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Value of p53 Expression in Oral Cancer and Adjacent Normal Mucosa in Relation to the Occurrence of Multiple Primary Carcinomas

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Paraffin embedded, formalin fixed tissue sections from patients suffering from a primary oral squamous cell carcinoma were immunohistochemically investigated for the presence of p53 expression using the Bp53-11 antibody. The aim of this study was to determine the predictive value of p53 expression as a biomarker for the development of a second primary tumour (SPT) in the respiratory and upper digestive tract. In a nested case control study, neoplastic and normal tissue sections of 44 patients who had a previous history of cancer were used. 15 of the 44 had developed a SPT, while the other 29 were minimally 7 years free of disease. Additionally, nine SPTs were included in this study to establish whether concordance exists in tumours that develop in the same field. 10 of the 29 patients (34%) free of tumour during follow-up had p53 positive tumours. 8 of 15 patients (53%) who developed a SPT had a p53 positive primary tumour. This difference is not statistically different (χ^2-test). Forty per cent of the total group of primary oral cavity tumours showed p53 positivity. When comparing the first and the second tumours, discordance in p53 expression between the first and second tumours was seen in 4 out of 9 cases. None of the cases showed p53 positivity in adjacent normal mucosa. In conclusion, p53 immunoreactivity in neoplasia, dysplasia and normal tissue does not predict the development of a SPT. In addition, multiple primary tumours do not have identical p53 expression.

Keywords: p53, multiple primary tumours, oral squamous cell cancer, immunohistochemistry

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INTRODUCTION

A REVIEW of treatment outcomes over the last 30 years in head and neck squamous cell carcinoma (HNSCC) reveals progress in initial treatment, but only a marginal increase in long-term survival. The development of a second primary tumour (SPT) in the respiratory and upper digestive tract is the chief cause of treatment failure and death in patients who present with early stage disease [1]. Head and neck carcinogenesis has been proposed to be a multistep process in an anatomic field repeatedly exposed to carcinogens. Recent findings of geno- and phenotypic alterations in histologically normal epithelium of cancer patients support the "field cancerisation" theory [2–4].

Activation of cellular protooncogenes or inactivation of tumour suppressor genes are most probably genetic alterations, involved in this multistep process of carcinogenesis. The transcription factor p53 is the product of a tumour suppressor gene. The wild-type form has a half-life of 6–30 min. In contrast, proteins from mutated genes form complexes with a heat shock protein (hsp70) that result in a metabolically stable protein, that has a half-life of many hours [5]. This forms the basis for the detection of mutated p53 by immunohistochemistry, because wild-type p53 does not accumulate in high enough levels for detection by immunohistochemistry [6]. Recently, Bartek and colleagues described Bp53-11 as a useful antibody for immunohistochemistry on paraffin sections [7, 8]. In HNSCC, as in a number of other human solid tumours [9], p53 gene mutations have been reported to be a common implicated genetic event. By immunohistochemical detection techniques increased p53 levels have been found in 35–90% of HNSCCs [6, 10–16]. In studies concerning oral cavity carcinomas, percentages of 35 [10] and 54 [13] p53 positivity have been reported. Moreover, p53 has recently been proposed to be an early event in head and neck carcinogenesis, since immunohistochemical studies showed p53 positivity in histologically inconspicuous epithelia at a significant distance from the primary tumour and

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premalignant lesions [17, 18]. The forementioned studies did not report on the prognostic value of early p53 expression in the clinical development of SPTs. Recently, it has been shown that p53 positivity in surgical margins, histopathologically free of tumour, was correlated with local tumour recurrence [19]. As there is a great need for early markers to identify patients who are at high risk of developing a SPT, we applied the immunohistochemical staining procedure against p53 on both tumours and the normal mucosa of patients who were radically surgically treated for their primary oral cavity tumour. From these patients the smoking and alcohol history was known and they had not undergone any previous cancer therapy. We performed this study as a nested case control study to establish the possible predictive value of p53 expression for the development of a SPT in both primary tumours and clinicopathologically normal tissue, obtained during surgery for the index tumour.

PATIENTS AND METHODS

From our archives we selected 15 formalin-fixed paraffin embedded tissue blocks of primary oral carcinomas of patients that were initially treated between 1978 and 1988. These patients eventually developed a SPT. The control group consisted of 29 patients from the same period that had not developed a SPT during the follow-up time of minimally 7 years. Patients with a local recurrence were excluded from the study. None of the patients had been exposed to previous radio- or chemotherapy. Individual patient characteristics are reported in Table 1. From 9 patients, who had developed a second primary tumour during follow-up, the second tumour was also investigated. All tumour specimens and normal tissues were reviewed on haematoxylin and eosin stained histological sections by one of the authors (IvdW). Dysplasia was identified by criteria previously described [20]. Paraffin sections of 5 μm on poly-L lysine (Sigma, St. Louis, Missouri, U.S.A.) coated slides were deparaffinised and rehydrated before immunohistochemistry was performed. p53 staining was performed using the immunoperoxidase technique with an avidin biotin complex (Vectastain ABC-kit, Vector laboratories, Burlingame, U.S.A.). The monoclonal mouse Bp53-11 antibody is a highly sensitive antibody recognising an antigenic determinant in the aminoterminal region of the p53 protein and reacting with both wild-type and mutant forms of p53 [17], and was kindly provided by Dr Bartek. The antibody was diluted 1:10 in PBS to which 1% bovine serum albumin (BSA, Sigma) was added. The slides were incubated with the Bp53-11 antibody for 60 min at room temperature. Non-specific staining was blocked with normal horse serum (Vector Laboratories) before incubation with the primary antibody and endogenous peroxidase activity by 0.006% H_2O_2 in methanol, before incubation with the secondary antibody. After incubation with the primary antibody, biotinylated horse-anti-mouse immunoglobulin (Vector Laboratories) was used and the peroxidase label was developed with diaminobenzidine tetrachloride (DAB, Sigma). Counterstaining was performed with haematoxylin (Merck, Darmstadt, Germany). The stained slides were finally mounted with "Kaiser's glycerine gelatine" (Merck). A negative control was made using PBS/BSA (1%) instead of the primary antibody.

Evaluation of p53 positivity was performed by light microscope on coded slides by two independent observers. Nuclei with a clear brown colour were regarded as positive for p53.

Statistical analysis was performed by the chi square test and by the Spearman rank correlation test.

RESULTS

In a nested case control study p53 expression has been determined in tumour and normal mucosa of 44 patients with an oral cavity tumour. No statistical difference in frequency of p53 positivity between patients who eventually developed a SPT and patients who did not, could be observed, as shown in Table 2. We also found discrepancies between p53 expression in the index tumour and the corresponding SPT in 4 out of 9 patients (Table 1). In all, but one, cases ($n=13$), where the invasive tumour exhibited p53 protein expression, the adjacent dysplastic areas also showed p53 positivity in the basal cell layer. In 3 of the 12 cases the dysplastic area showed only sparse p53 positive cells. The others showed an overall p53 positive basal cell layer. When the tumours were p53 negative, the corresponding areas were also negative.

No expression of p53 protein was found in adjacent, clinicopathological normal-looking mucosa in all cases.

A significant correlation between smoking habits and p53 expression has previously been reported in HNSCC [14]. The most common mutation in p53, a G to T transversion has been linked to tobacco usage, which implicates that specific carcinogens may induce mutations in the p53 gene [15]. However, a significant correlation between p53 expression and smoking habits ($P=0.28$), alcohol consumption ($P=0.32$), tumour T and N stage ($P=0.8$ and $P=0.46$, respectively) and age ($P=0.91$) could not be found in our study.

DISCUSSION

The purpose of this study was to establish whether immunohistochemical expression of mutated p53 in the tumour or peritumorous normal mucosa of HNSCC patients could be correlated with the eventual development of a SPT. In 1993 and 1994 two reports about p53 expression in histologically normal epithelium adjacent to the tumour of HNSCC patients appeared and it was proposed that mutations in the p53 gene are an early event in head and neck carcinogenesis [17, 18]. More recently, Brennan *et al.* showed that detection of p53 positivity by molecular analysis in histopathological tumour-free surgical margins and lymph nodes was correlated with the development of local tumour recurrence [19].

In general, we also found p53 positivity in the basal cell layers of dysplastic tissue adjacent to positive tumours. Thus, our study supports the concept that p53 mutation may occur in an early phase of carcinogenesis. However, it must be added that in a significant proportion of tumours (60%) p53 does not occur at all. This percentage of lack of p53 immunohistochemical staining is in agreement with reports of other authors [11, 21].

In our study, in histopathologically normal mucosa of 44 HNSCC patients, obtained during surgical removal of the index tumour, p53 protein could not be detected. Nees *et al.* [17] showed p53 positivity in several tumour distant biopsies of normal mucosa of HNSCC patients, using the same antibody as in our study. Also, Shin *et al.* [18] found p53 expression in histologically normal epithelium adjacent to the tumour in 21% of the specimens. In contrast to these latter two studies, in our study, patients with local recurrences were excluded.

Table 1. Patient characteristics and individual p53 data

No.	Gender	Age	Primary tumour			p53 positivity						
			Loc.*	TNM†	SPT‡	Normal	Dyspl.§	PrimT¶	SPT‡	psm‖	csm**	alc††
1	F	79	Rtongue	pT1N0	Ltongue	−	n.a.	+	+	−	−	+
2	F	57	Ltongue	pT2N0	Rtongue	−	n.a.	−	−	+ +	+	+ +
3	M	45	Ltongue	pT2N0	Rtongue	−	±	+	+	+ +	+	+ +
4	M	38	Rtongue	pT3N1	Sinus pf	−	n.a.	−	−	−	−	−
5	M	67	Ltongue	pT1N1	Lung	−	n.a.	−	n.a.	+	?	+
6	F	49	Rtongue	pT2N0	Pharynx	−	±	+	−	+	+	+
7	M	38	Rtongue	pT3N2	Lung	−	n.a.	+	n.a.	+ +	?	+
8	M	50	Ttongue	pT1N0	Glottis	−	n.a.	−	−	+	+	+
9	M	59	Fom	pT3N2c	Lung	−	n.a.	−	n.a.	+ +	?	+ +
10	M	42	Palatum	pT1N0	Tongue	−	+	+	−	+ +	−	−
11	M	48	Ltongue	pT2N0	Lung	−	n.a.	+	n.a.	+	?	−
12	F	57	Fom	pT2N1	Rtongue	−	n.a.	+	−	+	+	−
13	M	56	Trig.	pT2N0	Bronch.	−	−	−	n.a.	+ +	?	+ +
14	M	55	Rtongue	pT3N0	Btongue	−	n.a.	−	+	+ +	?	+ +
15	M	71	Fom	pT1N0	Lung	−	n.a.	+	n.a.	+ +	?	+ +
16	F	43	Tongue	pT1N0	−	−	n.a.	+	n.ap.	−	?	−
17	M	56	Tongue	pT3N0	−	−	n.a.	−	n.ap.	+ +	?	+ +
18	F	59	Tongue	pT1N0	−	−	n.a.	−	n.ap.	+	?	+
19	M	58	Tongue	pT3N0	−	−	n.a.	+	n.ap.	+	?	+
20	F	77	Tongue	pT2N0	−	−	n.a.	−	n.ap.	−	?	+
21	M	60	Tongue	pT1N0	−	−	n.a.	−	n.ap.	+	?	−
22	F	71	Tongue	pT2N0	−	−	n.a.	+	n.ap.	−	?	−
23	M	74	Tongue	pT2N1	−	−	−	−	n.ap.	+	?	+
24	M	79	Tongue	pT1N0	−	−	n.a.	+	n.ap.	−	?	−
25	M	75	Tongue	pT2N1	−	−	−	+	n.ap.	+	?	+
26	M	66	Tongue	pT1N0	−	−	−	−	n.ap.	−	?	+
27	M	50	Tongue	pT3N1	−	−	n.a.	+	n.ap.	+ +	?	+ +
28	M	40	Tongue	pT3N0	−	−	−	−	n.ap.	+ +	?	+ +
29	F	65	Tongue	pT1N0	−	−	−	−	n.ap.	+	?	+
30	M	52	Tongue	pT3N1	−	−	±	+	n.ap.	+ +	?	+ +
31	M	46	Tongue	PT3N0	−	−	−	−	n.ap.	+ +	?	+ +
32	M	71	Tongue	pT2N0	−	−	−	−	n.ap.	+	?	+
33	M	54	Tongue	pT3N0	−	−	n.a.	−	n.ap.	+ +	?	+ +
34	M	50	Tongue	pT3N1	−	−	n.a.	−	n.ap.	+ +	?	+ +
35	F	79	Tongue	pT1N0	−	−	n.a.	+	n.ap.	−	?	−
36	F	45	Tongue	pT1N0	−	−	n.a.	−	n.ap.	−	?	+
37	M	69	Fom	pT3N1	−	−	n.a.	−	n.ap.	+ +	?	+
38	M	51	Fom	pT2N0	−	−	n.a.	−	n.ap.	+ +	?	+ +
39	F	64	Fom	pT1N0	−	−	−	−	n.ap.	−	?	−
40	F	82	Fom	pT2N1	−	−	n.a.	+	n.ap.	−	?	−
41	M	60	Tongue	pT2N0	−	−	n.a.	−	n.ap.	+	?	+
42	M	59	Fom	pT1N0	−	−	n.a.	−	n.ap.	+ +	?	+
43	F	58	Fom	pT2N0	−	−	n.a.	+	n.ap.	+	?	+
44	M	62	Tongue	pT1N0	−	−	n.a.	−	n.ap.	+	?	+

*Loc, localisation; †TNM, pTNM stage of the primary tumour, according to the new TNM classification [26]; ‡SPT, second primary tumour; §Dyspl., dysplasia, by previously described criteria [15]; ¶PrimT, primary tumour; ‖psm, previous smoking habit; **csm, current smoking habits; ††alc., alcohol habits. Rtongue, right side of tongue; Ltongue, left side of tongue; Btongue, base of tongue; Trig., retromolar trigonum; Fom, floor of mouth; Bronch., bronchus. n.a., not available. n.ap., not applicable. Smoking habits: + + > 20 cig/day; + < 20 cig/day; − never smoked. Alcohol habits: + + > 4 units/day; + 1–4 units/day; − no alcohol usage; p53 positivity score: − no positive cells, ± sparse positive cells, + fields with positive cells.

Table 2. p53 staining of the primary oral cavity tumour of patients who developed a second primary tumour and patients who did not

	p53 positive	p53 negative
SPT* + ($n = 15$)	8 (53%)	7 (47%)
SPT* − ($n = 29$)	10 (34%)	19 (66%)

*SPT, second primary tumour.

The key finding of our study is that the immunohistochemical expression of p53 by Bp53-11 in neoplastic, dysplastic and normal peritumorous mucosa has no value in predicting the development of a SPT. However, the absence of antibody reactivity does not rule out genetic alterations of p53. Frameshift mutations, as well as nonsense mutations, that may result in the production of truncated forms of p53, can lead to altered p53 undetectable by available monoclonal antibodies [22]. Recently, a 40% discrepancy between single strand conformational polymorphism analysis for the detection of

p53 mutations in exons 5–9, the most common sites for p53 abnormalities, and immunohistochemistry, has been shown [23]. Both overexpression without apparent mutations and mutations without detectable p53 immunostaining were observed. Recognising mutations in tumour-surrounding biopsies, always obtained during radical surgical resections, could otherwise be of great value in identifying patients at high risk of developing a SPT and could eventually improve their prognosis. However, the now known discrepancy between molecular genetic and antibody-based studies caution against drawing a definitive conclusion about the value of p53 overexpression by using a single detection modality. The approximately positive predictive value of p53 detection in tumours by immunohistochemistry is 63% [24]. It is still an option that detecting p53 mutation at the genomic level has some value in predicting the development of a SPT.

We also observed that p53 mutations, detected by the Bp53-11 antibody, in multiple primary tumours within one individual, are not always similar. This shows a biological diversity in tumour suppressor genes, that are probably involved in the development of a SPT in HNSCC. This finding of differences in the mutation pattern of multiple primary tumours in HNSCC supports the "field cancerisation" theory, that states that dietary or environmental agents cause wide-spread genetic damage leading to multifocal neoplasia [2]. The discordance of p53 mutations in multiple primary tumours strongly favour a multifocal, polyclonal process, as earlier reported by Chung *et al.* [25].

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