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SSCP Pattern Indicative for *p53* Mutation is Related to Advanced Stage and High-grade of Tongue Cancer

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***p53* and *bcl-2* are involved in the control of cell cycling and apoptosis. Environmental factors such as smoking and radiation can disturb *p53* function and predispose a cell to malignant transformation. To investigate the role of *p53* mutations, as well as p53 and bcl-2 protein expression in squamous cell carcinoma of the tongue, 39 samples were analysed. Since neck metastasis is the most important prognostic factor of this disease, samples from patients both with and without nodal disease were selected to find out whether there was any difference between the groups. Non-radioactive single-stranded conformation polymorphism (SSCP) was used to screen *p53* mutations; an altered SSCP pattern indicating *p53* mutation was found in 21 samples (54%). A significant correlation between tumour size, histological differentiation and *p53* mutations was found ($P<0.01$). Immunocytochemically, nuclear expression of p53 was moderate or strong in 18 (46%) samples. No correlation between altered *p53* SSCP pattern and p53 immunoreactivity was seen. bcl-2 expression was cytoplasmic; moderate or strong staining was detected in only six of the carcinoma samples (15.5%). Interestingly, there was a significant correlation between smoking and bcl-2 expression ($P<0.01$): all six samples with moderate or strong staining were taken from heavy smokers. Furthermore, all those patients died within 32 months. Copyright © 1996 Elsevier Science Ltd**

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INTRODUCTION

The single most important prognostic factor in tongue cancer is probably the presence of neck metastases. Owing to the rich lymphatics of the tongue, 35% of tongue cancers have spread into cervical lymph nodes at the time of diagnosis [1]. Without lymph node metastasis, the 3-year survival rate is over 60% for small cancers; the presence of clinical metastases lowers it by 50% [2]. The TNM-classification (UICC 1987) does not individually predict the clinical behaviour of each tumour and even small tongue cancers can behave surprisingly aggressively. The discovery of more accurate predictive factors for tongue cancer would be of importance in planning the treatment of patients.

The tumour suppressor gene *p53* has a dual action on the normal cell: first, it acts at a cell cycle checkpoint inhibiting proliferation of the cell by arresting it at the G1-phase following DNA-damage. In addition, *p53* has an important role in apoptosis following genotoxic damage. Both these mechanisms are also thought to suppress tumour growth [3]. Cells expressing normal p53 protein respond to gamma irradiation, as well as to cytotoxic drugs, better than cells without normal *p53* gene function [4]. Thus, a loss of the *p53* gene may also contribute to the treatment failure of cancer patients through an elevated threshold to apoptosis [5].

The functions of the wild-type *p53* gene and its protein can be blocked by mutations and interactions with viral products. For example, the E6 protein of HPV types 16 and 18 is known to bind to p53 protein disturbing its normal function and promoting degradation [6]. Furthermore, co-expression of the oncogenes *myc* and *bcl-2* can inhibit *p53* effects [7]. Alterations in *p53* functioning and protein overexpression have been found in about 50% of oral cancers in most reports [8, 9]. However, even higher rates have been reported in head and neck cancer patients [10].

It is well documented in lung cancer that environmental factors, such as cigarette smoking and asbestos exposure, can disturb the *p53* function [11, 12]. In the oral cavity, exogenic factors can act on cells more directly than in almost any other human tissues. However, there are some controversial reports concerning the role of cigarette smoking in deregulation of p53 protein [13, 14].

The *bcl-2* gene codes for an intracellular membrane protein.

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It is known to inhibit apoptosis, induced by a variety of stimuli. Therefore, it has a protective effect on the cell by preventing triggering of the apoptotic pathway too easily [15]. Abnormally high bcl-2 protein expression, as well as an aberrant pattern of protein production, have been found in many tumours, including nasopharyngeal carcinoma [16] and small cell lung carcinoma [17]. High expression of bcl-2 protein has been associated with worse response to therapy in some malignancies [18]. Additionally, an increased rate of translocations of *bcl-2* has been reported in the peripheral blood of heavy smokers [19] and a significant correlation was found between smoking and bcl-2 expression in head and neck tumours [20].

Because of the known functions and potential roles of *p53* and *bcl-2* in the aetiology and pathogenesis of cancer, we examined the state of *p53* in two groups of tongue cancer patients, those with and those without neck metastases at the time of diagnosis by means of mutation screening using single-stranded conformation polymorphism (SSCP) and immunocytochemistry. In parallel, we also studied immunocytochemical expression of bcl-2. The results were correlated with several clinical and histopathological parameters to examine their prognostic potential.

MATERIALS AND METHODS

This study includes 39 formalin-fixed paraffin-embedded primary squamous cell carcinomas of the mobile tongue. Altogether 19 consecutive patients with neck metastases at the time of diagnosis (N+) and 20 consecutive patients without metastatic nodes (N0) were selected. Apart from the clinical evaluation, the lymph node status had been confirmed by computer tomography in 23 patients and by ultrasound in 3 patients. There were 21 males and 18 females. The mean age was 64 years, ranging from 25 to 86 years. All the specimens analysed had been taken before any treatment. The histological diagnosis of squamous cell carcinoma (SCC) was reconfirmed in haematoxylin–eosin stained sections.

According to TNM staging, 29 patients had T1-T2 and 10 patients T3-T4 disease. The tumours were either well (19), moderately (17) or poorly (3) differentiated carcinomas according to the WHO classification. The detailed clinical data are shown in Table 1.

The patients were treated at the Department of Otorhinolaryngology at Turku University Hospital during 1986–1993. All patients were treated with pre-operative radiotherapy to the tumour site (60–65 Gy) and to the neck (50 Gy), followed by radical surgery of the primary tumour and supraomohyoidal, functional or radical neck dissection in 75% of the patients 3–6 weeks later. The surgery was not carried out in 5 patients due to their poor physical condition or refusal; for the same reasons 3 patients did not receive curative radiation treatment. The smoking history was available in 36 patients. The smoking habits were divided into three groups: non-smokers (also those who had stopped smoking over the previous 10 years; 14 patients), moderate smokers (under 20 cigarettes per day; 3 patients) and heavy smokers (over 20 cigarettes per day for more than 10 years; 19 patients). Data of alcohol consumption were available in 31 patients. The patients were divided into two groups: those who did not consume alcohol at all or consumed less than 250 ml of absolute alcohol weekly (25 patients) and those who consumed more than 250 ml of absolute alcohol weekly (6 patients).

Polymerase chain reaction (PCR)

Several 5-μm-thick sections of paraffin blocks were cut with a total area of 1 cm^2. The sections were placed directly in Eppendorf tubes and deparaffinised with xylene and ethanol. After adding acetone the samples were dried and treated with 30 μg/μl proteinase K solution (Boehringer Mannheim, Germany) at 37°C overnight. The samples were then heated at 95°C for 10 min to inactivate proteinase K. After centrifugation, the supernatant was used for PCR reaction. We were interested in exons 5–9 of *p53* since the vast majority of *p53* mutations in human cancer are located in these regions. Four sets of oligonucleotide primers specific for the intronic sequences were used, one set for each of exons 5, 6 and 7 and one set for the exon-pair 8/9. The sequences were as follows. 5A, 5′-TTC CTC TTC CTG CAG TAC TC-3′ and 5B, 5′-GCC CCA GCT GCT CAC CAT CG-3′. 5/6A (starting from the end of exon 5), 5′-ACC ATG AGC GCT GCT CAG AT-3′ and 6B, 5′-AGT TGC AAA CCA GAC CTC AG-3′. 7A, 5′-GTG TTG TCT CCT AGG TTG GC-3′ and 7B, 5′-CAA GTG GCT CCT GAC CTG GA-3′. 8A, 5′-CCT ATC CTG AGT AGT GGT AA-3′ and 9B, 5′-CCC AAG ACT TAG TAC CTG AA-3′. The length of the exons were as follows: exon 5, 214 base pairs (bp); exon 6, 236 bp; exon 7, 138 bp; exon 8/9, 331 bp. The PCR was performed in 50 μl reaction containing the PCR buffer (10 mM TRIS-Cl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100) (Finnzymes OY, Espoo, Finland), 15 μl of the target DNA solution, 1.25 mM deoxynucleotide triphosphate, 20 pmol of each primer and 1.0 U of DNA-polymerase (Finnzymes OY, Espoo, Finland). After the addition of mineral oil, the amplification was carried out in an automated DNA thermal cycler (Perkin-Elmer Cetus, Norrwalk, Connecticut, U.S.A.) using either 35 cycles (exon 7) or 40 cycles (exons 5, 6, 8/9). The programme was as follows: the initial denaturation at 95°C for 4 min 30 s, followed by denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min (exon 7) and 2 min (exon 5, 6, 8/9). The final extension step was carried out at 72°C for 10 min.

The successful amplification was confirmed by agarose gel electrophoresis (Nusieve, FMC BioProducts, Rockland, Maine, U.S.A.) under ultraviolet light. Reamplification was carried out for the samples where no PCR product was visible on the gel. The PCR was performed in the same way except 1 μl of the previous PCR product was used instead of 15 μl of DNA solution. This method was applied to 20 samples for the amplification of exon 8/9.

Single-stranded conformation polymorphism (SSCP)

For SSCP, 2 μl of the PCR product were mixed with an equal volume of formamide loading dye (98% deionised formamide, 0.05% bromophenol blue and 0.05% xylene cyanol). DNA was denaturated by heating at 95°C for 5 min followed by cooling on ice for a few minutes. Electrophoresis was carried out using the PhastSystem™ and gels were silver stained in its development unit as optimised recently by our group [21]. One microlitre of the mixture was immediately loaded on to the gel. The single-stranded PCR products were separated on PhastGel™ homogeneous polyacrylamide gels with PhastGel™ native buffer strips (0.88 M L-alanine,

Table 1. Clinical data and results of each patient

No.	Sex/Age (years)	TN-state	Grade	Smoking	Clinical outcome	Survival or follow-up in months	Mutations of p53	p53 staining	bcl-2 staining
1	F/77	T2N1	I	2	DOD	11	No	1	1
2	M/44	T2N0	II	1	NED	16	Exon 5	3	0
3	F/77	T2N2	I	1	DOD	11	No	3	0
4	F/91	T3N0	II	0	DOD	17	Exon 7	3	0
5	F/69	T2N0	II	1	NED	19	No	0	0
6	F/59	T2N0	I	3	NED	13	No	2	0
7	F/82	T2N0	I	1	NED	16	Exon 7	1	0
8	F/37	T2N0	I	1	NED	14	Exon 5	1	0
9	F/86	T1N0	III	0	NED	22	No	3	0
10	F/72	T2N0	I	1	NED	26	Exons 6 & 8/9	0	0
11	F/91	T2N0	I	0	NED	11	No	1	0
12	M/56	T3N0	III	3	DOD	7	Exon 5	1	0
13	F/75	T2N2	I	1	NED	16	No	1	0
14	F/67	T2N0	I	1	DOD	16	No	1	0
15	M/76	T4N2	II	2	DOD	3	Exon 5	1	1
16	M/64	T2N0	I	3	PD	19	No	1	0
17	M/85	T1N0	II	1	NED	32	Exon 5	3	0
18	F/61	T1N0	I	1	NED	67	No	1	0
19	M/75	T2N1	II	3	NED	63	No	1	0
20	F/73	T2N1	II	1	DOD	3	Exons 5 & 7	3	0
21	F/71	T2N0	I	3	NED	40	No	0	0
22	M/39	T3N1	II	3	NED	47	Exons 5 & 8/9	2	0
23	M/68	T4N2	II	3	DOOD	24	Exon 7	2	2
24	M/48	T4N1	II	3	DOD	23	Exon 6	2	2
25	F/66	T2N0	I	1	NED	48	Exon 7	3	0
26	M/65	T2N1	III	2	NED	52	Exon 5	2	0
27	F/69	T2N2	I	3	NED	58	No	3	0
28	M/52	T3N0	I	3	NED	74	No	2	1
29	M/25	T3N1	II	1	DOD	8	Exon 5	1	0
30	M/58	T4N1	II	3	DOD	15	Exon 7	1	3
31	M/50	T2N0	I	1	DOD	19	Exon 7	1	0
32	M/51	T1N0	II	3	DOD	18	No	1	2
33	M/51	T1N0	I	1	DOD	23	Exon 6	0	0
34	F/77	T3N1	II	1	DOD	9	Exon 7	2	0
35	M/58	T1N1	II	1	NED	76	Exon 5	1	0
36	M/72	T2N1	II	3	NED	46	Exon 6 & 7	3	0
37	M/65	T2N1	II	1	NED	63	No	0	0
38	M/59	T2N1	I	3	DOD	32	No	3	2
39	M/45	T2N2	I	3	DOD	19	No	3	3

Abbreviations: Smoking: 0 = data not available, 1 = non-smoker, 2 = moderate smoker, 3 = heavy smoker; DOD: dead of disease, DOOD: dead of other disease; PD: persistent disease; NED: no evidence of disease. Staining pattern: 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining.

0.25 M Tris, pH 8.8) (Pharmacia Biotech, Uppsala, Sweden). The DNA fragments containing exon 5 were run in a 12.5% gel at 15°C for 200 Vh, exon 6 in a 12.5% gel at 15°C for 250 Vh, exon 7 in a 20% gel at 15°C for 200 Vh and exon 8/9 in a 12.5% gel at 20°C for 300 Vh.

Immunocytochemistry for p53 and bcl-2

Five micrometre thick sections on organosilan slides were deparaffinised and dehydrated through xylene and graded alcohol. For bcl-2 staining but not for p53 staining, the sections were treated in a microwave oven in citric acid buffer (pH 6.0) for 5 min to intensify the antibody reaction. Endogenous peroxidase activity was blocked using 5% hydrogen peroxide in phosphate buffered saline (PBS) for 5 min. For p53 staining, the samples were incubated in normal goat serum (Vectastain ABC kit, Vector Laboratories, Burlingame, California, U.S.A.) for 15 min followed by incubation with rabbit polyclonal antibody for p53 (dilution 1:1000) (CM1, Novocastra Laboratories Ltd, Newcastle upon Tyne, U.K.) at 4°C, overnight. For bcl-2 staining, the sections were incubated in normal horse serum (Vectastain ABC kit) followed by incubation in 1:100 diluted bcl-2 primary monoclonal antibody (DAKO-bcl-2, DAKO, Denmark) at 4°C, overnight. After rinsing in PBS, the sections were incubated with biotinylated secondary antibody (Vectastain ABC kit) at room temperature (RT) for 30 min and after washing, treated with ABC complex (Vectastain ABC kit). The samples were then incubated in diaminobenzidine at RT for 5 min, washed in running water and counterstained with haematoxylin. For p53 staining, human breast carcinoma was used as a positive control and tongue cancer was used as a negative control by omitting the primary antibody. For bcl-2 staining, tonsil

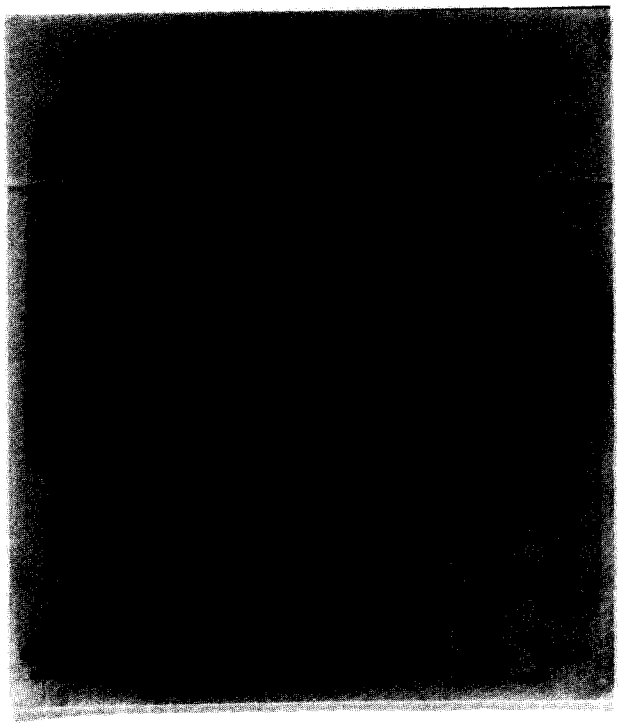

Fig. 1. A PhastSystem™ electrophoresis gel showing altered pattern in exon 7 in lanes 4 and 7 (arrows). Lane 1, normal control (DNA from CaSki cells).

sections were used as positive controls and the negative control was a carcinoma section, without primary antibody incubation.

The immunoreactivity in cancer tissue was classified as follows: 0 = no staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining. Also, the location of positive signals was recorded either as basal, parabasal or throughout the epithelium.

For statistical analysis, chi-square and Fisher's exact tests were used.

RESULTS

Thirty-nine primary tongue cancers were analysed. All samples were screened by SSCP with PhastSystem™ to detect mobility shifts suggesting *p53* mutations. The PCR analysis was unsuccessful in two samples, because of the small size or the inhibitory factors present in the sample. Moreover, exon 8/9 could not be amplified in four additional samples. The electrophoretic pattern was altered in 21 out of 39 (54%) samples suggesting the presence of a *p53* mutation. In total 10 mutations were found in exon 5; exon 7 harboured 9 mutations (Fig. 1). There were five mutations in exon 6 and two mutations in exon 8/9 (Table 1). In four specimens, two mutations were found in the same sample but those patients did not differ from other patients by any other parameter.

The association between altered SSCP pattern indicating *p53* mutation and the tumour size was statistically significant (Fisher's exact test, $P<0.01$): *p53* mutations were present in only 41% (12/29) of T1-T2 tumours but in 90% (9/10) of T3-T4 tumours (Table 2). *p53* mutations could be found in 75% of the high grade (grade 2 and 3) cancers but only in 32% of the low grade cancers, which was also statistically significant (chi-square test, $P<0.01$). No correlation was found between *p53* mutations and the occurrence of cervical metastases, smoking or alcohol consumption (Tables 3 and 4). Also, there was no relation between *p53* mutation and protein overexpression (Table 3).

Immunoreactivity with p53 was strong in 11 (28%), moderate in seven (18%), weak in 16 (41%) and absent in five (13%) tumours. p53 staining was nuclear and expressed in the basal cell layer of normal epithelium. In squamous cell carcinoma, p53 expression was found throughout the epithelium (Fig. 2). However, in several well-differentiated carcinomas, immunopositivity was restricted to basal and parabasal cells as shown in Fig. 3.

The tumour size, the histological grading, smoking habits or alcohol consumption did not correlate with immunohistological staining of p53 (Tables 2 and 4). Also the expression patterns of p53 and bcl-2 did not correlate.

bcl-2-staining was strong in two (5.1%) and moderate in four (10.4%) tumours; it was weak in three (7.7%) and absent in 30 (76.8%) of the cancer sections. The staining was always cytoplasmic. Strong bcl-2 expression is shown in Fig. 4; the proliferative epithelium adjacent to dysplastic and carcinoma epithelium is totally negative for bcl-2. In most samples, scattered basal cells in normal epithelium showed bcl-2 expression. In a few samples, the atrophic and hyperkeratotic epithelium adjacent to the carcinoma area showed strong bcl-2 expression in all basal cells. Interestingly, there were also totally negative areas in the same sample (Fig. 5). Lymphocytes showed strong bcl-2 staining (Figs 4 and 5). In two specimens, where strong staining was observed, carcinoma tissue seemed to be more aggressive and patches of infiltration were seen.

A statistically significant correlation was found between bcl-2 expression and smoking (Fisher's exact test, $P<0.01$): all 6 heavy smokers presented with moderate or strong bcl-2 immunoreactivity; no expression was found in the cancer tissue of non-smokers (Table 1). Furthermore, those 6 patients died within 32 months; 1 of them due to another disease. No correlation was found between bcl-2 staining and other parameters studied.

DISCUSSION

In this study, the incidence of *p53* mutations was 53% and mutational hot-spots were exons 5 and 7, which resemble previous reports of oropharyngeal cancers [8]. The mutation analysis was performed by means of SSCP analysis, which is only a screening method. Recently, however, SSCP has been shown to detect 90% of the mutations of *p53* [22]. In our hands, we have been able to confirm all mobility shifts in SSCP suggestive for *p53* mutation by direct sequencing.

p53 mutations often precede metastatic spread [23] and *in vitro* experiments have shown that *p53* mutations increase the opportunity of metastasising [24]. However, in our material no correlation between metastasising and *p53* mutations or protein expression could be found. It is likely that tongue cancer, as other cancers, is a sum of several related or independent environmental and genetic factors gathering into the same cell, thus leading stepwise to malignancy and finally metastasis [25]. The finding that T3-T4 cancers harboured more *p53* mutations than T1-T2 cancers supports the theory of accumulation of mutations in the course of cancer. On the other hand, *p53* mutations of these advanced diseases might be an early event which contribute to their more aggressive

Table 2. p53 immunopositivity, mutations and their correlation to tumour size and gradus

p53 immunopositivity grade	T1-T2	T3-T4	Low grade	High grade
No	5	0	3	2
Slight	12	4	9	7
Moderate	2	5	2	5
Strong	10	1	5	6
Total no.	29	10	19	20
p53 mutations present	12 (41%)	9 (90%)	6 (32%)	15 (75%)

For size of tumour, T-staging according to the UICC 1987 was used. Low grade refers to WHO I and high grade to WHO II and WHO III classification.

Table 3. Correlation of p53 *mutations with p53 immunopositivity and lymph node status*

p53 immunopositivity grade	Total no. (%)	No metastasis No	Neck metastasis No	*p53* mutations present No
No	5 (13%)	4	1	2
Slight	16 (41%)	9	7	8
Moderate	7 (18%)	2	5	5
Strong	11 (28%)	5	6	6
Total no.	39 (100%)	20	19	21
No. of *p53* mutations		10	11	

Table 4. p53 immunopositivity, mutations and their correlation to smoking and alcohol consumption

p53 immunopositivity grade	Smoking habits			Alcohol consumptions	
	No	Moderate	Heavy	Moderate	Abundant
No	3	0	1	5	0
Slight	8	2	5	12	2
Moderate	1	1	5	4	1
Strong	5	0	4	4	3
Total no.	17	3	15	25	6
p53 mutations present	12 (70%)	2 (67%)	6 (40%)	13 (52%)	3 (50%)

behaviour compared with more innocent tumours without genetic lesions [23]. This could also explain the increased rate of mutations of moderate and high grade cancers in this study.

The present results show moderate or strong p53 staining in 46% of the cancer sections which is in concordance with other reports of tongue cancer [9]. Evidence is gathering that the p53 antibody reaction and *p53* mutations are not related [26, 27], which is supported by this study. The antibody used, CM1, recognises wild-type and most mutant forms of p53. Thus, it is unlikely that undetected mutated p53 proteins could remarkably change the results. These findings suggest that post-translational mechanisms are at least partly responsible for the dysregulation of p53 protein.

The low bcl-2 immunoreactivity in our material is supported by the recent report studying head and neck cancer patients [20]. It is assumed that bcl-2 protein protects mitotic cells from apoptosis [28]. That is in line with the observation that bcl-2 was expressed in the normal basal cell layer of the epithelium. It is obscure why the bcl-2 expression in most samples ceased as the epithelium turned out into the carcinoma tissue. Interestingly, all moderate or strong bcl-2 expression patterns detected were found among heavy smokers; the result is similar to the recent report by Gallo *et al.* [20]. Thus, it is possible that carcinogens of tobacco, such as benzo(α)pyrene and methylating agents, disturb *bcl-2* function and promote protein overexpression. Subsequently, the apoptosis is abnormally inhibited, enhancing malignant transformation. Moreover, all 6 patients died within 32 months. Even though one of them died of another disease, bcl-2 overexpression combined with smoking could also predict a more aggressive behaviour of some squamous cell carcinomas. However, the number of the positive cases was too limited to make any final conclusions.

No correlation with bcl-2 and p53 expressions could be found in this study. Recently, other gene products, such as bax, have been found to counteract the effect of bcl-2 protein [29]. *p53*, *mdm-2*, *bcl-2* and *bax* are known to be mutually regulated [3, 15], along with other putative genes. Thus, it is

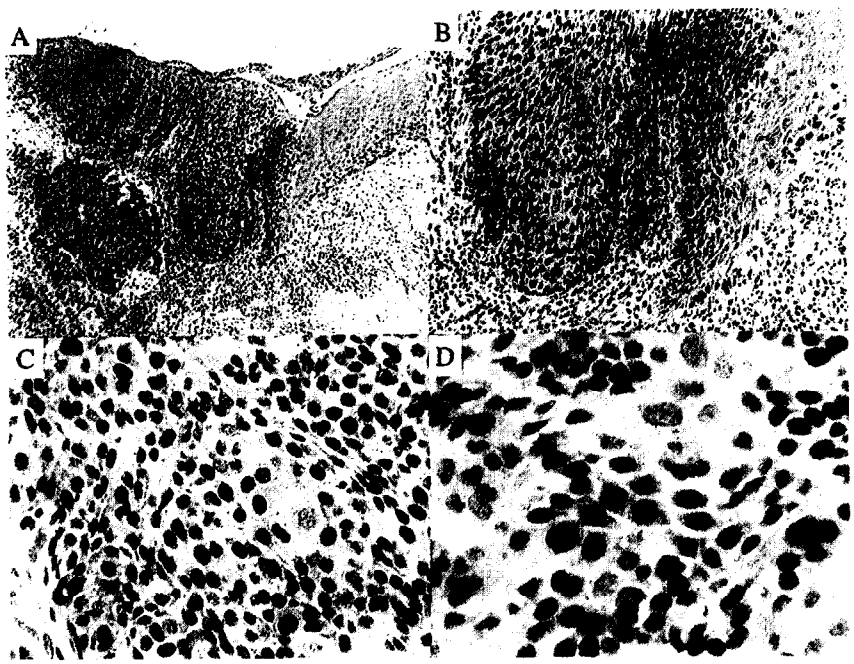

Fig. 2. A micrograph of p53 immunohistostaining showing normal hyperkeratotic epithelium changing into a malignant one. Strong nuclear staining (brown) of p53 protein is seen in most dysplastic and cancer cells. p53 expression is increased in malignant cells. (Original magnification: panel A: 16 ×, panel B: 40 ×, panel C: 250 ×, panel D: 400 ×).

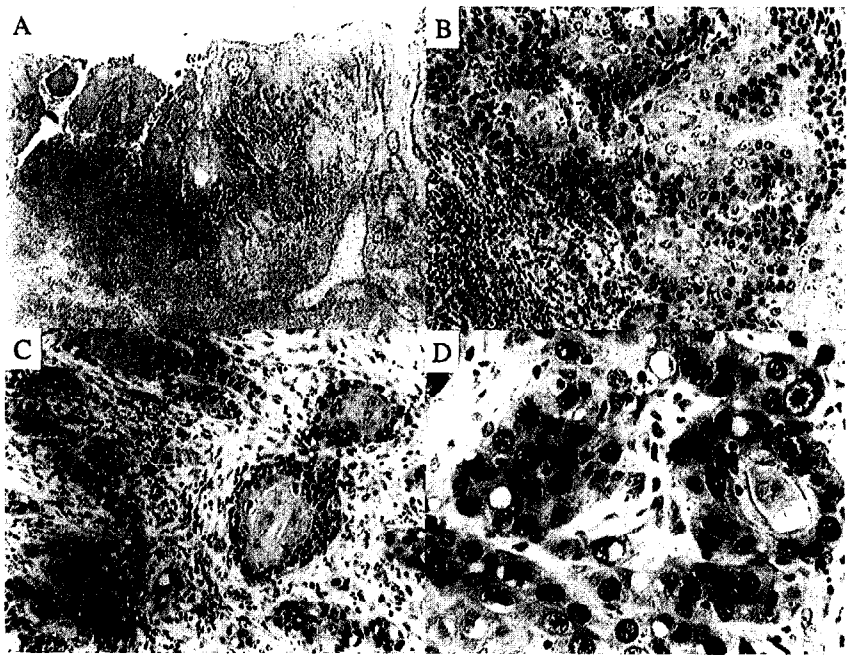

Fig. 3. Well-differentiated squamous cell carcinoma of the tongue. Strong immunopositivity for p53 is seen in the basal and parabasal cells. Infiltrations also show the same staining pattern. (Original magnification: panel A: 16 ×, panel B: 250 ×, panel C: 250 ×, panel D: 400 ×).

obvious that the regulatory system involved in the malignant transformation and progression of cancer is much more complex than described herein.

The mutational hot-spot for *p53* in tongue cancer seems to be exon 5, as reported in this study and by others [30]. This result is, however, somewhat controversial with reports of oral cancers from Asia and the U.S.A. [31, 32]. In those works, mutations in exons 6 and 8 seemed to be at least as common as in exons 5 and 7. Most of the mutations in a non-small cell lung cancer have also been found to lie in exon 5 [33]. Knowing that the non-small cell lung cancer is a tobacco-related disease, one could suppose that *p53* mutations in tongue cancer are also related to smoking. However, our results do not support the association between smoking and *p53* mutation. In a recent report, a correlation between smoking and *p53* mutations in head and neck cancers was found [34]. Most of their head and

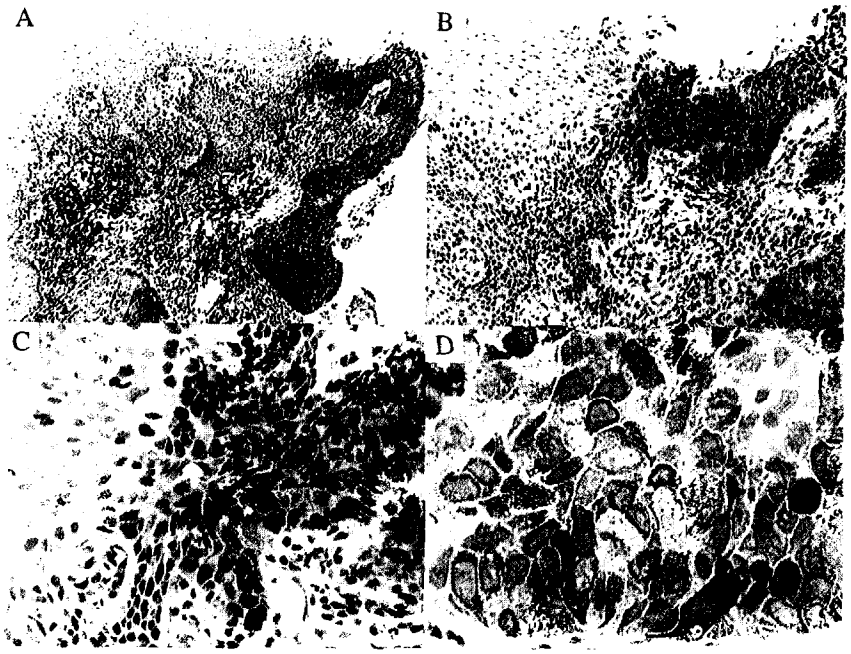

Fig. 4. Strong cytoplasmic staining for bcl-2 is detected in dysplastic and carcinomatous cells. The hyperplastic epithelium with mild dysplasia is totally negative. (Original magnification: panel A: 16 ×, panel B: 40 ×, panel C: 250 ×, panel D: 400 ×).

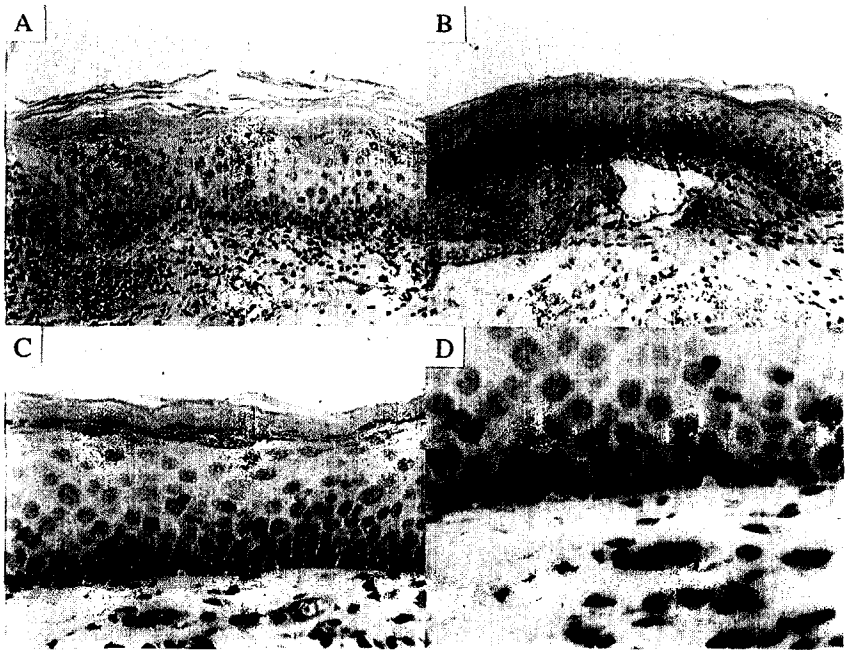

Fig. 5. Hyperkeratotic and atrophic epithelium adjacent to a carcinoma lesion. Part of the epithelium shows strong bcl-2 expression while other areas are totally negative. (Original magnification: panel A: 40 ×, panel B: 40 ×, panel C: 250 ×, panel D: 400 ×).

neck cancer patients presented with stage III-IV disease. All of our samples were collected from tongue cancer patients and about half of them had stage I-II disease, which could explain the differences in the results. Also, we could not find any association with p53 protein overexpression and smoking. Interestingly, Matthews *et al.* [14] found a negative relationship between smoking and p53 expression in tongue cancer patients. These contradictory results indicate that the interaction between smoking and *p53* is still unsettled in oral cancer.

This study included 5 patients who were both heavy-smokers and heavy alcohol consumers, and 4 of them had moderate to high expression of p53 in the immunohistochemical staining. Although the number of patients is too small for any definitive conclusions, it is possible that carcinogens of tobacco and alcohol might have a mutual effect on *p53* function. This is also supported elsewhere [35].

For further illumination of molecular changes involved in metastasising, it would be necessary to analyse the mutations

of metastatic lymph nodes. If original mutations also prevail in the neck metastases, their involvement in the spread of cancer might be more important than demonstrated in this study.

1. Lindberg RD. Distribution of cervical lymph node metastases from squamous cell carcinoma of the upper respiratory and digestive tract. *Cancer* 1972, **29**, 1446–1449.
2. Ildstad ST, Bigelow ME, Remensnyder JP. Squamous cell carcinoma of the tongue: a comparison of the anterior two thirds of the tongue with its base. *Am J Surg* 1983, **146**, 456–461.
3. Chang F, Syrjänen S, Syrjänen K. Implications of the *p53* tumor-suppressor gene in clinical oncology. *J Clin Oncol* 1995, **13**, 1009–1022.
4. Lowe SW, Bodis S, McClatchey A, *et al. p53* status and the efficacy of cancer therapy *in vivo*. *Science* 1994, **266**, 807–810.
5. Lowe SW, Ruley HE, Jacks T, Housman DE. *p53*-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993, **74**, 957–967.
6. Park N-H, Li S-L, Xie J-F, Cherrick HM. *In vitro* and animal studies of the role of viruses in oral carcinogenesis. *Oral Oncol, Eur J Cancer* 1992, **28B**, 145–152.
7. Ryan JJ, Prochownik E, Gottlieb CA, *et al. C-myc* and *bcl-2* modulate *p53* function by altering p53 subcellular trafficking during the cell cycle. *Proc Natl Acad Sci USA* 1994, **91**, 5878–5882.
8. Zariwala M, Schmid S, Pfaltz M, Ohgaki H, Kleihues P, Schäfer R. *p53* gene mutations in oropharyngeal carcinomas: a comparison of solitary and multiple primary tumours and lymph-node metastases. *Int J Cancer* 1994, **56**, 807–811.
9. Leedy DA, Trune DR, Kronz JD, Weidner J, Cohen JI. Tumor angiogenesis, the p53 antigen, and cervical metastasis in squamous cell carcinoma of the tongue. *Otolaryngol Head Neck Surg* 1994, **111**, 417–422.
10. Ahomadegbe JC, Barrois M, Fogel S, *et al.* High incidence of p53 alterations in head and neck primary tumors and metastases; absence of correlation with clinical outcome. Frequent protein overexpression in normal epithelium and in early non-invasive lesion. *Oncogene* 1995, **10**, 1217–1227.
11. Suzuki H, Takahashi T, Kuroishi T, *et al. p53* mutations in non-small cell lung cancer in Japan: association between mutations and smoking. *Cancer Res* 1992, **52**, 734–736.
12. Wang X, Christiani DC, Wiencke JK, *et al.* Mutations in the *p53* gene in lung cancer are associated with cigarette smoking and asbestos exposure. *Cancer Epidemiol Biomarkers Prev* 1995, **4**, 543–548.
13. Field JK, Spandidos JA, Malliri A, Gosney JR, Yiagnisis M, Stell PM. Elevated p53 expression correlates with a history of heavy smoking in squamous cell carcinoma of the head and neck. *Br J Cancer* 1991, **64**, 573–577.
14. Matthews JB, Scully C, Jovanovic A, Van der Waal I, Yeudall WA, Prime SS. Relationship of tobacco/alcohol use to p53 expression in patients with lingual squamous cell carcinomas. *Oral Oncol, Eur J Cancer* 1993, **29B**, 285–289.
15. Miyashita T, Krajewski S, Krajewska M, *et al.* Tumor suppressor *p53* is a regulator of *bcl-2* and *bax* gene expression *in vitro* and *in vivo*. *Oncogene* 1994, **9**, 1799–1805.
16. Lu Q-L, Elia G, Lucas S, Thomas JA. *Bcl-2* proto-oncogene expression in Epstein–Barr virus-associated nasopharyngeal carcinoma. *Int J Cancer* 1993, **53**, 29–35.
17. Ikegaki N, Katsumata M, Minna J, Tsujimoto Y. Expression of bcl-2 in small cell lung carcinoma cells. *Cancer Res* 1994, **54**, 6–8.
18. Yunis JJ, Mayer MG, Arnesen MA, Aeppli DP, Oken MM, Frizzera G. *Bcl-2* and other genomic alterations in the prognosis of large-cell lymphoma. *N Engl J Med* 1989, **320**, 1047–1054.
19. Bell DA, Liu Y, Cortopassi GA. Occurrence of bcl-2 oncogene translocation with increased frequency in the peripheral blood of heavy smokers. *J Natl Cancer Inst* 1995, **87**, 223–224.
20. Gallo O, Bianchi S, Porfirio B. Bcl-2 overexpression and smoking history in head and neck cancer. *J Natl Cancer Inst* 1995, **87**, 1024–1025.
21. Kurvinen K, Hietanen S, Syrjänen K, Syrjänen S. Rapid and effective detection of mutations in the *p53* gene using nonradioactive single-strand conformation polymorphism (SSCP) technique applied on PhastSystem™. *J Virol Meth* 1995, **51**, 43–54.
22. Moyret C, Theillet C, Puig PL, Moles JP, Thomas G, Hamelin R. Relative efficiency of denaturing gradient gel electrophoresis and single strand conformation polymorphism in the detection of mutations in exons 5 to 8 of the *p53* gene. *Oncogene* 1994, **9**, 1739–1743.
23. Sameshima Y, Matsuno Y, Hirohashi S, *et al.* Alterations of the *p53* gene are common and critical events for the maintenance of malignant phenotypes in small-cell lung carcinoma. *Oncogene* 1992, **7**, 451–457.
24. Pohl J, Goldfinger N, Radler-Pohl A, Rotter V, Schirrmacher V. *p53* increases experimental metastatic capacity of murine carcinoma cells. *Mol Cell Biol* 1992, **8**, 2078–2081.
25. Vogelstein B. A deadly inheritance. *Nature* 1990, **348**, 681–682.
26. Scarpa A, Capelli P, Mukai K, *et al.* Pancreatic adenocarcinomas frequently show *p53* gene mutations. *Am J Path* 1993, **142**, 1534–1543.
27. Kennedy SM, MacGeogh C, Jaffe R, Spurr NK. Overexpression of the oncoprotein p53 in primary hepatic tumors of childhood does not correlate with gene mutations. *Hum Pathol* 1994, **25**, 438–442.
28. Lu Q-L, Hanby AM, Hajibagheri MAN, *et al.* Bcl-2 protein localizes to the chromosomes of mitotic nuclei and is correlated with the cell cycle in cultured epithelial cell lines. *J Cell Sci* 1994, **107**, 363–371.
29. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, bax, that accelerates programmed cell death. *Cell* 1993, **74**, 609–619.
30. Burns JE, Baird MC, Clark LJ, *et al.* Gene mutations and increased levels of p53 protein in human squamous cell carcinomas and their cell lines. *Br J Cancer* 1993, **67**, 1274–1284.
31. Sakai E, Tsuchida N. Most human squamous cell carcinomas in the oral cavity contain mutated *p53* tumor-suppressor genes. *Oncogene* 1992, **7**, 927–933.
32. Brachman DG, Graves D, Vokes E, *et al.* Occurrence of *p53* gene deletions and human papilloma virus infection in human head and neck cancer. *Cancer Res* 1992, **52**, 4832–4836.
33. Mitsudomi T, Steinberg SM, Nau MM, *et al. p53* gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of *ras* mutations and clinical features. *Oncogene* 1992, **7**, 171–180.
34. Brennan JA, Boyle JO, Koch WM, *et al.* Association between cigarette smoking and mutation of the *p53* gene in squamous-cell carcinoma of the head and neck. *N Engl J Med* 1995, **332**, 712–717.
35. Field JK, Spandidos DA, Stell PM. Overexpression of *p53* gene in head-and-neck cancer, linked with heavy smoking and drinking. *Lancet* 1992, **339**, 502–503.

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