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Expression of biomarkers (p53, transforming growth factor alpha, epidermal growth factor receptor, c-erbB-2/neu and the proliferative cell nuclear antigen) in oropharyngeal squamous cell carcinomas

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Abstract

Using immunohistochemistry, expression of p53, transforming growth factor-alpha (TGF-α), epidermal growth factor receptor (EGFR), c-erbB-2/neu and proliferating cell nuclear antigen (PCNA) was examined in 26 fresh frozen tissue specimens of oropharyngeal squamous cell carcinomas (SCCs). *p53* gene mutations were examined by polymerase chain reaction (PCR)/DNA sequencing methods in 22 carcinomas. The findings were examined for correlations with patients' clinicopathological parameters. Expressions of p53 and PCNA were also examined in 21 formalin-fixed corresponding tissues. Of the fresh frozen tissue specimens, 77% (20/26) showed expression and 68% (15/22) showed mutations (substitutions) of the p53, with significant clustering of the mutations in exons 5 (8/22; 36%), 7 (4/22; 18%) and 8 (5/22; 23%). No mutations were found in exon 6. There was a discordance between expression of p53 protein and mutations of the gene. Parallel to expression and mutations of the p53 found in most of the specimens, expression of TGF-α, EGFR, c-erbB-2/neu and PCNA was found in 88% (22/25), 92% (23/25), 58% (14/24) and 91% (21/23) of the specimens, respectively. For the formalin-fixed tissue specimens, 62% (13/21) and 90% (19/21) expressed p53 and PCNA, respectively. Examining for correlations with patients' clinicopathological parameters, expression of p53, TGF-α, EGFR and c-erbB-2/neu seemed to negatively correlate with the increase of the tumour grade. The present work suggests that: (1) lack of negative growth regulation due to inactivation of the *p53* gene together with activation of other proto-oncogenes are necessary genetic events in the carcinogenesis of oropharyngeal SCCs; (2) in oropharyngeal SCCs, *p53* gene mutations were clustered in exons 5 (codons 130–186), 7 (codons 230–248) and 8 (codons 271–282) which perhaps suggests that tobacco carcinogens probably affect the mutational hot spots of the *p53* gene at codons 157, 175, 186, 248, 273 and 282; and (3) fresh frozen and formalin-fixed tissue specimens give similar results when an immunohistochemical method is applied. The importance of p53, TGF-α, EGFR, c-erbB-2/neu and PCNA as biomarkers in oropharyngeal SCCs deserves particular attention because it might offer further understanding of the development of these carcinomas.

Keywords: Squamous cell carcinoma; *p53*; TGF-α; EGFR; c-erbB-2/neu; PCNA; immunohistochemistry; PCR

1. Introduction

Worldwide, incidence of oral/pharyngeal squamous cell carcinoma (SCC) is on the increase [1, 2]. In the West, smoking of cigarettes and/or drinking of alcohol have been etiologically associated with the development of these carcinomas [1]. Carcinogenesis is a multi-step process that involves a number of aberrant genetic events. Among these, negative (tumour suppressor genes) and positive (oncogenes) regulators of the transformed state as well as growth factor pathway lesions have been suggested [3]. Tumour suppressor genes and oncogenes are thought to be susceptible to environmental carcinogens and mutagens [1, 3–5]. In carcinogenesis, activation

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of a single gene usually does not lead to immediate transformation, but it is likely that multiple activations and events are involved [1, 3–5].

Mutations of the *p53* gene have been found in more than 50% of diverse human tumour types [4]. These mutations induce changes, probably conformational, that prolong the half-life (6–24 h) of the p53 protein [4]. The resulting mutant protein accumulates within the nuclei of malignant cells in amounts that can be detected by immunohistochemistry. *p53* gene mutations have been reported in about 40–50% of oral/pharyngeal SCCs with codons 238–248 and 278–281 as probable hotspots for these mutations [6, 7]. A number of studies have examined association between expression and mutation of the p53 and different tobacco- and/or alcohol-related oral/pharyngeal SCCs [8–11]. p53 protein overexpression has been found to positively correlate with the patient's history of heavy cigarette smoking and/or alcohol drinking [8, 9], betel and tobacco chewing [10] and snuff dipping [11]. In patients with oral/pharyngeal SCCs, however, there is a great heterogeneity in relating the status of the p53 expression and mutation to clinical and/or histopathological parameters of these patients (reviewed in [5–7]). In fact, most of the published results relating to this are conflicting. Studies on correlation between p53 expression and tumour site, size, histopathological grading and/or patients age and/or sex have reported conflicting results (reviewed in [5–7]). Both no correlations and positive correlations have been reported (reviewed in [5–7]). Reports on the exact role played by expression and mutation of the p53 found in oral/pharyngeal SCCs in relation to patients' clinical and/or histopathological parameters were inconclusive [6, 7].

The transforming growth factor-alpha (TGF-α) is a member of a growth factor family of which the epidermal growth factor (EGF) was the first to be described [3, 12, 13]. Epithelial tumours often express the transmembrane glycoprotein receptor to TGF-α, namely the epidermal growth factor receptor (EGFR, or c-*erb*B-1) [3, 12, 13]. EGFR is a 170-kD transmembrane glycoprotein with tyrosine-specific protein kinase activity [3, 12, 13]. EGF and TGF-α interact with the target cell through binding to the EGFR [3, 12, 13]. The c-*erb*B-2/*neu*, mapped to human chromosome 17q21, encodes a 185-kD transmembrane glycoprotein with tyrosine kinase activity, and is closely related to the EGFR [3, 12, 13]. In vitro studies have suggested the existence of an integrating *erb* receptor family network claiming c-erbB-2/neu as the key molecule in receptor heterodimerization [14].

Several studies have examined overexpression of TGF-α, EGFR, c-erbB-2/neu in oral/pharyngeal SCCs in relation to patients' clinicopathological parameters, and have reported contradictory results [15–25]. A study of 91 cases of oral/pharyngeal SCCs has shown that expressions of TGF-α and EGF appear to be among the most important prognostic factors yet identified for patients with these carcinomas [17]. Another study on 68 cases of laryngeal carcinoma has found that the survival rate in patients whose tumours expressed EGFR and TGF-α was significantly lower compared to that found in patients whose tumours were EGFR and TGF-α negative [18]. On the contrary, a study of 43 cases of oral/pharyngeal SCCs has suggested that increased production of TGF-α and EGFR in the carcinomas might serve as a marker for tumour progression [19]. However, simultaneous expression of TGF-α and EGFR was only found in three tumours, which harboured the highest levels of EGFR expression [19]. It was then concluded that simultaneous expression of TGF-α and EGFR indicate poor prognosis, prompting the suggestion that increased production of these growth factors in oral/pharyngeal SCCs might serve as a target for therapy [19]. On the other hand, overexpression of EGFR alone has also been suggested to be of prognostic value for predicting either shorter disease-free survival or shorter overall survival in oesophageal carcinoma [20]. A significant positive association between expression of c-erbB-2/neu and tumour progression in cases of oral SCCs has been reported [26]. Furthermore, it has also been suggested that overexpression of the c-erbB-2/neu in oral SCCs was correlated negatively with survival, thus indicating its importance to serve as biomarker for poor prognosis [27]. Other studies have reported that expression of c-erbB-2/neu in oral/pharyngeal SCCs did not correlate with patients' survival [28, 29].

The proliferating cell nuclear antigen (PCNA) plays an important role in nucleic acid metabolism, DNA replication, DNA excision repair, chromatin assembly, and in several instances it has been shown to be involved in RNA transcription [30]. In immunohistochemistry, expression of PCNA is used as a marker to study the state of cell proliferation in normal and neoplastic tissues [30].

Previously, it has been suggested that overexpression of p53, TGF-α, EGFR and c-erbB-2/neu in oral/pharyngeal SCCs in relation to patients' clinicopathological parameters remained contradictory and controversial [6, 7, 15–25]. There are also wide variations in the incidence of biomarkers overexpression, intensity and staining localisation. Thus, further studies are needed on these biomarkers in oral/pharyngeal SCCs for uniformity and consistency. This might enhance our understanding of their role in regulation of cell proliferation during carcinogenesis and tumour progression. To better understand the relative importance of these biomarkers in the development of oral/pharyngeal SCCs, it was therefore the objective of the present work to: (1) examine the expression and mutation (using PCR/DNA sequencing methods) of p53, and the expression

of TGF-α, EGFR, c-erbB-2/neu and PCNA using avidin–biotin immunohistochemistry in fresh frozen tissue specimens of oral/pharyngeal SCCs; (2) to examine if there are correlations between expression of these biomarkers and/or patients' clinicopathological parameters, history of cigarette smoking and/or alcohol drinking; and (3) compare results obtained by immunohistochemistry on expression of p53 and PCNA in fresh frozen tissue with those in corresponding formalin-fixed ones.

2. Materials and methods

2.1. Patients and tissue specimens

From the period September 1991 to November 1993, surgical fresh biopsy tumour samples (approximately 0.5–1 cm^3) were obtained from 26 consecutive patients with previously untreated oral/pharyngeal SCCs operated on at the Department of Otolaryngology/Head & Neck Surgery at the Haukeland University Hospital, Bergen, Norway. There were 18 males and eight females (age range 34–82 years, mean 65.6 ± 2.5 SE, SD 12.83 years). The diagnosis of the tumours was based on clinical examination and histopathological analysis of the tissue specimens. All tumours were staged following the 1987 UICC staging system. Accordingly, five tumours were T1, seven tumours were T2 and 14 tumours were T3 (Table 1). Regional lymph node involvement in the neck was found in eight patients, while evidence for distant metastasis was found in only one patient. Using lesional tissue sections including also adjacent epithelium from those selected for the current study, all the cases had their histopathologic diagnosis confirmed by one of the authors (A.C.J.) using cryostat sections of all biopsies stained with haematoxylin and eosin (H&E), at the Department of Odontology–Oral Pathology and Forensic Odontology at the Haukeland University Hospital, Bergen, Norway. The tumours were histologically graded as highly, moderately or poorly differentiated according to Cawson and Eveson [31] (Table 1). Tissue specimens were obtained at the time of biopsy or primary surgery and were always taken from the interface between the tumour and normal tissue using a knife and a scalpel. The tumour tissue samples were bisected (approximately 0.2–0.5 cm^3). One-half was fresh frozen in isopentane pre-chilled in liquid nitrogen and stored at $-70°C$. The other half was used for diagnostic reporting following fixation in 10% buffered formalin, and routine processing. The samples consisted of SCCs from oral (15 cases), laryngeal (seven cases) and pharyngeal (four cases). The various subsites of oral carcinomas included: gingiva (four cases), tongue (seven cases), floor of the mouth (two cases) and hard palate (two cases). Fresh punch biopsy specimens of normal oral buccal mucosa were obtained from five volunteer subjects. All the normal control biopsy specimens were treated in the same way, as were the tumour specimens.

From the stored fresh frozen tissue samples, several sections (6 μm thick) were prepared, mounted on glass slides and stored at $-20°C$ until use. Twenty-one corresponding formalin-fixed tissue carcinoma specimens were available from the 26 frozen tissue specimens. From these formalin-fixed tissues, several sections (6 μm thick) were also prepared and stored until use. For the 26 patients, only data on anatomical site of the carcinoma, histopathological diagnosis, clinical characteristics, history of tobacco use and/or drinking of alcohol were available (Table 1). Smoking was quantified by number of cigarettes smoked per day, and accordingly patients were grouped into non-smokers, light smokers (1–10 cigarettes per day) moderate smokers (11–20 cigarettes per day) and heavy smokers (>20 cigarettes per day) (Table 1). Data were also collected on cigar, pipe and snuff use. For alcohol drinking, patients were grouped into non-drinkers, light drinkers (1–2 drinks per day), moderate drinkers (3–5 drinks per day) and heavy drinkers (>5 drinks per day) (Table 1).

2.2. Immunohistochemistry

The available numbers of the fresh frozen tissue sections investigated for each biomarker were not equal. Two standard protocols previously described from our laboratory were used. The avidin–biotin–peroxidase complex (ABC) protocol used for fresh frozen tissue immunohistochemistry and that used for formalin-fixed tissue immunohistochemistry have been described in detail elsewhere [32, 33]. The monoclonal antibodies (MAbs) used, their sources, dilutions and incubation times are summarised in Table 2.

As positive controls, fresh frozen tissue specimens of papillary thyroid carcinomas ($n=5$) previously found positive for TGF-α, EGFR and c-erbB-2/neu were included. In addition, formalin-fixed, paraffin-embedded carcinomas of the uterine cervix ($n=5$) previously found positive for p53 and PCNA were included.

For each specimen (fresh frozen and formalin-fixed), negative control incubations were performed where the primary antibody was replaced with phosphate-buffered saline (PBS) and/or normal rabbit serum, normal mouse serum of the same isotype and application of the ABC substrate alone (to determine the quenching of endogenous peroxidase activity).

2.3. Evaluation of the immunohistochemistry

Whole-tissue sections (including epithelium adjacent to the non-malignant lesional area, pre-malignant and malignant areas when present in the specimen) were examined with a light microscope at a final magnification

Table 1
Patient's clinicopathological parameters and immunohistochemical expression of p53, transforming growth factor-alpha (TGF-α), epidermal growth factor receptor (EGFR), c-erbB-2/neu and proliferating cell nuclear antigen (PCNA) in the oropharyngeal squamous cell carcinomas (SCCs)

Patient clinicopathological parameters								Immunohistochemical expression and distribution of the intensity of staining						
Tumour No.	Site	H.D.	TNM	T.G.	Cigarette smoking	Other tobacco	Alcohol consumption	Fresh frozen tissue specimens					Formalin-fixed tissue specimens	
								p53	TGF-α	EGFR	c-erbB-2/neu	PCNA	p53	PCNA
1	La	H	T1N0M0	I	NS	–	ND	+, c	+, b	+, a	+, b	+, b	+, b	+, c
2	La	P	T3N3M1	III	HS	–	HD	–	+, a	–	–	–	–	–
3	La	H	T1N0M0	I	HS	–	nd	+, c	+, b	+, b	+, b	+, b	nd	nd
4	La	M	T2N0M0	II	MS	Snuff	MD	+, a	+, a	+, a	nd	+, b	nd	nd
5	La	P	T3N0M0	III	MS	–	nd	–	+, b	+, b	+, b	+, c	–	+, b
6	La	H	T1N0M0	I	HS	–	LD	+, b	nd	nd	nd	nd	nd	nd
7	La	M	T2N0M0	II	NS	–	nd	–	+, c	+, c	–	+, b	–	+, b
8	Ph	P	T3N1M0	III	HS	–	LD	+, c	+, a	+, b	+, b	+, b	+, a	+, c
9	Ph	P	T3N1M0	III	HS	–	LD	+, b	+, c	+, a	+, c	+, b	+, b	+, b
10	Ph	P	T3N0M0	III	MS	–	MD	+, b	+, a	+, b	–	+, a	+, a	+, b
11	Ph	P	T3N1M0	III	MS	–	MD	+, a	+, a	+, a	–	+, c	+, b	+, c
12	Or	P	T3N0M0	III	LS	–	ND	+, c	+, b	+, a	+, a	+, c	+, a	+, b
13	Or	H	T1N0M0	I	HS	–	nd	+, a	+, a	+, b	+, c	+, b	+, c	+, c
14	Or	M	T2N0M0	II	NS	–	ND	+, b	+, c	+, a	–	+, b	+, b	+, b
15	Or	P	T3N0M0	III	NS	–	nd	+, c	–	+, a	+, a	+, c	+, a	+, b
16	Or	M	T2N0M0	II	NS	–	ND	+, b	+, a	+, c	+, a	+, b	+, c	+, b
17	Or	M	T2N0M0	II	NS	–	ND	+, c	+, b	+, a	+, b	+, b	–	+, c
18	Or	M	T2N0M0	II	NS	–	ND	–	+, a	+, b	+, a	+, c	–	+, b
19	Or	P	T3N0M0	III	HS	–	HD	+, a	+, c	+, a	+, b	+, b	+, c	+, b
20	Or	P	T3N0M0	III	HS	–	LD	–	+, a	+, c	–	+, b	+, a	+, c
21	Or	P	T3N1M0	III	HS	–	LD	+, c	+, b	+, a	+, b	+, c	+, b	+, b
22	Or	M	T2N0M0	II	HS	Snuff	nd	+, c	–	+, a	–	+, b	–	+, c
23	Or	P	T3N1M0	III	HS	–	ND	+, a	+, b	+, b	+, a	nd	nd	nd
24	Or	P	T3N2M0	III	HS	–	nd	–	–	–	–	+, b	–	+, c
25	Or	H	T1N0M0	I	LS	–	nd	+, a	+, b	+, a	–	–	–	–
26	Or	P	T3N1M0	III	NS	Pipe	ND	+, a	+, a	+, b	–	nd	nd	nd
Total %								77% (20/26)	88% (22/25)	92% (23/25)	58% (14/24)	91% (21/23)	62% (13/21)	90% (19/21)

Anatomical site: La, larynx; Ph, pharynx; Or, oral; H.D., histological differentiation; H, high; M, moderate; P, poor; TNM; tumour grading Broder's classification system; T.G., tumour grade; HS, heavy smoker; MS, moderate smoker; LS, light smoker; NS, non-smoker; HD, heavy drinker; MD, moderate drinker; LD, light drinker; ND, non-drinker; nd, not done. Assessment of cigarette smoking and alcohol drinking is presented in the Materials and methods section. Staining intensity: a, weak; b, moderate; c, intense.

Table 2
The primary monoclonal antibodies (MAbs) used, their dilutions, times of incubations and sources

Antigen	Mouse MAb	Dilution[a]/times of incubation (min) at room temperature	Source
p53	1801	1:1000/90/overnight	Oncogene Science®, Mannhasset, USA
p53	DO-7	1:500/90/overnight	DAKO®, Copenhagen, Denmark
p53	DO-1	1:1000/90 min/overnight	Santa Cruz Biotechnology®, USA
TGF-α	MAb Ab-2	1:50/60	Oncogene Science®, Mannhasset, USA
EGFR	RPN.513	1:20/60	Amersham®, Bucks, UK
c-*erb*B2/c-*neu*	Anti-(Ab-3)*erb*B-2	1:40/60	Oncogene Science®, Mannhasset, USA
PCNA	PC10	1:1000/60/overnight	Santa Cruz Biotechnology®, Santa Cruz, CA, USA

TGF-α, transforming growth factor-alpha; EGFR, epidermal growth factor receptor; PCNA, proliferating cell nuclear antigen.
[a] Diluted in phosphate-buffered saline (PBS) containing 5% bovine serum albumin.

of ×400 for p53 and PCNA-positive nuclear staining. For the TGF-α, EGFR and c-erbB-2/neu, the staining was also assessed in the tissue sections at the same magnification by determining its presence (membrane, cytoplasmic and/or mixed membrane/cytoplasmic) or absence. For all the biomarkers investigated, the staining was recorded as positive (+, >10% of the entire tumour cells positive) and as negative (−, <10% positive cells). The intensity of staining for all the biomarkers examined was scored as: (a) weak, (b) moderate and (c) intense staining.

2.4. DNA extraction

Of the 26 fresh frozen tissue carcinomas investigated, specimens from 22 were available for total cellular DNA extraction which was carried out using proteinase K (Sigma, St. Louis, MO, USA) and phenol–chloroform, using a standard protocol as described in detail elsewhere [34]. Purified DNA was quantified spectrophotometrically using a spectrophotometer (Beckman, Fullerton, CA, USA DU· 530, Life Science).

2.5. Polymerase chain reaction (PCR)

Exons 5–9 of the *p53* gene were amplified in vitro from each tumour using primers described earlier by Ryberg et al. [35]. The primer sequences and PCR fragment sizes were as follows: exon 5 (299 bps): 5′TTCAACTCTGTCTCCTTCCT3′ (sense), 5′GCAATCAGTGAGGAATCAGA3′ (antisense); exon 6 (224 bps): 5′TGGTTGCCCAGGGTCCCCAG3′ (sense), 5′TGTGAGGGCCACTGACAACCA3′ (antisense); exon 7 (218 bps): 5′AGGCGCACTGGCCTCATCTT3′ (sense), 5′AGGGGTCAGCGGCAAGCAGA3′ (antisense); exons 8/9 (445 bps): 5′TTGGGAGTAGATGGAGCCT3′ (sense), 5′AGTGTTAGACTGGAAACTTT3′ (antisense). The primers were obtained from MedProbe, Oslo, Norway. The PCR was carried out in the GeneAmp® PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA). The 50-μl PCR-mixture reaction consisted of 1 μl genomic DNA solution, 200 μM of each of the four deoxynucleotide triphosphates (dNTPs), 0.25 U of AmpliTaq®Gold DNA polymerase (5 U/μl, PE Applied Biosystems, Foster City, CA, USA), 1.25 (for primer exon 5), 1 (for primer exons 6 and 7) and 2.5 (for primer exons 8/9) mM $MgCl_2$, respectively, and 10 pmol of each of the primers. A 'hot start' at 94°C for 10 min was followed by 40 cycles of amplification, where each cycle consisted of 40 s denaturation at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C. The last PCR cycle was followed by a final extension at 72°C for 7 min. Human placental DNA was used as normal control. PCR reactions without DNA as a template were included as negative controls. Determination of the PCR amplification product was done by electrophoresis and staining with 0.5 μg/ml ethidium bromide in a 1.5% agarose gel, and the amplified samples were recorded. Before further analysis, PCR amplification products were purified using either the QIAquick Gel Extraction Kit or the QIAquick PCR Purification Kit Protocols using a microcentrifuge as described in QIAquick Spin Handbook (QIAGEN®, QIAquick™, QIAvac).

2.6. DNA sequencing

After the initial PCR amplification analysis of all the tumours, purified product of only the tumours that showed amplification reaction with the PCR were subjected to sequencing in a non-radioactive cyclic sequencing reaction carried out following the manufacturer's protocol described for the commercially available kit: the ABI PRISM™ BigDye Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). In this 10-μl PCR-mixture reaction, 2 μl from the purified PCR products of the tumour, 4 μl from the BigDye reaction mixture and 1.5 pmol from the sense or antisense primers of the PCR used for exons 5, 7 and 8/9 as described above, were amplified. Sequencing was done on the automated ABI PRISM™ 377 DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA). The software packages Vector NTI 5.1 and Align X 1.0 (InforMax BioSuite™) version 5.0 for Windows™ (InforMax, Inc., North Bethesda, USA) were used to analyse the results of sequencing.

2.7. Statistical analysis

Either Chi-square or two-tailed Fischer's exact tests (at $p < 0.05$ significance levels) were used to examine the association between expression of biomarkers: p53, TGF-α, EGFR, c-erbB-2/neu and the PCNA gene products in the fresh frozen tissue samples of the oral/pharyngeal SCCs, and the patients' clinicopathological parameters, history of cigarette smoking and drinking of alcohol (separate and in combination).

3. Results

3.1. Immunohistochemistry

3.1.1. Expression of p53

A summary of the expression/intensity of staining for the nuclear p53 (Fig. 1A) found in the fresh frozen and formalin-fixed tissue carcinoma specimens is shown in Table 1. Both fresh frozen and formalin-fixed carcinomas showed expression of the p53 in the periphery of the carcinoma cell nests located in its most invasive regions, and in the dysplastic cells of the basal region of the squamous epithelium. This finding was most prominent in the highly differentiated carcinomas, but for the low differentiated ones, heterogeneous intense staining was found distributed throughout the carcinoma cell nests. All the areas with cells that showed expression of p53 in the formalin-fixed tissue carcinomas were found comparable to those found in the fresh frozen ones regarding location, intensity as well as pattern of staining. The normal epithelium located adjacent to the carcinomas in the fresh frozen and formalin-fixed tissue carcinomas as well as the five normal control oral mucosal epithelial specimens were negative for p53.

3.1.2. Expression of TGF-α

A summary of the expression/intensity for the cytoplasmic TGF-α (Fig. 1B) found in the fresh frozen tissue carcinoma specimens is shown in Table 1. In 17 out of the 22 carcinomas that showed expression of TGF-α, additional weak staining for TGF-α was also seen in the inflammatory cell infiltrate. The normal epithelium located adjacent to the carcinomas as well as the five normal control oral mucosal epithelial specimens showed weak cytoplasmic staining in the basal/intermediate cell layers of the epithelium. When found in the normal epithelium, expression of TGF-α varied in all the layers of the epithelium.

3.1.3. Expression of EGFR

A summary of the expression/intensity of staining for the membrane EGFR (Fig. 1C) found in the fresh frozen tissue carcinoma specimens is shown in Table 1. In

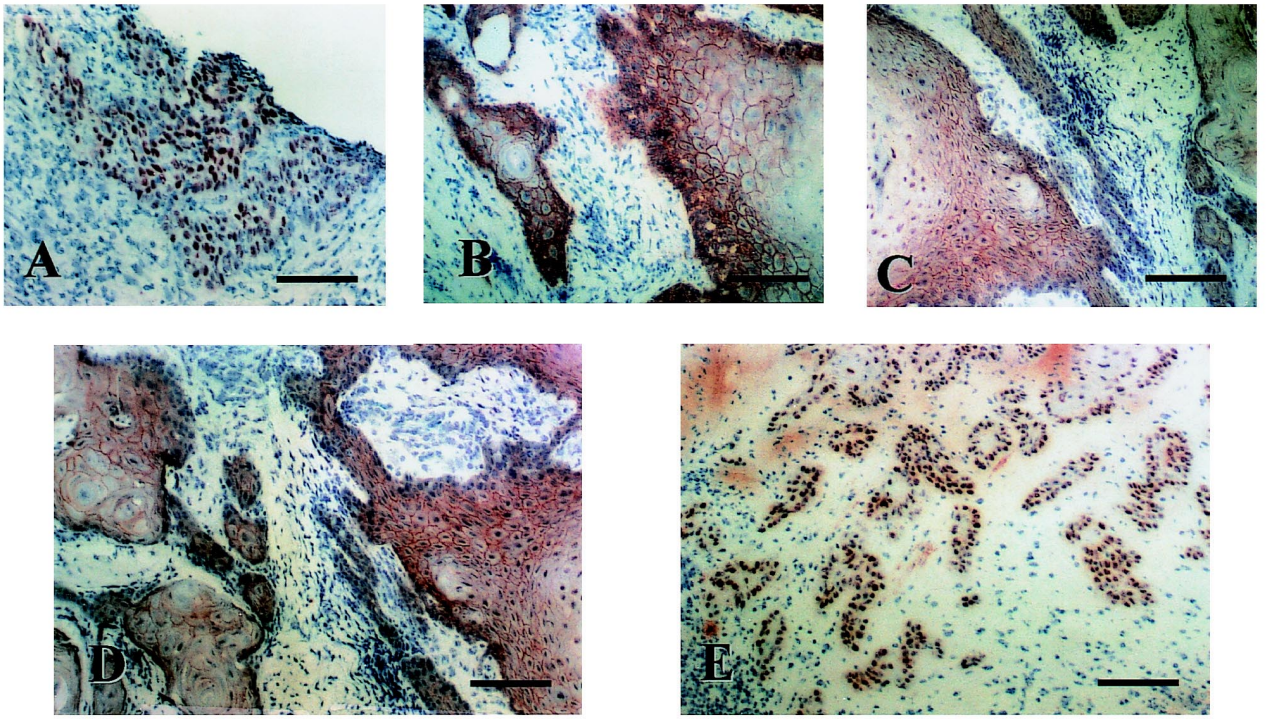

Fig. 1. Immunohistochemical detection of (A) p53, (B) TGF-α, (C) EGFR, (D) c-erbB-2/neu and (E) PCNA in a fresh frozen tissue specimen of oropharyngeal squamous cell carcinoma (SCC) (×250).

nine out of the 23 carcinomas that showed expression of EGFR, weak membrane staining was also found in the basal/intermediate cell layers of the adjacent normal epithelium. In the low and moderately differentiated carcinomas, more pronounced expression of EGFR was found in nearly all the basal epithelial cell layers and tumour tissues. With the increase of the tumour grade, expression of the EGFR confined to the basal or suprabasal cell layers of the epithelium. The five normal control oral mucosal epithelial specimens showed weak membrane staining of EGFR in the basal/intermediate cell layers of the epithelium.

3.1.4. Expression of c-erbB-2/neu

A summary of the expression/intensity of staining of mixed membrane/cytoplasmic c-erbB-2/neu (Fig. 1D) found in the fresh frozen tissue carcinoma specimens is shown in Table 1. In all the carcinomas that showed expression of c-erbB-2/neu, except for two cases, the expression was found in the same areas of the carcinomas that showed expression of the EGFR. However, the total cell areas of the carcinomas that showed expression of the c-erbB-2/neu were smaller when compared to those which showed expression of the EGFR in the same carcinomas. The five normal control oral mucosal epithelial specimens showed weak mixed membrane/cytoplasmic staining of the c-erbB-2/neu in the basal/intermediate cell layers of the epithelium.

3.1.5. Expression of PCNA

A summary of the expression/intensity of staining of nuclear PCNA (Fig. 1E) found in the fresh frozen and formalin-fixed tissue carcinoma specimens is shown in Table 1. Within each tumour, the intensity of PCNA staining ranged from intense to weak. Both normal, dysplastic and malignant epithelium in the fresh frozen and formalin-fixed tissue specimens showed nuclear staining of PCNA in large areas of the basal cell layers of the epithelium. PCNA staining in the formalin-fixed tissues followed the same pattern as that found in the fresh frozen ones.

The possible correlations between expression of the biomarkers investigated and tumour site, tumour grade and patient's history of cigarette smoking and/or alcohol drinking in the fresh frozen tissue carcinomas was examined. The data (Table 3) showed no statistically significant correlations to tumour site, tumour grade, history of cigarette smoking and drinking of alcohol alone, or combined history of cigarette smoking and drinking of alcohol ($p > 0.05$). The expression of these biomarkers (except for the

Table 3
Proportions of the positive findings on the expression of p53, transforming growth factor-alpha (TGF-α), epidermal growth factor receptor (EGFR), c-erbB-2/neu and proliferating cell nuclear antigen (PCNA) in the oropharyngeal squamous cell carcinomas (SCCs) according to tumour site and grade, and patients' history of cigarette smoking and/or alcohol drinking

	p53	TGF-α	EGFR	c-erbB-2/neu	PCNA
Tumour site					
Oral ($n = 15$)	12/15 (80%)	14/15 (93%)	9/15 (60%)	12/15 (80%)	12/15 (80%)
Larynx/pharynx ($n = 11$)	8/11 (72%)	10/11 (91%)	5/9 (56%)	10/10 (100%)	9/11 (82%)
	$p = 1.00$	$p = 1.00$	$p = 1.00$	$p = 0.25$	$p = 1.00$
Tumour grade					
I/II ($n = 12$)	10/12 (83%)	10/11 (91%)	11/11 (100%)	6/10 (60%)	10/11 (91%)
III ($n = 14$)	10/14 (71%)	12/14 (86%)	12/14 (86%)	8/14 (57%)	11/12 (92%)
	$p = 0.65$	$p = 1.00$	$p = 0.49$	$p = 1.00$	$p = 1.00$
Cigarette smoking					
NS ($n = 8$)	6/8 (75%)	7/8 (88%)	8/8 (100%)	5/8 (63%)	7/7 (100%)
LS/MS ($n = 6$)	5/6 (83%)	6/6 (100%)	6/6 (100%)	2/5 (40%)	5/6 (83%)
HS ($n = 12$)	9/12 (75%)	9/11 (82%)	9/11 (82%)	7/11 (64%)	9/10 (90%)
	$p = 0.91$	$p = 0.54$	$p = 0.25$	$p = 0.64$	$p = 0.55$
Alcohol drinking					
ND ($n = 8$)	7/8 (88%)	8/8 (100%)	8/8 (100%)	6/8 (75%)	6/6 (100%)
LD ($n = 5$)	4/5 (80%)	4/4 (100%)	4/4 (100%)	3/4 (75%)	4/4 (100%)
MD/HD ($n = 5$)	4/5 (80%)	5/5 (100%)	4/5 (80%)	1/4 (25%)	4/5 (80%)
	$p = 0.91$	–	$p = 0.28$	$p = 0.20$	$p = 0.34$
Cigarette smoking and alcohol drinking					
NS and ND ($n = 6$)	5/6 (83%)	6/6 (100%)	6/6 (100%)	4/6 (67%)	5/5 (100%)
LS and ND/MS and MD ($n = 4$)	4/4 (100%)	4/4 (100%)	4/4 (100%)	1/3 (33%)	4/4 (100%)
HS and ND/HS and LD ($n = 6$)	5/6 (83%)	5/5 (100%)	5/5 (100%)	4/5 (80%)	4/4 (100%)
HS and HD ($n = 2$)	1/2 (50%)	2/2 (100%)	1/2 (50%)	1/2 (50%)	1/2 (50%)
	$p = 0.49$	–	$p = 0.47$	$p = 0.59$	$p = 0.073$

HS, heavy smoker; MS, moderate smoker; LS, light smoker; NS, non-smoker; HD, heavy drinker; MD, moderate drinker; LD, light drinker; ND, non-drinker.

PCNA) seemed to negatively correlate with the tumour grade, but with no statistically significant differences ($p > 0.05$).

3.2. DNA sequencing analysis

A summary of the substitution mutations (transitions and transversions) of the *p53* gene found in the oral/pharyngeal SCCs investigated is shown in Table 4. An example (in exon 5) of the sequencing results for both (1) the normal placental DNA and (2) the tumour DNA is illustrated in Fig. 2. Of the 22 carcinomas investigated, 68% (15/22) showed mutations in exons 5 (8/22; 36%), 7 (4/22; 18%), 8 (5/22; 23%) and 9 (2/22; 9%) (Table 4). No mutations were found in exon 6. The incidence of *p53* mutations was found higher in laryngeal tumours (5/6; 83%) compared to those of pharyngeal (2/3; 67%) and/or oral ones (8/13; 62%). However, the association was not statistically significant ($p > 0.05$). The incidence of *p53* mutations was found to be greater in higher grade (III) tumours (8/15; 53%) compared to lower grade (I/II) tumours (7/15; 47%), but this difference was also not statistically significant ($p > 0.05$). There were 22 substitution mutations found in the carcinomas investigated. These 22 mutations were distributed as 13 (59%) transversions (three A→T in exons 7 and 9, two C→A in exons 5 and 7, three T→G in exon 5, one A→C, one C→G and one T→A in exon 5, two G→T in exons 5 and 8), nine (41%) transitions (three G→A in exons 7 and 8; four C→T in exons 5 and 8; two T→C in exons 5 and 7).

3.3. Relationship between p53 mutations and history of cigarette smoking and/or alcohol drinking

In tumours from three patients (Tables 1 and 4) there were three G:C→A:T mutations. In two of these three patients (one heavy smoker/light drinker, one non-smoker/non-drinker), these *p53* gene mutations were found at the CpG sites (248 and 273) (Tables 1 and 4). In the third patient (heavy smoker/light drinker), *p53* gene mutation was found at a non-CpG site (codon 271) (Tables 1 and 4). In tumours from two other patients (one moderate smoker/moderate drinker/snuff dipper, one heavy smoker/snuff dipper), *p53* gene mutations were found at the CpG sites (157, 186, respectively). There were also four C:G→T:A transitions in tumours from four patients (one non-smoker, one heavy smoker/light drinker, one moderate smoker/moderate drinker,

Table 4
Mutations of the *p53* gene (exons 5, 7–9) found in the oropharyngeal squamous cell carcinomas (SCCs)

Tumour No.	Exon 5 codon/mutation	Exon 7 codon/mutation	Exon 8 codon/mutation	Exon 9 codon/mutation	Expression and intensity of staining for p53 protein
1	–	–	–	–	+, c
2	182/TGC→CGC	–	–	–	–
4	157/GTC→GTA	–	–	–	+, a
5	–	–	–	–	–
6	–	230/ACC→TCC	271/GAG→AAG	–	+, b
7	130/CTC→GTC 142/CCT/CTT	–	–	–	–
8	–	248/CGG→CGA (S)	–	–	+, c
10	147/GTT→GTG (S)	–	282/CGG→TGG	–	+, b
11	–	–	–	–	+, a
12	–	–	271/GAG→TAA[a]	–	+, c
13	166/TCA→TCC	–	–	–	+, a
14	147/GTT→GTG (S)	–	273/CGT→CAT	–	+, b
15	–	236/TAC→CAC	–	312/ACC→TCC	+, c
17	–	–	282/CGG→TGG	–	+, c
18	–	–	–	–	–
19	–	–	–	–	+, a
20	–	–	–	320/AAG→TAG[a]	–
21	174/AGG→AGT 175/CGC→CGT	247/AAC→AAA	–	–	+, c
22	147/GTT→GTG (S) 186/GAT→GAA	–	–	–	+, c
23	–	–	–	–	+, a
25	–	–	–	–	+, a
26	–	–	–	–	+, a
Total %	36% (8/22)	18% (4/22)	23% (5/22)	9% (2/22)	77% (17/22)

All tumours from Table 1 (except tumours no. 3, 9, 16 and 24) were investigated for *p53* gene mutations. (S), silent.
[a] Stop codon.

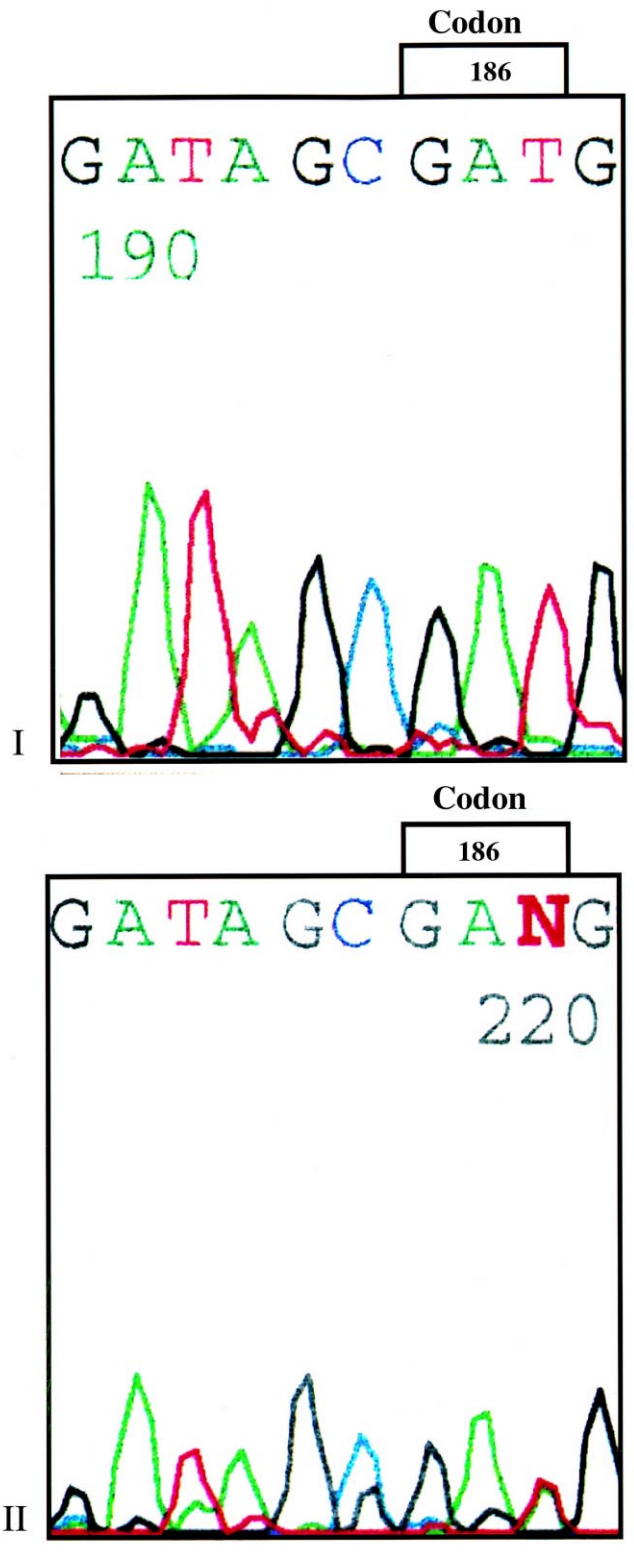

Fig. 2. An example of polymerase chain reaction (PCR) sequence analysis for a squamous cell carcinoma (SCC) and normal DNA in exon 5. ABI PRISM™ electrophotograms of normal DNA (I) and carcinoma DNA (II) are shown in 5′→3′ (sense) directions. Multiple alignments of the tumour mutation has shown T→A transversion (Asp–Glu) in codon 186.

one non-smoker/non-drinker). These transition mutations were found at codon 142, and CpG sites 175 and 282, respectively (Tables 1 and 4). In only two patients (one light smoker, one heavy smoker/light drinker), we found two G:C→T:A transversions, one at codon 174 and other at codon 271 (Tables 1 and 4).

4. Discussion

The *p53* tumour suppressor gene plays a fundamental role in the development of human tumours. Many other proto-oncogenes including the *erb*B family, are involved in growth regulation, and when expressed wrongly, generate growth signals that might prevail other cellular controls. Immunohistochemical markers have been found helpful in routine histopathological evaluation of oral/pharyngeal SCCs. The present study was undertaken to examine the expression of five immunohistochemical markers (p53, TGF-α, EGFR, c-erbB-2/neu and PCNA) in oral/pharyngeal SCCs. The hypothesis was that oropharyngeal SCCs demonstrating lack of negative growth regulation due to inactivation of p53 (stable protein and/or mutated gene), might also demonstrate activation of some other proto-oncogenes involved in the process of carcinogenesis. If this prevails, it might lead to a better understanding of the development of these carcinomas and may emerge as a useful prognostic indicator. We found high and similar levels of expression of p53 protein (77%; 62%) in both fresh frozen and formalin-fixed tissues of the oral/pharyngeal SCCs examined by anti-p53 MAbs 1801, DO-7 and/or DO-1. There were no differences in the expression of p53 between fresh frozen and formalin-fixed tissue specimens. These findings agree with other studies on oral/pharyngeal SCCs [36, 37]. We also found that immunolocalization of the p53 protein staining was similar to that of the PCNA, indicating that p53 protein expression was found increased in areas with proliferative activity. This might indicate involvement of the mutated form of the p53 protein in the alteration of the cell cycle regulation process. In our study, p53-positive immunoreaction was found in the periphery of the tumour cell nests and in the dysplastic cells of the basal region of the squamous epithelium. Both of these are considered to be areas of active cell division. This might support the suggestion of a correlation between positive p53 staining and proliferative activity of these cells as reported by others [38, 39].

There is a prolonged dispute as to whether p53 expression detected by immunohistochemistry harbours a mutated event (reviewed in [6, 7]). PCR/DNA sequencing methods were used to analyse *p53* gene mutations in 22 matched carcinomas (Table 4). For these carcinomas, 17 were positive for p53 expression with immunohistochemistry and five were negative. However, mutations

in the *p53* gene were found in only 11 out of the 17 carcinomas that showed expression of the p53 protein. In the remaining five carcinomas which were negative for p53 expression, three showed mutations in the *p53* gene (Tables 1 and 4). These findings suggest that use of immunohistochemistry can provide indication for presence of mutation to a reasonable degree. Thus, results of p53 expression require further analysis by molecular techniques. *p53* gene mutations were detected in specimens that appeared negative by immunohistochemistry in our study. This might be related to presence of point mutations leading to increased instability of the p53.

In human tumours, most of the *p53* gene mutations occur in the highly conserved regions (exons 5–9) of the gene [6, 7, 40]. Epidemiological data from the West have linked cigarette smoking and/or drinking of alcohol to the development of oral/pharyngeal SCCs [1, 40]. Differences in p53 expression and mutation found in oral/pharyngeal SCCs with or without history of tobacco use and/or drinking of alcohol have been reported [6, 7, 40]. In our study, some of the *p53* gene mutations found were associated with exposure to tobacco and alcohol. In addition, locations of the changes within the *p53* gene regarding the CpG and non-CpG sites were also associated with exposure to tobacco and alcohol. In tumours from some patients who neither smoked nor drank, we also found mutations at the CpG sites, and other mutations associated with cigarette smoking. The total number of carcinomas investigated in the present study ($n = 22$) was relatively small compared to those investigated by others [8, 9, 36, 37]. Nevertheless, our findings support the suggestion that tobacco carcinogens probably affect the CpG sites in the mutational hot spots of exons 5 (codons 157, 175, 186), 7 (codon 248) and 8 (codons 273 and 282) of the *p53* gene [6, 8, 9, 40]. These observations, however, must be regarded as preliminary and need to be further clarified by examining sufficiently large patient cohorts to determine the significance of the *p53* status and tobacco in carcinogenisis of oral/pharyngeal SCCs. In this study, some of the tumours showed multiple mutations in the *p53* gene (Table 4), which is in line with other studies [41, 42]. In one of the tumours (No. 2), no p53 protein expression or mutations were found in the tumour metastasis.

In this work, in all areas of malignancy as well as the dysplastic basal epithelium that previously showed expression of p53 and PCNA, expressions of TGF-α, EGFR and c-erbB-2/neu were found. In contrast to p53, EGFR and c-erbB-2/neu expressions, expression of TGF-α was also found in the inflammatory infiltrate in some of the carcinomas investigated. Increased expression of the TGF-α and EGFR reported in this study agree with findings of others in tumours and cell lines from patients with oral/pharyngeal SCCs [43, 44]. We found simultaneous expression of TGF-α and EGFR in 21 out of the 25 carcinomas investigated, and even in the adjacent normal epithelium. This finding also agrees with others [15, 16], and it might also support the suggestion of TGF-α–EGFR autocrine loop involvement in the neoplastic progression of human tumours [15, 16, 45–47]. Nevertheless, neither others [15, 16, 45–47], nor our own research group could conclude on whether TGF-α and EGFR have any fundamental role in the development of oral/pharyngeal SCCs. It has been suggested that EGFR can be activated by TGF-α only when the growth factor receptor reaches a critical concentration, conferring a clonal growth advantage of cells expressing EGFR [48, 49]. This finding has also been suggested to indicate that development of elevated TGF-α and EGFR levels might signal an early event in carcinogenesis [50].

In this study, expression of the c-erbB-2/neu was found in the normal epithelium adjacent to the carcinomas as well as in the control oral epithelium. This finding agrees with a previous report [28]. In breast tumours, membrane staining of the c-erbB-2/neu has been suggested to be associated with c-erbB-2/neu amplification [48]. Cytoplasmic staining of c-erbB-2/neu has been reported [46, 49], but its interpretation has been controversial. However, findings of mixed cytoplasmic/membrane staining were suggested to identify the c-erbB-2/neu [46]. In line with that, we found mixed cytoplasmic/membrane staining in 14 out of the 24 carcinomas investigated, and the staining was blocked using the specific blocking peptides. Thus, cytoplasmic staining observed in our study might support the suggestion of EGFR role in papillary thyroid carcinoma, which has suggested incomplete degradation of the c-erbB-2/neu receptor [47, 49]. The findings of high cytoplasmic expression of TGF-α, membrane staining of EGFR and mixed membrane/cytoplasmic staining of c-erbB-2/neu in the present study agree with others [15, 16, 28]. Such an observation was suggested to relate either to "field cancerization" or "condemned mucosa" [50, 51]. In these two concepts, multiple foci of premalignant or malignant changes may occur as a result of exposure of the entire epithelium to initiating agents like tobacco smoke and alcohol [50, 51]. In our study, however, no correlations were found between expression of these biomarkers and patients' clinicopathological parameters, perhaps due to the small number of carcinomas examined.

In conclusion (1) lack of negative growth regulation due to inactivation of the *p53* gene, together with activation of other proto-oncogenes, are necessary genetic events in the carcinogenesis of oral/pharyngeal SCCs, (2) in oral/pharyngeal SCCs, *p53* gene mutations were clustered in exons 5 (codons 130–186), 7 (codons 230–248) and 8 (codons 271–282) which might suggest that tobacco carcinogens probably affect the mutational hot spots of the *p53* gene at codons 157, 175, 186, 248, 273 and 282, and (3) fresh frozen and formalin-fixed tissue

specimens give similar results when an immunohistochemical method is applied. The importance of p53, TGF-α, EGFR, c-erbB-2/neu and PCNA as biomarkers in oral/pharyngeal SCCs deserves particular attention. This might offer further understanding of the development of these carcinomas based on specific p53, TGF-α, EGFR, c-erbB-2/neu and PCNA tumour targeting. In oral/pharyngeal SCCs, however, the need for carefully controlled studies comparing qualitative/quantitative determinants of frequency and identity of oncogenes, proto-oncogenes and/or tumour suppressor genes and examination of sufficiently large patient cohorts to determine potential prognostic significance is apparent.

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