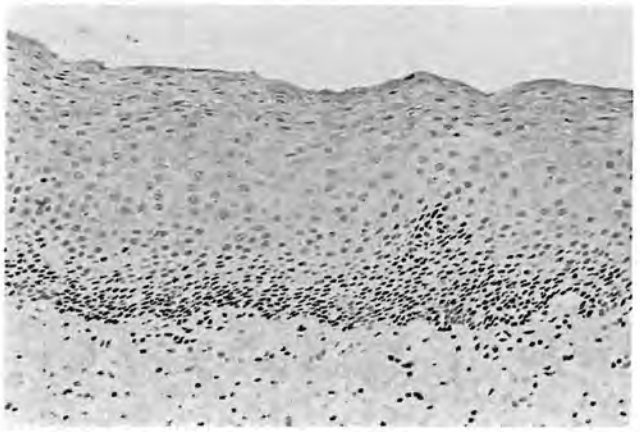

Fig. 1. Normal stratified squamous epithelium showing prominent Fhit staining in the stratum spinosum and stratum superficiale. Note the scarcity of staining in the basal and parabasal cells (×60).

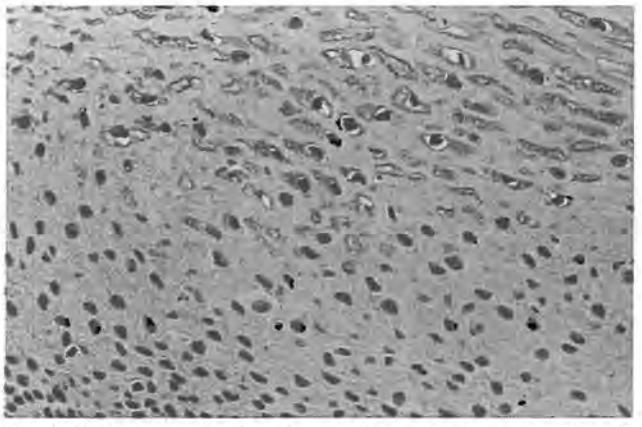

Fig. 2. Higher magnification than in Fig. 1 shows the cytoplasmic staining of the more superficial epithelial cells (×125).

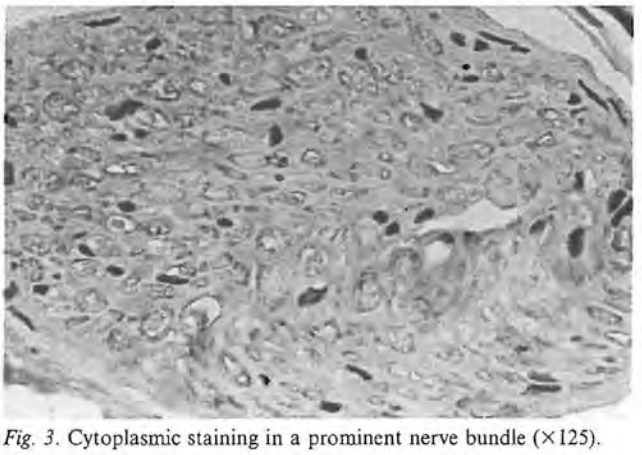

Fig. 3. Cytoplasmic staining in a prominent nerve bundle (×125).

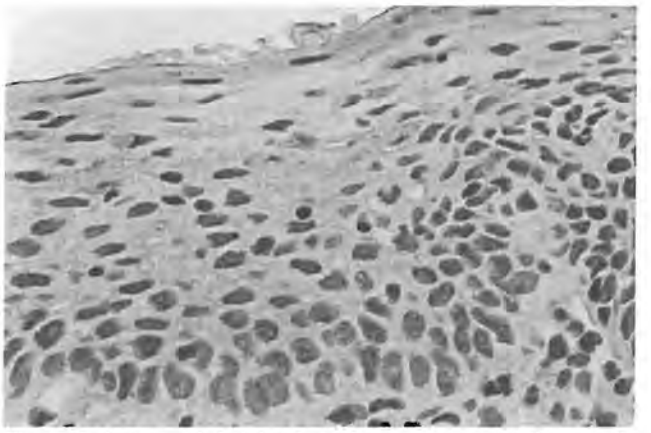

Fig. 4. Moderate epithelial dysplasia showing staining in the superficial cells but no staining in the basal and parabasal dysplastic cells (×250).

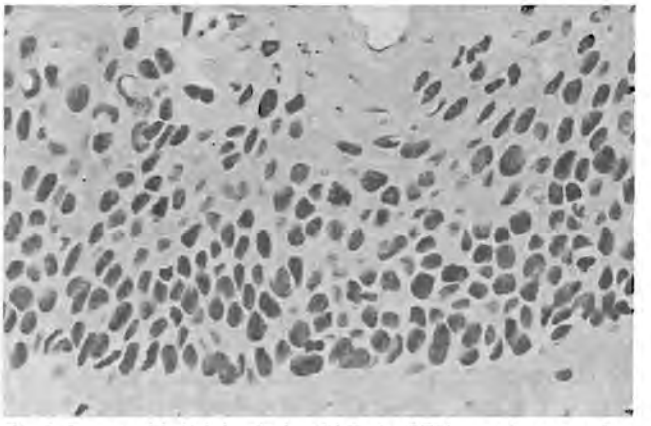

Fig. 5. Severe epithelial dysplasia with lack of Fhit protein expression in all cell layers (×250).

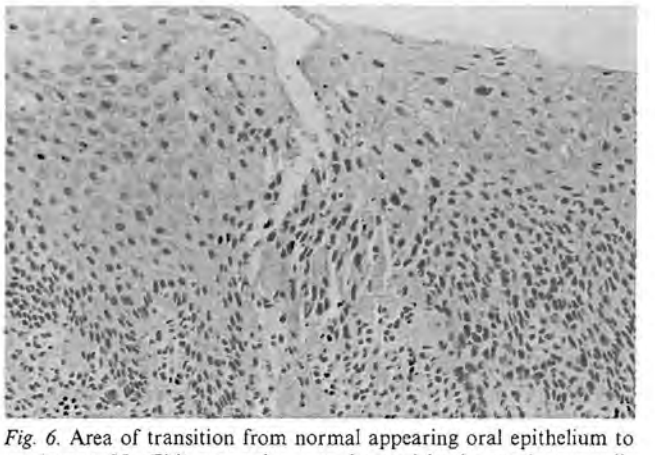

Fig. 6. Area of transition from normal appearing oral epithelium to carcinoma. No Fhit expression was detected in the carcinoma cells (×125).

demonstrating a low level of *FHIT* transcript, undetectable by Northern analysis. Six of the nine cell lines (66%) showed no Fhit protein or trace amounts. All of the primary tumours expressed very little Fhit, which could be due to contaminating non-tumour cells (12). This question would need to be pursued by immunohistochemical detection of Fhit to determine the actual level of Fhit expressed in the primary cells. It is important to resolve this question because evidence is accumulating in other tumour systems that Fhit can be inactivated in a large fraction of a number of tumour types (13,14) and at a very early stage in some, such as lung carcinomas (15).

Abnormalities of the *FHIT* gene and its expression have been reported in digestive tract (4), cervix (16), lung (15, 17), and kidney (18, 19) carcinomas.

The Fhit protein has dinucleoside 5′,5‴-P^1,P^3-triphosphate hydrolase activity *in vitro* (6) but its cellular function is thus far unknown (13).

To evaluate the possible role of Fhit

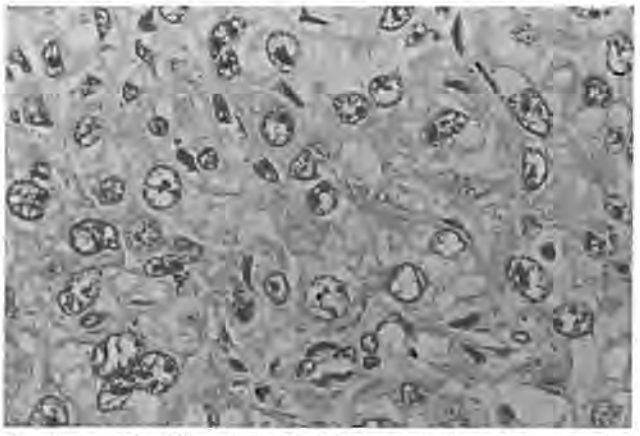

Fig. 7. A poorly differentiated OSCC without Fhit protein expression (×300).

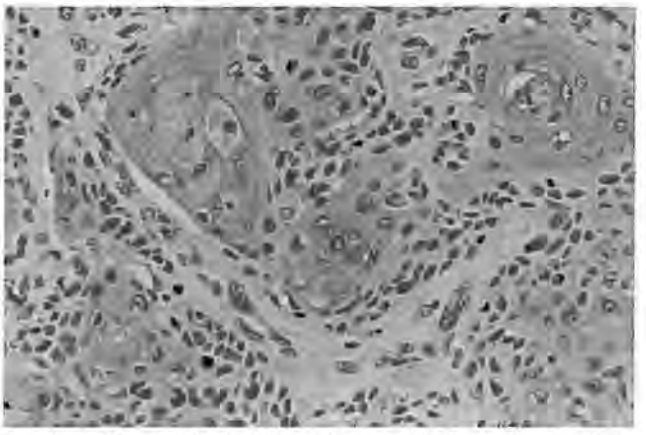

Fig. 8. Positive staining in the carcinoma cells. Note the prominent staining in the better-differentiated areas (×250).

protein in oral squamous cell carcinomas, we have studied its expression in normal and carcinoma tissues. The pattern of expression of Fhit protein in oral squamous epithelium, adjacent epithelial dysplastic lesions (when present) and squamous cell carcinoma was evaluated by immunohistochemistry using Fhit-specific antiserum (6, 20).

Material and methods

Intraoral squamous cell carcinomas were retrieved from the archives of the Departments of Oral Pathology and Anatomical Pathology, University of Pretoria. Blocks containing squamous cell carcinoma with adjacent epithelium were selected. Thirty-two cases were included in this study.

These formalin-fixed, paraffin-embedded tissue blocks were sectioned at 3 μm onto 3-aminopropyl-triethoxysilane coated slides. (SIGMA A3648), dried overnight at 37°C and then deparaffinised in two changes of fresh xylene for 5 min each. Sections were hydrated into dH2O through a series of graded alcohols. Antigen enhancement was performed by microwave heating of the sections in a saturated lead thiocyanate solution in a microwave pressure cooker. Slides were subsequently cooled for 20 min. Slides were washed three times in phosphate buffer and then treated with hydrogen peroxide for 5 min at 37°C to reduce endogenous peroxidase activity. Following additional phosphate buffer washes the slides were incubated with PBA (Coulter 0599 Universal Immunostaining kit) for 5 min at 37°C. Slides were then incubated overnight at 25°C with primary rabbit polyclonal anti-GST-FHIT antiserum at 1:600 (17, 19, 20). This antiserum is against the full length Fhit protein fused at the N-terminus to GST. Subsequent staining was performed with the Coulter 0599 Universal Immunostaining kit. Slides were then washed three times and incubated with secondary biotinylated antibody for 10 min at 37°C. After washing the slides three times in phosphate buffer they were incubated with the streptavidin-peroxidase reagent for 10 min at 37°C. Antibody localisation was effected by a 4-min incubation at 37°C with the chromogen, AEC. Finally, slides were washed in dH2O, lightly counterstained with haematoxylin, washed and then coverslipped using Faramount (Dako S3025) aqueous mounting medium. Negative controls were done by substituting the primary antibody with PBS buffer.

The intensity of Fhit protein expression was evaluated using criteria suggested by GREENSPAN *et al.* (17). The intensity of staining was recorded as: absent/weak, 1; moderate, 2; and strong, 3. The extent of immunostaining was scored based on the percentage of positive cells: <10%, 1; 10–50%, 2; and >50%, 3. The two scores were then multiplied to give a composite score (1–9) for each tumour. Composite scores of 1–3 were defined as marked reduction or absence of Fhit protein expression. The areas of the squamous cell carcinoma showing the poorest differentiation were used for evaluation of Fhit protein expression.

The sections were evaluated and scored independently by two oral pathologists. Those cases where the composite scores were not in concordance (<3 versus>3) were reviewed and scored on consensus opinion.

Results

The normal stratified squamous epithelium showed strong staining for the Fhit protein. This staining was more prominent in the stratum spinosum and in areas of keratin differentiation, with almost no staining in the basal and parabasal cells (Figs. 1 & 2). The staining was seen as a granular cytoplasmic chromogen. Staining was also seen in the excretory ducts of the underlying minor salivary glands and in nerve bundles (Fig. 3).

The intensity of staining in the normal appearing epithelium could be classified as strong in the majority of cases. Three cases showed only a moderate intensity of staining in the normal epithelium. A decrease in staining intensity was seen where the normal epithelium was associated with a dense chronic inflammatory cell infiltrate in the upper lamina propria and with exocytosis of lymphocytes into the epithelium.

Mild and moderate dysplastic changes did not appear to influence the Fhit expression of the epithelium (Fig. 4). Severe dysplastic changes, though, consistently showed absence of Fhit expression (Fig. 5). Most cases, however, showed a sudden transition from normal appearing epithelium to carcinoma, resulting in dysplastic lesions being present in only a few cases (Fig. 6).

Twenty-one (66%) of the squamous cell carcinomas had scores of three or less, indicating a distinct reduction or loss of Fhit protein expression (Fig. 7). Agreement with regard to the extent of staining for the two types of Fhit expression (≤3 and>3) was reached by the two oral pathologists in all the cases. A heterogeneous staining pattern

was observed in some of the tumours. When carcinoma cells were positive, the staining was found to be more prominent in the better-differentiated areas, especially the areas of keratinisation. (Fig. 8). Tumour grading, however, did not influence Fhit expression. Moderately to well-differentiated tumours were found to be negative, while some poorly differententiated tumours expressed Fhit protein.

Discussion

Fhit expression in tumours may be evaluated by quantification of *FHIT* mRNA expression. This may be impeded by lack of enough RNA, poor RNA quality and contaminating non-neoplastic cells in the tumour specimen (17). Immunohistochemical techniques directed against the protein product are also suitable to evaluate Fhit expression in different tumours (15, 17, 19).

The presence of Fhit protein in adjacent normal appearing epithelium served as a positive internal control for the immunohistochemical technique in this study. The extent of Fhit protein expression in various tissues has been assessed in various studies by analysis of RNA and protein levels. Epithelia of all major organs tested express Fhit protein strongly (breast, stomach, distal colon, kidney, cervix, liver) to moderately (lung, pancreas); lymphoid and brain tissue express Fhit protein less strongly and only in subsets of cells (13, 15–19, 21). The moderate staining observed in the normal epithelium in three of our OSCC cases may be either fixation or processing related.

The lack of influence of mild and moderate dysplastic changes on Fhit protein expression was to be expected. Marked Fhit protein expression in oral epithelium was consistently observed in the stratum spinosum and superficial epithelial areas. The basal and parabasal cells, which are phenotypically altered in mild and moderate epithelial dysplasia, were uniformly negative. Fhit protein expression in mild and moderate epithelial dysplasias would therefore be expected to be negative. Severe epithelial dysplasia, with atypical cells through the full thickness of the epithelium, including stratum spinosum and superficial layers, showed no Fhit protein expression. The absence of Fhit protein expression in the severe dysplastic lesions suggested that *FHIT* inactivation might occur at an early phase of oral squamous cell carcinogenesis. The number of carcinomas with adjacent dysplastic epithelium was unfortunately too small to correlate the staining pattern of the dysplasia with the carcinoma. Absence of Fhit protein expression was also found in dysplastic lesions of the lung (15).

The prominent positivity of the better differentiated squamous cell carcinoma cells may be linked to the expression pattern of the overlying epithelium, where strong positivity was absent from the basal and parabasal cells. The negative basal and parabasal cells in normal epithelium might be an indication that Fhit protein was not expressed in large enough quantities to be detected with immunohistochemistry. These negative cells had no correlation with Fhit-negative tumours, since no Fhit protein was expressed in the basal and parabasal epithelium cells of Fhit-positive tumours. This is in contrast to squamous cell carcinomas from the lung where SOZZI *et al.* (15) observed a prominent basal pattern of expression in lung squamous cell carcinomas. No staining was detected in the differentiated tumour cells in their study.

It has been suggested that *FHIT* gene involvement in a variety of malignancies is a consequence of its location in a genetically unstable region (22). All the sections in this study included normal epithelium as well as neoplastic cells. Absent or reduced Fhit protein expression was observed only in the malignant cells and in severe dysplastic lesions compared to the normal squamous epithelium. This would suggest the inactivation of both *FHIT* alleles.

The loss of Fhit protein may affect oral carcinogenesis by causing genetic instability, perhaps through loss of DNA replication control *via* its Ap4A hydrolase function. Alteration of the normal Ap4A levels in cells may compromise their ability to respond to environmental stresses caused by carcinogenic agents. A common feature of the different tumours presenting with abnormal *FHIT* is that they are carcinomas from areas directly exposed to environmental carcinogens (4).

The alterations in Fhit protein expression in many oral squamous cell carcinomas, but not in normal tissues, suggest that *FHIT* gene alterations may play a role in oral carcinogenesis. These results confirm and complement the study by KISIELEWSKI *et al.* (12), who showed that 6 of 9 (66%) HNSCC cell lines expressed no Fhit protein or only trace amounts. This fraction of Fhit-negative HNSCC cell lines is the same as our fraction of 32 Fhit- negative/reduced primary OSCCs. The former report also investigated protein expression in 32 primary HNSCCs by Western blot analysis and found that all tumours expressed low levels of Fhit. These authors discuss the fact that the low level of Fhit protein in primary tumours, and indeed the light RT-PCR bands from RNA templates of primary tumours, may indicate that *FHIT* RNA and protein expression are actually absent and that they are detecting expression from normal infiltrating stromal cells. Immunoblotting results cannot resolve this issue. Our immunohistochemistry study confirms that the majority of OSCCs have lost Fhit protein expression. Since by now hundreds of tumour cell lines and primary tumours of various types have been shown to harbour independent *FHIT* deletions in both alleles (13, 14), it is likely that the majority of OSCCs carry *FHIT* alleles that have been damaged directly by exposure to carcinogenic agents.

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FHIT RNA and Protein Expression in Oral Squamous Cell Carcinomas

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Abstract. *Background: To investigate the possible role of FHIT, a possible tumour suppressor gene, in oral carcinogenesis, we examined 17 oral squamous cell carcinomas (OSCCs) for genetic alterations. Materials and Methods: Fresh tissue was obtained during surgery, snap-frozen in liquid nitrogen and stored at -70°C. Nested PCR amplification to examine the integrity of FHIT mRNA was performed on the reverse transcribed complementary DNA obtained from the frozen normal and tumour tissue. Immunohistochemistry was done on formal in-fixed paraffin-embedded tissue protein from the same cases using a polyclonal antiserum against the full length Fhit. Results: Twelve out 17 (71%) OSCCs showed reduced or absent Fhit protein and half of the cases with reduced Fhit protein exhibited aberrant RT-PCR products. Conclusion: Immunohistochemical detection of Fhit protein expression in OSCCs is the more sensitive method to determine the status of Fhit in these tumours, in agreement with previous studies of other tumour types.*

Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity and appears to be on the increase in developing countries (1). Oral cancer rank 6th behind cancer of the prostate, lung, oesophagus, bladder and colon in males in South Africa. In combination with cancers of the pharynx, these malignancies are the 4th most common in South African males (2).

Previous cytogenetic and molecular studies of oral cancer have indicated that malignant transformation in the carcinogenic pathway results from progressive accumulation of genetic changes, including inactivation of tumour-suppressor genes, amplification or over-expression of oncogenes or both, and specific chromosomal abnormalities (3). Tobacco and alcohol have been implicated as the most important aetiological agents in the majority of head and neck malignancies (4). Exposure to tobacco carcinogens may also lead to characteristic mutational events in head and neck malignancies, as have been observed with the p53 gene (5). Particular genetic alterations have also been demonstrated in other tobacco- related malignancies such as lung cancer, where alterations involving the *FHIT* gene are significantly increased in smokers compared to non-smokers (6). It has also been shown that differences in genetic changes of squamous cell carcinoma from different sites in the head and neck region do exist, suggesting intrinsic tumour properties regardless of a common aetiological agent (7).

The *FHIT* gene at 3pl14.2 covers the renal carcinoma-associated chromosome t(3;8) translocation breakpoint and the FRA3B fragile site (8). This gene encodes a protein that shows homology to diadenosine 5',5'''-P^1, P^3-triphosphate (Ap3A) hydrolase from the yeast Schizosaccharomyces pombe (9) and is itself a diadenosine polyphosphate hydrolase. Support for the tumour suppressor function of the *FHIT* gene is based on homozygous deletions within the gene in numerous tumour cell lines (8, 13), the presence of aberrant *FHIT* transcripts in different tumours (10-12), a high incidence of allelic imbalance at 3p14.2 (13, 14) and loss of Fhit protein expression in cancers.

Abnormalities of the FHIT gene and its protein expression have been reported *inter alia* in digestive tract (8), lung (15, 16), cervical (17, 18) and kidney (19, 20) carcinomas. A reduction or loss of Fhit protein expression was also found in 66% of oral squamous cell carcinomas (OSCC) (21). The aim of this study was to evaluate the pattern of Fhit protein expression in OSCC and to compare its protein expression status with detectable genetic abnormalities.

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Key Words: Oral cancer, oral squamous cell carcinoma, *FHIT* gene, Fhit expression.

Materials and Methods

Tissues. Seventeen fresh OSCC tumour samples were obtained from resection specimens, snap-frozen in liquid nitrogen and stored at -70°C to be used for isolation of RNA followed by RT-PCR analysis. The remainder of the material was fixed in 10% buffered formalin and used

for routine histological examination, followed by immunohistochemical analysis.

RT-PCR. RNA was prepared from frozen oral tissue using the RNeasy protocol for the isolation of total RNA from animal tissues (Qiagen GmbH, Germany). RT-PCR was carried out using the Access RT-PCR system (Promega Corporation, Madison WI, USA). The reaction mixture contained 5µl RNA, 200 µM of each nucleotide, 0.4 µm of each primer 5U2 (5'-CATCCTGGAAGCTTTGAAGCTC) and 3D2 (5'-CCTGTATTCTCAACCAGTGA), 1U of *Taq* DNA polymerase (Promega Corporation), 10 µl of Promega 10x bufferl and 1.5 M of $MgCl_2$ made up in a final volume of 100 µl. Amplification cycles were carried out using a Perkin-Elmer PCR 9600 machine as follows: 48°C for 45 minutes; 94°C for 2 minutes; 40 cycles denaturing at 94°C for 1 minute, annealing at 50°C for 1 minute, and extension at 68°C for 1.5 minutes and one cycle extension at 68°C for 7 minutes. A nested PCR was performed using 2 µM of RT- PCR product and primers 5U1 (5'-TTCCGTAGTGCTATCTACAT) and 3D1 (5'-CAAGAGGAACTGAATCAGCATG). PCR amplification was as follows: one cycle of denaturing at 94°C for 2 minutes forty-five cycles denaturing at 94°C for 1.5 minutes annealing of primers 50°C for 1.5 minutes; and extension of the annealed primers at 72°C for 3 minutes and one cycle extension at 72°C for 7 minutes. The 700 base pair (bp) PCR products were visualised with ultraviolet light after electrophoresis through 2% agarose gels and ethidium bromide staining.

Sequencing. The PCR products of four samples yielded more than one band on agarose gel electrophoresis, namely samples 2, 8, 12 and 15. They were run on a 20% polyacrylamide gel and the bands were cut out. The DNA was extracted using the Qiaex II Gel Extraction kit (Qiagen GmbH, Germany) and the PCR was repeated using the nested primer pair. The nested PCR products were directly sequenced on the 373 DNA Sequencer (Perkin Elmer, California, USA) using the ABI Prism Dye Terminator Cycle sequencing kit (Perkin Elmer, California, USA). 5Ul and 3Dl were used as sequencing primers. The sequences were translated into amino acids using the DNAMAN computer program (Lynnon Biosoft, Canada, 1994). Alignment of the amino acid sequences was conducted using the CLUSTAL V software program.

Immunohistochemistry (IHC). Formalin-fixed paraffin-embedded tissue blocks of the same OSCCs were used for immunohistochemical evaluation of Fhit protein expression. These formalin-fixed, paraffin-embedded tissue blocks were sectioned at 3 µm and mounted onto 3-aminopropyltriethoxysilane-coated slides (SIGMA A3648), de-paralfinized in xylene and rehydrated in graded alcohol. Antigen enhancement was performed by microwave heating of the sections in a microwave pressure cooker in citric acid buffer (pH 6). The slides were subsequently cooled for 20 minutes and then washed three times in phosphate buffer and treated with hydrogen peroxide for 5 minutes at 37°C to reduce endogenous peroxidase activity. Immunohistochemical detection of Fhit protein was carried out using a polyclonal antibody (ZR44) that detects the full length Fhit protein (Zymed Laboratories, South San Francisco, CA, USA). The tissue sections were treated with the non-immune blocking serum of the Zymed Histostain^{TM}-Plus detection kit (85-9043) for 10 minutes at room temperature. The sections were then incubated with the antibody for 60 minutes at room temperature. Detection of the antibody reactivity was done with the above mentioned Zymed detection kit according to the manufacturer's instructions. AEC was the chromogen of choice. Finally, the slides were washed in distilled water, lihtly counterstained with haematoxylin, washed and then coverslipped using Faramount (Dako S3025) aqueous mounting medium. Negative controls were performed by substituting the primary antibody with PBS buffer. Both the extent and intensity of Fhit protein expression was evaluated. The intensity of staining was recorded as: absent/weak, 1; moderate, 2; and strong, 3. The extent of immunostaining was scored based on the percentage of positive cells: <10%, 1; 10-50%, 2; and >50%, 3. The two scores were then multiplied to give a composite score (1-9) for each tumour. Composite scores of 1-3 were defined as marked reduction or absence of Fhit protein expression while scores >3 were considered positive for Fhit. The tumour cells at the invasive tumour front were used for evaluation of Fhit protein expression.

Table I. *RT-PCR results compared with immunohistochemistry findings. Concordant findings were observed in ten cases* and discordant findings in six cases. Simple could not be sequenced.*

Sample number	Fhit protein composite score	Interpretation of RT-PCR results
1	1 (neg)	Normal
2*	3 (neg)	Deletion within one *FHIT* allele
3	1 (neg)	Normal
4*	6 (pos)	Normal
5	2 (neg)	Normal
6	3 (neg)	Normal
7	4 (pos)	Not evaluable
8*	3 (neg)	Deletion within one *FHIT* allele
9*	6 (pos)	Normal
10	3 (neg)	Normal
11 *	1 (neg)	Deletion within one *FHIT* allele
12*	1 (neg)	Deletion within one *FHIT* allele
13*	3 (neg)	Deletion within one *FHIT* allele
14*	6 (pos)	Normal
15*	1 (neg)	Deletion within one *FHIT* allele
16*	6 (pos)	Normal
17	1 (neg)	Normal
19 (positive control)		Normal
20 (positive control)		Normal

Results

Heterogeneity or a mixed pattern of protein expression, with co-existing posotive and negative tumour cells occurred in most of the tumours. Staining was also seen in nerve bundles, skeletal muscles, inflammatory cells and promlnent in the excretory ducts of minor salivary glands when present. Fhit protein expression at the invasive front was detected in 30%(5/17) of the tumours.

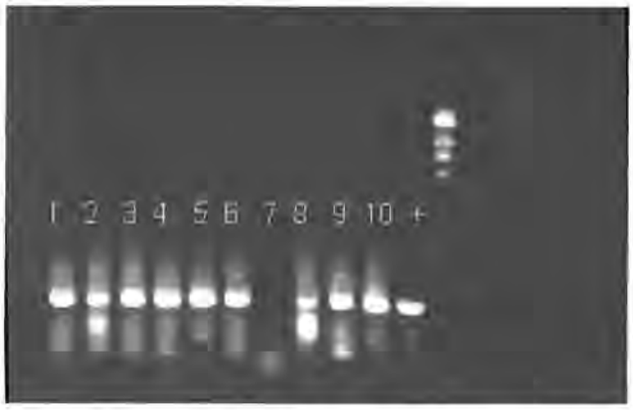

Figure 1. *Ethidium bromide-stained agarose gel of nested PCR products. All specimens 1- 'b demonstrated a normal band except 7, where no band was found. Specimens 2 and 8 also demonstrated aberrant RT-PCR products.*

Figure 2. *RT-PCR products from OSCCs. Cases 11 and 13 showed only aberranl-sized RT-PCR lel products, suggesting deletion within both FHIT alleles, cases I2 and 15 exhibited both normal IQd and aberrant-sized amplification products, suggesting a mixed population oJ cells with intact and deleted FHIT alleles. Cases 14, 16 and 17 showed only Ihe normal sized FHIT RT-PCR product.*

The agarose gels of the nested RT-PCR products are pictured in Figures 1 and 2. Aberrant transcripts were observed in 6 (35%) of the cases. All RT-PCR amplifications were repeated 3 times with similar results. All the samples had an 11 bp deletion representing an alternative splice form, when compared with the FHIT consensus sequence. Samples 2, 8, and 11 had a 296 bp deletion within the cDNA, while a 458 bp cDNA deletion was observed in samples 12 and 15. A summary of the RT-PCR amplification results and the corresponding immunohistochemistry analysis of *FHIT* protein expression are shown in Table I.

Discussion

RNA was used for genetic analysis because the *FHIT* genomic locus is very large (more than a 1000 kilobases) while the coding region (mRNA) is small (about 1 kilobase in size). Perfect correlation between the RT-PCR and immunohistochemistry results was observed in 10 cases. Immunohistochemistry for the Fhit protein was negative in all the cases where RT-PCR results were abnormal. Six cases showed normal RT-PCR results with no detection of Fhit protein expression. Several explanations were possible: it might have been that very low levels of the RNA were expressed in these cases, too low for immunohistochemical detection of the Fhit protein, thereby reflecting the difference in sensitivity of the two methods. It might also be possible that Fhit protein was absent in tumours and that the RT-PCR products detected derived from amplification of FHIT messages from normal stromal and inflammatory cells. The Fhit epitopes recognised by the antibody might also have been altered by processing or fixation (22), although this seems an unlikely explanation since all samples were treated similarly.

The *FHIT* gene is inactivated by deletion rather than mutation (8, 23, 24). The complexity of DNA lesions observed in *FHIT* gene studies may point towards protein detection as the best method to assess the level of involvement of Fhit in various human cancers. More sensitive Western blots could be used, although only immunohistochemistry can evaluate the fraction of cells expressing and their level of protein expression. Five of the cases (30%) showed Fhit protein expression by immunohistochemistry. The method of evaluating Fhit expression by immunohistochemistry is very important. The invasive tumour front was used as the preferred site to evaluate Fhit expression. This site was selected because of its appropriateness in predicting prognosis in OSCC (25). This evaluation of selected sites in a tumour overlooked the presence of heterogeneity of Fhit expression results and should be considered when comparing immunohistochemical results from different studies.

Abnormal RT-PCR products were amplified in 6/17 (35%) cases. Four of the six cases showing aberrant transcripts on RT-PCR had normal transcripts as well. This may reflect the presence of different clones in the tumour, some of which may have homozygous deletions, or contamination with normal stromal cells as the source of normal transcripts. The frequency of aberrant sized *FHIT* products in our study was higher than that reported for head and neck squamous cell carcinomas (HNSCC) in several studies. Kisielewski *et al* (26) found aberrant products in 2/9 HNSCC-derived cell lines and 4/42 primary HNSCC (three of which failed to give amplified fragments), similar to the 4/16 from Mao *et al* (12) and the 1/8 HNSCC-derived cell lines and 1/11 primary OSCC from the study by Pateromichelakis *et al* (27). Virgilio *et al* (23) observed aberrant transcripts in 15/25 (55%) in their HNSCC-derived cell lines. These differences could be attributed to different RT-PCR methods used or different cell culture conditions.

These results indicated that FHIT aberrations were frequently found in OSCC in the population sample studied. Loss of Fhit expression at the invasive tumour front was also common but may not necessarily correlate with detectable gene alterations.

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Fhit Protein Expression in Oral Epithelium: Immunohistochemical Evaluation of Three Antisera

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Abstract. *Background: A number of studies have shown that the Fhit tumour suppressor protein is abundantly expressed in normal epithelial cells of human organs and that this expression is lost or reduced in the majority of cancers arising in these epithelial tissues. A variety of antiFhit sera have been used but a systematic comparison of the different antisera has not yet been reported. Materials and Methods: We compared the Fhit expression pattern in the epithelium of fibrous epuli, oral lichen planus, oral epithelial dysplasia and oral squamous cell carcinomas (OSCC) using three different Fhit antisera. Results: The antigstFhit sera from two sources gave very similar results for all types of oral lesions except for lichen planus and showed that about 60% of OSCCs have lost Fhit expression. Conclusion: Although different staining patterns were found for the three antisera, all three could be used for evaluation of Fhit expression in OSCC.*

The *FHIT* (fragile histidine triad) gene, a candidate tumour suppressor gene, has been mapped to 3p14.2 and covers the renal carcinoma-associated chromosome t(3;8) translocation breakpoint, the FRA3B fragile site and homozygous deletions in many human cancer cell lines (1). This gene encodes a protein that shows homology to diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap3A) hydrolase from the yeast *S. pombe* (2) and the human and murine Fhit proteins are diadenosine polyphosphate hydrolases. Support for the tumour suppressor function of the *FHIT* gene is based on the presence of aberrant *FHIT* transcripts in different tumours (1, 3-5), homozygous deletions in the cDNA of many tumour cell lines (1, 6), a high incidence of allelic imbalance at 3p14.2 (1, 6, 7) and functional studies showing suppression of tumourigenicity after exogenous expression of Fhit in cancer cells (8-10).

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The evaluation of Fhit protein expression using immunohistochemistry has been reported *inter alia* in lung (11-13), cervical (14-16), breast (17), kidney (18) and oral squamous carcinomas (19). Fhit protein is also expressed in the normal epithelia of all major organs tested (12, 13, 15, 19-21).

Although most immunohistochemical studies have reported a large fraction of cancers with reduced or absent *Fhit* protein relative to Fhit-positivity in corresponding normal tissues, there have been variations in the percentages of negative tumours of specific types, for example lung cancer (12, 13). Since different antibodies against Fhit protein have been used in the reported studies (16, 18, 19), differences in affinity, avidity or other variables could contribute to these discrepancies. To evaluate the staining patterns of Fhit protein in oral epithelium in different disease states and, at the same time, to compare different antisera against *Fhit*, we studied its expression using three different Fhit antisera.

Materials and Methods

Tissues. Formalin-fixed paraffin-embedded blocks of fibrous epuli, oral lichen planus, epithelial dysplasia and oral squamous cell carcinoma (OSCC) were retrieved from the files of the Department of Oral Pathology, University of Pretoria, South Africa. Fibrous epuli are reactive connective tissue lesions of the gingiva covered by normal stratified squamous epithelium. No sub-epithelium inflammatory cell infiltrate was present in the fibrous epuli included in this study. These served as the normal epithelium control. Lichen planus is an immune-mediated skin disease characterised by a dense sub-epithelial lymphocytic infiltrate and was included to evaluate the possible influence of an inflammatory cell infiltrate on the staining properties of the overlying epithelium. Ten blocks each of the fibrous epuli and lichen planus and twenty blocks each of epithelial dysplasia (varying from mild to severe) and OSCC were selected.

Immunohistochemistry. Immunohistochemical detection of Fhit protein was carried out using three different antibodies recognising human *Fhit* protein. Two Zymed polyclonal rabbit anti-*Fhit* sera (Zymed Laboratories, South San Francisco, CA, USA) were used. One of these sera (ZP54) was raised against a synthetic peptide derived from a sequence near the C-terminus of the human Fhit protein and detects

Table I. *Composite staining scores for Fhit expression in oral epithelium. The loss or reduction of Fhit expression in OSCC is expressed as a percentage.*

Antisera	Fibrous epulis (n=10)	Lichen planus (n=10)	Epithelial dysplasia (n=20)	OSCC (n=20)	%OSCC with loss/reduction
Zymed:					
GST-*Fhit*	5.4 ± 2.9	2.7 ± 1.3	6.1 ± 2.3	3.6 ± 2.2	66
Zymed:					
Fhit C-terminus	2.0 ± 1.1	0.7 ± 0.6	1.7 ± 2.2	4.1 ± 2.9	60
KCC:					
GST-*Fhit*	4.6 ± 2.4	6.2 ± 1.9	6.2 ± 2.7	4.9 ± 2.7	60

full-length Fhit protein in Western blots. The other Zymed antiserum (ZR44) also detects full-length *Fhit* protein and the full-length human Fhit fusion protein with glutathione amino S-transferase (GST) was used as immunogen. The other serum was received from the Kimmel Cancer Center (KCC) and consisted of a rabbit polyclonal anti-GST-Fhit fusion protein. Three sections (3 mm each) of each paraffin-embedded tissue block were cut and mounted onto aminopropyltriethoxysilane coated slides (SIGMA A3648), de-paraffinized in xylene and rehydrated in graded alcohol. Antigen enhancement of all sections was performed in a microwave using a pressure cooker and citric acid buffer (pH 6). Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in distilled water for 5 minutes at 37°C. The two sections of each case on which the two Zymed antisera were tested were treated with the non-immune blocking serum of the Zymed HistostainTM-Plus detection kit (85-9043) for 10 minutes at room temperature. The sections were then incubated with the two antisera (ZP54 at 1µg/ml dilution and ZR44 at 1:50) for 60 min at room temperature. Detection of the antibody reactivity was done with the above-mentioned Zymed detection kit according to the manufacturer's instructions. AEC was the chromogen of choice. The remaining section on which the antiserum from KCC was tested was treated with non-immune serum of the Vectastain Universal Elite ABC detection kit (PK-6200) for 20 minutes at room temperature. The sections were incubated overnight at 25°C with the antiserum (1:600) (19). Detection of the immunostaining was carried out by the Vectastain Universal Elite ABC detection kit according to the manufacturer's instruction. AEC was the chromogen of choice (Immunotech 0599 A Coulter Company, BP 177-13276 Marseille Cedex 9- France). All the sections were counterstained with haematoxylin, washed in tap water and mounted with Dako Faramount aqueous mounting medium. Both the extent and intensity of Fhit protein expression were evaluated. The intensity of staining was recorded as: absent/weak, 1; moderate, 2; and strong, 3. The extent of immunostaining was scored based on the percentage of positive cells: <10%, 1; 10-50%, 2 and >50%, 3. The two scores were then multiplied to give a composite score (1-9) for each lesion. Composite scores of 1-3 were defined as marked reduction or absence of Fhit protein expression. The full epithelial thickness was used for evaluation of Fhit expression in the fibrous epuli, lichen planus and dysplastic lesions, while the invasive areas of the squamous cell carcinoma showing the poorest differentiation were used. The same areas on the different sections were used when evaluating the staining of different antisera.

The one-way repeated measures analysis of variance (Anova) was used to evaluate the staining properties of the four different antibodies. Pairwise multiple comparison procedures were used to isolate the antisera that differed from the others. The Tukey Test was used for normally distributed data while the Student-Newman-Keuls method was used where normality failed.

Results

The staining properties of the three antisera in the four different lesions are reflected in Table I. The extent and intensity of staining with the Zymed C-terminus antibody were significantly lower than that of the other two antisera in the cases of fibrous epuli and oral epithelial dysplasia. The degree of epithelial dysplasia did not affect the staining scores. The staining properties of both Zymed antisera were significantly lower than the KCC antiserum in the case of lichen planus. The difference between the two Zymed antibodies for lichen planus was also statistically significant. The differences in staining scores in the OSCC group among the three antisera was not statistically significant (Figures 1, 2). A more intense staining in the better-differentiated areas of the OSCC was observed with the KCC antiGST-Fhit (Figures 3, 4).

Subtle staining differences between the antisera were noted. The Zymed antiGST-Fhit serum demonstrated strong staining of all the inflammatory cells as well as strong staining

Figure 1. *Invasive tumour front of OSCC with a combined staining score of 6 (Zymed antiGST-Fhit).*

Figure 2. *Example of a combined staining score of 9 in OSCC (KCC antiGST-Fhit).*

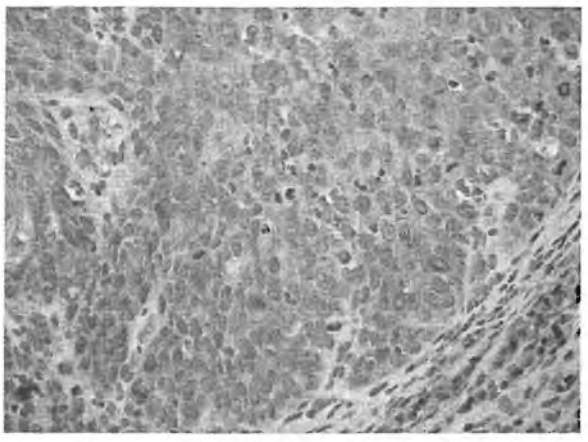
Figure 3. *Prominent staining of the better-differentiated area in OSCC with KCC antiGST-Fhit.*

Figure 4. *Same area as in Figure 3 with poor staining in the better-differentiated area with Zymed antiGST-Fhit.*

Figure 5. *Zymed antiGST-Fhit demonstrated strong staining in the inflammatory cells of lichen planus. The epithelial staining is more prominent in the basal cell region (total staining score of 6).*

Figure 6. *KCC antiGST-Fhit in the same case of lichen planus (Figure 5) showed a more prominent staining of the superficial epithelial cells and no staining in the inflammatory cells (total staining score of 9).*

Figure 7. *Strong staining in the excretory ducts of minor salivary glands with Zymed antiFhit C-terminus.*



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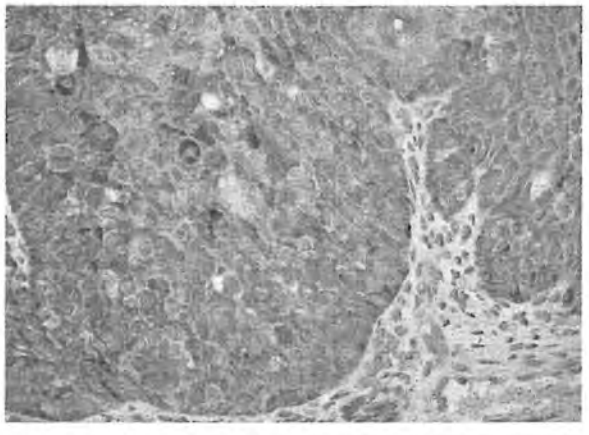

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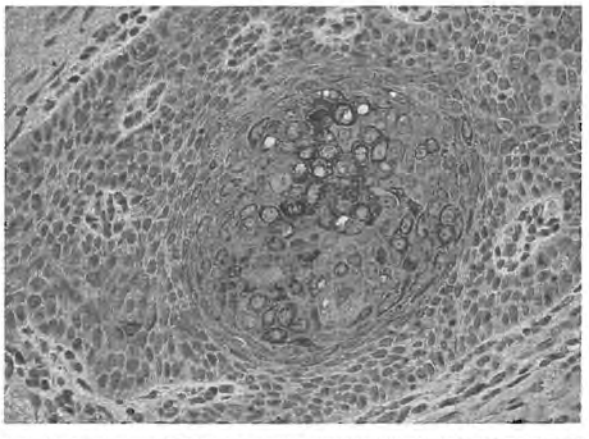

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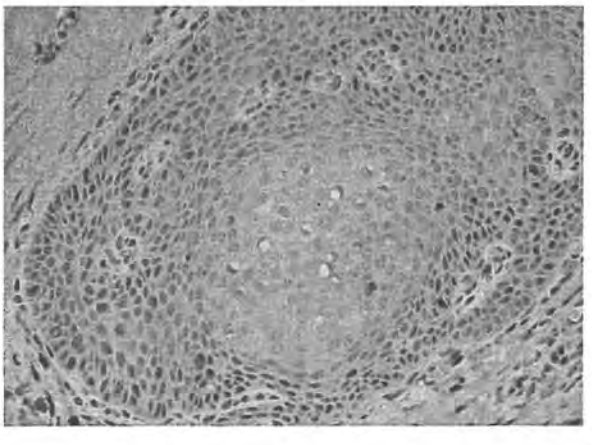

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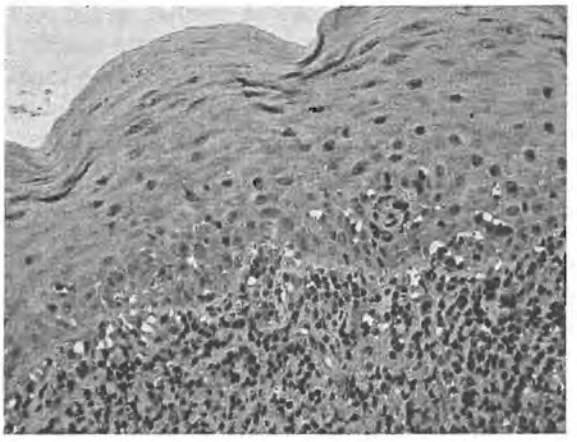

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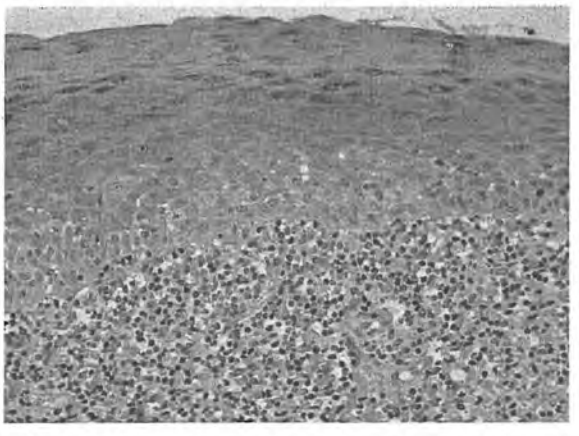

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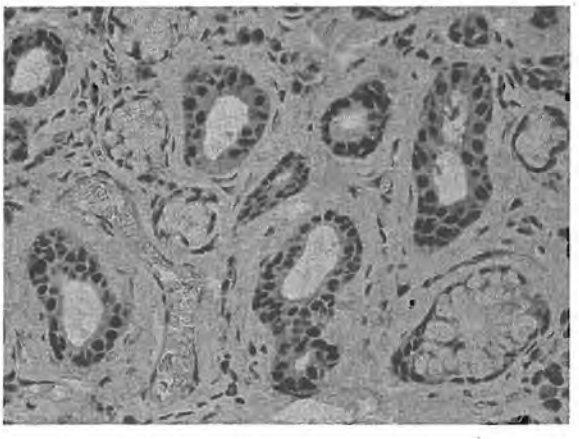

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of the basal and parabasal cells in contrast to the KCC antiGST-Fhit that showed stronger staining in the superficial layers of the epithelium. The presence of subepithelial inflammatory cells had no influence on the staining of the epithelial cells (Figures 5, 6). Although the Zymed Fhit C-terminus antiserum showed relatively poor staining of the oral epithelium, strong staining was detected in minor salivary glands with this (Figure 7), as well as the other two antisera.

Discussion

Immunohistochemistry is the best method to evaluate protein expression in a primary tumour because the proportion of cells expressing the protein, as well as their level of expression, can be determined. This is in contrast to Western blots where some positivity from non-tumour cells is often found, even if the tumour is negative. Immunohistochemistry is also a suitable method to determine *FHIT* inactivation in tumour cells because *FHIT* is inactivated by deletion rather than mutation.

The stronger staining of the basal and parabasal epithelium using the Zymed GST-Fhit antiserum, in contrast to the KCC GST-Fhit antiserum, is important when comparing results from studies using different antisera. Although the KCC GST-Fhit antiserum appeared to be related to keratin differentiation in normal epithelium, all three antisera showed strong staining in the better-differentiated areas of the OSCC.

The reason for the relatively weak epithelial staining of lichen planus using both Zymed Fhit antisera compared to the KCC antiGST-Fhit is difficult to explain. It may be related to the closely associated lymphocyte component, although strong epithelial staining was observed with the Zymed antiGST-Fhit in dysplastic epithelium when associated with a subepithelial inflammatory cell component. The nature of the infiltrate in lichen planus, consisting predominantly of T-lymphocytes with an imbalance between T-helper and T-suppressor activity (22), compared to the mixed infiltrate associated with oral epithelial dysplasia might have influenced the staining abilities of the Zymed antiGST-Fhit. The presence of inflammatory cells in the oral dysplasia group did not influence the staining of the antisera.

The staining score for the three Fhit antisera was slightly higher in the dysplastic epithelium compared to the normal epithelium in the fibrous epuli. The composite staining score of the OSCC for the antisera except the Zymed antiFhit C-terminus, was lower than the staining score in normal oral epithelium and dysplastic oral epithelium. This difference was statistically significant for the Zymed and KCC full-length antisera. The invasive tumour front area of the OSCC was used for Fhit evaluation and generally showed less intense and less extensive expression of Fhit than the rest of the tumour. The reduction or loss of Fhit protein expression in about 60% of OSCC in our study was similar to previous reports of OSCC (19, 23) but differed from results reported by Götte *et al* (24).

Tissue manipulation, including excessive fixation and antigen retrieval, has a considerable influence on the outcome of IHC studies (25). Since all the material was retrieved from the archives of a diagnostic histopathology laboratory, most of the specimens were fixed in 10% buffered formalin for less than 12 hours and similar heat-mediated antigen retrieval performed on all cases. It is therefore unlikely that the absence of immunoreactivity when evaluating certain antisera reflects the destruction or masking of that specific target epitope. The fact that significant differences were observed among the four antisera in the non-neoplastic lesions but not in the OSCC group can therefore not be attributed to tissue manipulation.

Both the qualitative and quantitative staining patterns of the different antibodies were evaluated. This was done to include more than one parameter for the antibody comparison. It may not necessarily be important to determine both aspects when evaluating Fhit expression in all tissues. This is especially relevant to OSCC, where new tumour cell clones with different behavioural characteristics and arguably different staining patterns may arise. This necessitates the use of a well-defined area when evaluating IHC staining in solid tumours. The invasive tumour front of OSCC is an appropriate site because it has been suggested that molecular and morphological characteristics at this site reflect tumour prognosis better than other parts of the tumour (26).

In conclusion, comparison of the three Fhit antisera showed that all three could be used for evaluation of Fhit expression in OSCC. The Zymed C-terminus antibody showed weaker staining characteristics in non-neoplastic oral epithelium than the other antibodies evaluated. It would appear that loss of Fhit expression might be important in the behaviour rather than in the initiation of OSCC.

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