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Relationship of Tobacco/Alcohol use to p53 Expression in Patients with Lingual Squamous Cell Carcinomas

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This study examined p53 expression immunocytochemically in 40 lingual squamous cell carcinomas from Dutch patients with known histories of smoking and/or drinking alcohol. 30% of neoplasms showed positive p53 reactivity, suggesting increased levels of p53 protein. No alcohol or tobacco risk factors were evident in 33.3% (4/12) of p53-positive neoplasms whereas only 7.1% (2/28) of p53-negative neoplasms showed an absence of these risk factors. 25% (3/12) of p53-positive neoplasms and 71.4% (20/28) of p53-negative neoplasms were found in patients who had been exposed to both alcohol and tobacco. A similar negative association with p53 reactivity was also found when either tobacco or alcohol were used in isolation. The results contrast with previous observations in head/neck and oral carcinomas and indicate that the association of alcohol/tobacco and p53 expression remains open to question.
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INTRODUCTION

TUMOUR SUPPRESSOR proteins function as negative regulators of cellular proliferation. Deletion and/or mutation of the p53 gene not only leads to unrestrained cellular growth [1] but is common in carcinomas of the head and neck [2, 3] and purportedly, correlates with a history of heavy smoking and drinking [4, 5].

Gene mutations may produce a more stable p53 protein than the wild-type product [6]. This observation has resulted in the use of immunocytochemical techniques to demonstrate abnormally high levels of p53 which, when detected, are assumed to reflect the presence of mutant product. Positive reactivity has been described in 34–100% of cancers of the head and neck [2, 4, 7]. Two recent studies, using a monoclonal antibody on frozen sections (PAb1801 [8]) or a polyclonal antiserum on paraffin sections (CM1 [9]), have suggested that between 35% [8] and 54% [9] of oral squamous cell carcinomas are p53 positive. The majority of these studies have been carried out using tissues from patients with and without previous treatment and often without recourse to the patients' social history. It is important, therefore, to extend the current observations to a specific group of malignant oral lesions where the risk factors are relatively well-defined.

The purpose of the present study was to examine p53 immunoreactivity in lingual squamous cell carcinomas from patients where a history of smoking and drinking was known and who had not undergone any previous cancer therapy.

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MATERIALS AND METHODS

Patients and tissues

Formalin-fixed paraffin embedded blocks of tissue from 49 Dutch patients diagnosed as having lingual squamous cell carcinoma during the period 1972–1986 were studied. None of the patients had been exposed to previous radiotherapy or chemotherapy, or had previous history of malignancy. Full patient details were known including habits such as tobacco smoking and alcohol intake and STNMP classification. Smoking was classified as non-smokers, incidental smokers (not daily), moderate smokers (<20 cigarettes per day) or heavy smokers (>20 cigarettes per day) or equivalent quantities of pipe tobacco. Alcohol intake was similarly classified as non-drinkers, incidental drinkers (not daily), moderate drinkers (1–4 units of alcohol per day) or heavy drinkers (>4 units of alcohol per day). Individuals with a history of smoking and/or drinking that had stopped either/both prior to presentation with lingual carcinoma were not included in the study.

Immunocytochemistry

Staining for p53 was performed using an improved biotin–streptavidin immunoperoxidase technique (StrAviGen: Biogenex) and a monoclonal antibody (PAb1801, Oncogene Science, [10]) reactive with a denaturation resistant epitope between amino acids 32 and 79 of normal and mutant p53. This antibody has been used extensively to detect p53 in frozen [7, 8, 11] and formalin-fixed, paraffin-embedded tissue sections [4, 12].

In brief, 5 μm deparaffinised sections were immersed in 0.3% hydrogen peroxide in buffer for 10 min to abolish endogenous peroxidase activity, washed in buffer and treated at room temperature for 1 h with 3 μg/ml anti-p53 antibody.

Table 1. *Summary of p53 staining results and patient details*

	No. (%)	Mean age (±S.D.)	Age range	% Female	Smoking and drinking habits (%) None*	Alcohol only	Tobacco only	Both†
p53+ve	12 (30)	62.3 (±14)	38–79	50	33.3	25	16.7	25
p53−ve	28 (70)	56.7 (±12.7)	37–84	35.7	7.1	17.9	3.6	71.4
All tumours	40 (100)	58.4 (±13.2)	37–84	40	15	20	7.5	57.5

*Includes individuals who *never* drank alcohol *or* smoked tobacco.
†Includes individuals who drank alcohol *and* smoked tobacco.

Table 2. *The grading of habits and the number of individuals within p53-positive* (n = 12) *and p53-negative* (n = 28) *tumour groups who smoked tobacco and drank alcohol*

Habit	Tumour group	Never	Incidental*	Moderate†	Heavy‡
Smoking	p53+ve	7	1	1	3
	p53−ve	7	2	6	13
Alcohol	p53+ve	6	2	3	1
	p53−ve	3	5	12	8

*Not daily. † < 20 cigarettes or between 1 and 4 units per day. ‡ > 20 cigarettes or > 4 units of alcohol per day.

After washing, the sections were treated with biotinylated anti-mouse immunoglobin (BioGenex; 1/100 dilution in buffer containing 1% normal human serum; 1 h at room temperature), washed and then overlaid with peroxidase-labelled streptavidin (BioGenex; 1/100; 1 h at room temperature). Reaction products were developed by immersing sections in 3,3′-diaminobenzidine reagent (5 min) and subsequently enhanced by treatment with 0.5% (w/v) copper sulphate in 0.01 mol/l phosphate-buffered saline (PBS), pH 7.6 for 5 min at room temperature. Stained sections were lightly counterstained in Meyer's haematoxylin and mounted in Xam. All reagent dilutions and washing were performed in PBS.

Negative controls included replacement of the primary layer with normal mouse immunoglobulin (3 and 10 μg/ml), monoclonal antibody of irrelevant specificity (MRC OX-6; IgG_1 anti-rat I-A; 3 and 10 μg/ml) and PBS. Adjacent sections of all tissues were also stained for keratin (clone LP34 [13], Dako, 1/200, 1 h) in order to more clearly define areas of tumour epithelium and to act as a positive 'tissue' control.

Evaluation of tissue sections

Nuclei with clear brown colour, regardless of the staining intensity, were regarded as positive for p53. Assessment of the immunostained sections was performed by one of us (JBM) without prior knowledge of the clinical details of the patients.

Statistical analysis

Data were analysed using Minitab (ver 8.2) and comparisons between p53-positive and p53-negative groups performed using χ^2 or, for age differences, the Mann–Whitney U test.

RESULTS

Of the 49 blocks of tissue examined immunocytochemically for keratin and by routine haematoxylin and eosin staining, nine contained very small islands of carcinoma ($n=3$) or no detectable carcinoma ($n=6$) and were excluded from the study. Thirty-five of the remaining 40 specimens contained histologically normal lingual epithelium in addition to invading squamous cell carcinoma. All 40 specimens stained intensely for keratin using the LP34 monoclonal antibody.

Tables 1 and 2 summarise the results of the immunostaining for p53 together with other patient details including tobacco and alcohol usage. Smoking tobacco was significantly more common in males (21/24) than females (5/16; $\chi^2=13.35$, $P<0.00005$). Although the level of alcohol usage was also higher in males (21/24) than females (10/16) this was not significant ($\chi^2=3.44$, $P=$N.S.).

Twelve (30%) of the carcinomas demonstrated nuclear reactivity for p53. Positive cells were not evenly distributed throughout tumours but tended to occur in foci and were most common within the basal layers (Fig. 1). No positive cells were detected in histologically normal overlying lingual epithelium, or in five specimens where there were areas of obvious epithelial dysplasia.

There were no significant differences between p53-positive and p53-negative groups in respect of age (Mann–Whitney), gender or TNM classification (χ^2). At the time tissues were obtained, neck lymph node metastases were associated with 25% (3/12) and 35.7% (10/28) of p53-positive and p53-negative carcinomas, respectively. In both groups well differentiated carcinomas predominated and accounted for 75% (9/12) of p53-positive and 71.4% (20/28) p53-negative neoplasms. From the post-surgery follow-up data (between 4 and 11 years), 25% (3/12) of individuals with p53-positive

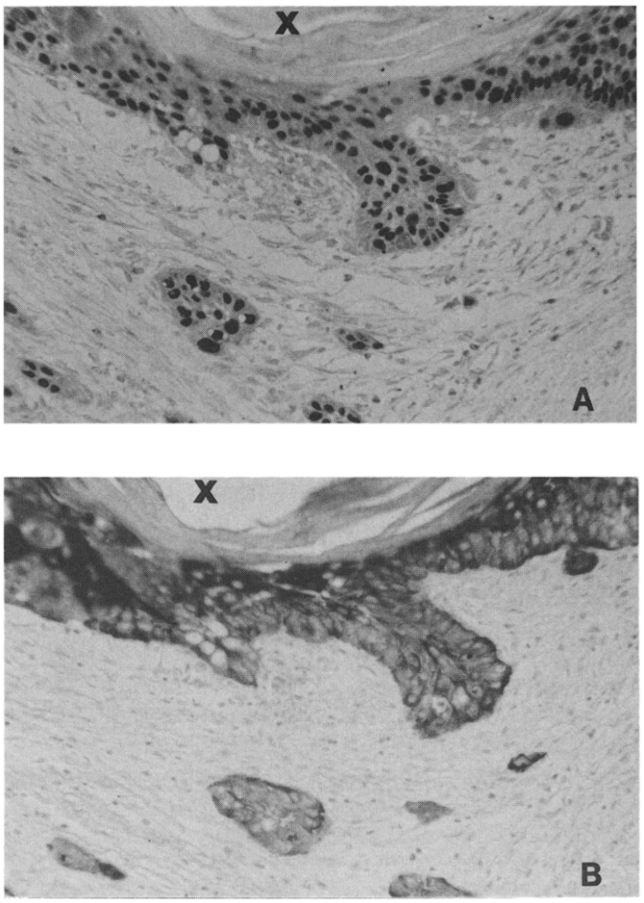

Fig. 1. Sequential paraffin sections of a cystic area of lingual squamous cell carcinoma immunohistochemically stained for p53 (PAb1801; panel A) and pan-keratin (LP34; panel B). p53 staining was localised to the nucleus. (X) indicates cystic space. Magnification ×250.

tumours have died as a direct result of recurrence/metastatic spread, 25% (3/12) have died of causes not related to the carcinoma and 50% (6/12) remain alive and free from cancer (4–10 year follow-up; mean, 5.7 years). Similarly, 39.3% (11/28) of individuals with p53-negative neoplasms have died as a direct result of recurrence/metastasis, 10.7% (3/28) have died of non-cancer related causes and 46.4% (13/28) remain alive and free from carcinoma (5–11 year follow-up; mean, 6.9 years).

Usage of alcohol and tobacco differed significantly ($P<0.01$ for alcohol, $\chi^2=7.4$; $P<0.05$ for smoking, $\chi^2=4.1$; $P<0.01$ for alcohol and smoking, $\chi^2=7.5$) between individuals within the p53-positive and p53-negative carcinoma groups. The biggest contributor to this difference was the high percentage of individuals in the p53-positive group who did not drink (50%; 6/12), smoke (58.3%; 7/12) or abstained from both habits (33.3%; 4/12). By contrast, 75% (21/28) of individuals in the p53-negative group smoked tobacco (19 at moderate to heavy levels), 89.3% (25/28) drank alcohol (20 at moderate to heavy levels) and 71.4% (20/28) used both tobacco and alcohol (16 at moderate to heavy levels for both habits).

DISCUSSION

Heavy smoking and drinking are well recognised risk factors for the development of upper aerodigestive tract malignancies, including lingual squamous cell carcinoma [14–16]. In such neoplasms, mutations in the p53 gene may be induced by specific carcinogens present in tobacco smoke [2, 4, 5, 17–19]. The most common mutation is G to T transversion which has been documented in head and neck [2], non-small cell lung [17, 19] and oesophageal [18] carcinomas.

In the present immunocytochemical study using monoclonal antibody PAb1801, 12/40 (30%) lingual carcinomas contained detectable amounts of the p53 gene product; a figure consistent with previous data on oral carcinomas [8, 9]. The surprising feature of the results, however, was the apparent negative association between immunocytochemical detection of p53 and tobacco smoking and/or alcohol intake. No alcohol or tobacco risk factors were evident in 33.3% of p53-positive carcinomas, whereas only 7.1% of p53-negative cases showed an absence of these risk factors. Furthermore, 25% of p53-positive and 71.4% of p53-negative carcinomas were found in patients who had been exposed to both alcohol and tobacco suggesting that overexpression/stabilisation of p53 is not necessarily associated with these two risk factors. This is in direct contrast to the previously reported positive link between p53 immunoreactivity and smoking [4, 9] and smoking and drinking [5] in cancers of the head and neck [4, 5] and the oral cavity [9].

Apart from the specific oral site of the carcinomas studied, there are two major differences between our studies and those of Field *et al.* [4] and Ogden *et al.* [9]. First, our material was obtained from a Dutch population whereas the other studies were based on British patients. The age standardised cumulative incidence rates of mouth and lingual cancer in the Netherlands and Britian are similar (approximately 1.2 and 0.6 per 100 000 for males and females, respectively [20]) indicating no obvious population differences. However, the incidence of bronchial cancer is higher in the Netherlands and shows a much greater difference between men (85.5 per 100 000) and women (8.5 per 100 000) than in England and Wales (65.4 and 20.5 per 100 000 for men and women, respectively) suggesting possible differences in smoking habits between these populations.

The second variable is that three different antibodies to p53 have been used. The initial studies on head and neck cancer of Field *et al.* [4] are based on combined data using two monoclonal antibodies which, individually, were only used on a proportion of the total number of specimens (PAb1801 on 40/73; PAb421 on 53/73). As one of the monoclonal antibodies (PAb421) is thought to cross-react with keratin [21], and as the published results do not allow determination of p53 positivity with the p53-specific PAb1801 alone, interpretation of this data requires caution. This may be important as only seven of the 73 carcinomas investigated were from non-smokers. By contrast, Ogden and co-workers [9] used a p53-specific polyclonal antibody (CM1) in their study of oral cancer and precancer. CM1, apparently able to detect smaller amounts of p53 than monoclonal antibodies [22], gave a higher rate of positives (54%) on formalin-fixed paraffin embedded oral carcinomas compared with PAb1801 on either frozen (35%; [8]) or routinely processed tissues (30%; this study). Their data clearly suggest a relationship between smoking and p53 expression in a group of 26 oral carcinomas, including 10 from non-smokers, but are dependent upon the specificity of the polyclonal antibody. Our contradictory data, based on the exclusive use of PAb1801 on paraffin sections, is dependent upon the sensitivity of p53 detection, which, when compared

to the results of Warnakulasuriya and Johnson [8] using the same antibody on frozen sections, appears satisfactory.

In this study it is not possible to determine whether the positive immunoreactivity for p53 found in 30% of carcinomas reflects the presence of stable mutant p53 species or stabilisation of normal p53 through binding to the products of cellular genes. For example, it has recently been shown that p53 protein is stabilised by cdc2 protein kinase [23], casein kinase II [24], the mdm2 gene product [25] and heat shock proteins [26]. Furthermore, the viral status of the lingual carcinomas in the present study is unknown, a significant factor as oncoproteins (SV40 large T antigen and adenovirus E1b) of DNA tumour viruses may stabilise cellular p53 [27, 28].

Ignoring technical problems there are a number of potential explanations for absence of immunocytochemical reactivity for the p53 protein in 70% of carcinomas, including those from patients with a background of tobacco and/or alcohol use. Negative carcinomas may have arisen independently of mutation of the p53 gene or, alterations in this locus, may have resulted in either deletion of both alleles or an alteration in transcription. Such changes have been detected in some lung tumour cell lines [29, 30]. A further possibility is that point mutations of the p53 gene, not resulting in the production of a sufficiently stable product to be detected immunocytochemically, are present. Such mutations have been detected in thyroid [31] and lung cancer [30] cell lines and, in one study of breast carcinoma, 7/10 specimens containing missense mutations were negative by frozen section immunocytochemistry [32]. We have shown variable stability of the p53 protein in human oral carcinoma cell lines harbouring a variety of p53 mutations (Yeudall *et al.*, submitted), suggesting that there may be alternative mechanisms of p53 stabilisation or regulation of expression in oral cancer. Finally, human papillomavirus (HPV) infection of oral carcinomas is common [33] and the HPV E6 protein has been shown to target the degradation of p53 via a ubiquitin-dependent protease system [34]. The HPV status of the carcinomas in the present study is unknown, but possibly could account for many of the p53-negative cancers.

There are no data available on the *direct* effects of tobacco and alcohol on steady state levels of p53 protein in the presence or absence of gene mutation. The interaction of viral and chemical carcinogens in cellular transformation is a significant area of cancer research at the present time and is likely to assume more importance in the future. Although the numbers are small, our data could indicate that oral cancer in patients with a background of tobacco and/or alcohol use is more likely to be immunocytochemically negative for p53 because of one or a combination of these factors. The corollary would be that an oral carcinoma arising in an individual not exposed to these risk factors would be more likely to have gene mutations leading to a stabilisation of the p53 protein.

In conclusion, the results of this study indicate that the purported correlation of tobacco/alcohol and increased steady state levels of p53 expression is questionable. Further investigations, combining p53 gene sequencing studies and immunocytochemistry, are required to more clearly define the relationship between this tumour suppressor gene product, oral cancer and the use of tobacco and alcohol.

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