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p53 Expression: a Potential Biomarker for Risk of Multiple Primary Malignancies in the Upper Aerodigestive Tract

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The concept of field cancerisation assumes that in head and neck cancer patients (HNCP) with multiple malignancies the second primary cancers may arise independently from the entire upper aerodigestive tract as a consequence of massive exposure to common carcinogens. Since mutations and/or overexpression of the p53 tumour suppressor gene represent a genetic alteration frequently occurring in HNCP, we analysed immunocytochemically p53 oncoprotein expression in first primary, second primary cancers and in macroscopically uninvolved normal epithelium from different sites of the upper aerodigestive tract from 12 HNCP with multiple malignancies, in comparison with p53 expression in biopsy specimens of the upper aerodigestive tract from 5 non-neoplastic heavy smokers, as controls. In patients with multiple malignancies 6 cases (50%) showed positive staining of both first and second primaries, whereas 3 (25%) had positive labelling of first primary cancer but not of the subsequent second primary, 2 (17%) patients showed p53 expression only in the second primary cancer, and finally only 1 patient (8%) showed no p53 immunoreactivity in both first and second primary tumours. Moreover, 10 out of 12 (83%) HNCP with multiple cancers showed p53-positive staining in the normal epithelium from different sites of the upper aerodigestive tract, also at a significant distance from the site of first and second primary malignancies. In contrast, sporadic p53 immunostaining was observed only in three out of 35 (8.5%) specimens from non-neoplastic controls. In addition, in 4 HNCP with multiple tumours the histological examination of apparently normal epithelium from the upper aerodigestive tract revealed signs of moderate or severe dysplasia, and in 1 case an *in situ* carcinoma. All these biopsy specimens showed strong p53 immunoreactivity. Thus, aberrant p53 oncoprotein expression seems to be an early event in the multistage process of head and neck multiple carcinogenesis and its expression in normal epithelium from HNCP would indicate an increased risk for transformation to second primary cancer.

Keywords: head and neck cancer, p53 tumour suppressor gene, immunohistochemistry, multiple tumours, risk assessment

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INTRODUCTION

In 1953 Slaughter *et al.* published a classic report describing the novel concept they called "field cancerisation" [1]. This term referred to the basic pathogenetic process that linked their oral cancer patients' originally diagnosed tumours with multiple other primary tumours in the oropharynx, larynx, oesophagus and lung. The authors postulated that in subjects at risk the entire aerodigestive epithelial surface, or "field", is exposed to repeated carcinogenic insults, usually from tobacco and alcohol use. Therefore, in this high-risk population, epithelial tumours could arise independently from multiple premalignant foci which develop and progress at variable rates.

Carcinogenesis of the aerodigestive tract epithelium is an extremely complex multistep process [2]. Evidence exists that aberrations of oncogenes and tumour suppressor genes are probably essential for this process, in which one of the most common changes at the gene level known so far is the p53 aberration [3–6]. Aberrant expression and mutations of the p53 tumour suppressor gene have been frequently reported in head and neck cancers (see review in [7]), but the stage of carcinogenesis at which the p53 gene mutation occurs has not been defined with certainty, even though recent reports indicate that the p53 mutation and/or overexpression could be an early event of tumour growth [8–10]. Because gene mutations may produce a more metabolically stable p53 protein than the wild-type product, p53 oncoprotein accumu-

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Table 1. Clinical characteristics of 12 head and neck cancer patients with multiple malignancies

Age, years	58
Median range	(46–70)
Male/female	11/1
Smoking history	
Non-smokers	2
Moderate smokers	3
Heavy smokers	7
Alcohol use	50%
Postoperative radiotherapy	33%

Average period to develop second primary 5.7 years (range 0.9–12).

lates in the nuclei of cells with the mutated form and becomes immunohistochemically detectable [11].

The aim of this study was to immunohistochemically analyse p53 tumour suppressor gene expression in primary and corresponding second primary cancers from 12 consecutive head and neck cancer patients (HNCP) with multiple malignancies and to correlate p53 immunoreactivity to tobacco use in these patients. Moreover, in this group of patients with multiple malignancies we studied p53 expression in tumour-distant macroscopically uninvolved epithelium from different areas of the upper aerodigestive tract, to assess the potential use of p53 immunostaining as a biomarker of intermediate end-points of multiple carcinogenesis of the head and neck.

MATERIALS AND METHODS

Between January 1993 and June 1993 at the Institute of Otolaryngology of the University of Florence 12 patients with previously treated squamous cell carcinomas of the head and neck who were found to have second primaries during the follow-up period were selected for our study. Clinical characteristics of the patients are shown in Table 1.

In this group of patients with multiple malignancies, all but 1 (patient no. 10, who presented with synchronous squamous cell carcinomas of the right and left trigonous), had metachronous cancers within a median period between first primary and second or third lesions of 5.7 years (range 0.6–13). Moreover, patient nos 2 and 4 experienced a third primary cancer (breast and urinary bladder, respectively). The sites of first primary and second primary cancers are shown in Table 2.

All these patients underwent surgical procedures for their second primary cancer at our institution within a few days of diagnosis. During these operations, with the consent of patients, specimens of the second primary cancers (in the only patient with synchronous lesions, of the both cancers) and biopsy specimens from mucosae of the upper aerodigestive tract (i.e. from floor of mouth, tonsil, base of tongue, hypopharynx, epiglottis, vocal cord and oesophagus) were collected under general anaesthesia.

As controls, we investigated p53 expression biopsy specimens of the same head and neck areas obtained with the consent of patients from 5 patients who underwent surgical head and neck procedures for non-neoplastic disease during the period of the study. All these controls were male, over 50 years of age and heavy smokers.

Tobacco exposure was documented retrospectively and recorded for all individuals. For those individuals who smoked cigarettes, pack-year history was calculated by multiplying the number of packs consumed per day by the number of years exposed. Accordingly, patients with a history of more than 30 pack-years and patients with less than 30 pack-years consumed, were considered heavy and moderate smokers, respectively.

Statistical analysis was performed according to Fisher's exact test, and values less than 0.05 were considered statistically significant.

Immunocytochemistry

Paraffin-embedded formalin-fixed specimens of the primary, second primary head and neck cancer and tumour-distant epithelium from each patient with multiple malignancies enroled in the study were available from the files of the Institute of Anatomic Pathology, for the immunocytochemical analysis. Immunocytochemistry was performed on deparaffinised, 5-μm sections after antigen retrieval using microwave oven heating [12]. A murine monoclonal antibody, DO-7 anti-human p53 protein, specific for a formalin-resistant epitope of the N-terminus of the human protein, reacting with both wild and mutant types of the p53 protein (Dako, Copenhagen, Denmark) was employed. For immunostaining, the avidin–biotin–peroxidase complex method was used. In brief, after deparaffinising and inactivating endogenous peroxidase activity, and blocking of cross-reactivity with pre-immune serum (Vectastain Elite Kit; Vector Laboratories, Burlingame, California, U.S.A.), the sections were incubated for 1 h at room temperature with the primary antibody diluted at 1:50. Localisation of the primary antibody was achieved by subsequent incubation of biotinylated anti-primary antibody, an avidin–biotin complex conjugated to horseradish peroxidase, and diaminobendizine (Vectastain Elite Kit). The slides were washed three times with phosphate-buffered saline after each incubation. As a negative control, some slides were subjected to normal serum blocking and omission of the primary antibody. In the immunohistochemical analysis each slide usually contained three to five serial sections from the same block. The staining pattern was assessed by one assessor and classified as: (−) for negative or equivocal staining, (+) 1–5% of positive cells, (++) 6–40% of positive cells, and (+++) > 40% of positive cells. Only nuclear staining was regarded as specific staining. Discordant cases were discussed and a consensus statement was reached.

RESULTS

A total of 143 specimens from the upper aerodigestive tract, including 108 first primary and second primary tumours and biopsies from apparently uninvolved epithelium of HNCP and 35 biopsy specimens from non-neoplastic controls, were immunohistochemically investigated for p53 expression (24 head and neck squamous cell carcinomas and 119 biopsy specimens of apparently uninvolved normal mucous areas of the upper aerodigestive tract). The overall rate of positive immunostaining was 59% (64 of 108) in tissues from HNCP with multiple malignancies, in comparison with 8.5% (three of 35) of p53 positivity observed in non-neoplastic specimens of the upper aerodigestive tract from controls. Moreover, among patients with multiple malignancies, 9 in the first primary (82%) and 8 (67%) in the second primary squamous cell carcinomas showed positive p53 immunostaining (82 versus 67%, P = n.s.).

Table 2. Second primary site by index tumour in 12 head and neck cancer patients with multiple malignancies

Index tumour	Oesophagus	Larynx	Oral cavity	Pharynx
Larynx ($n=5$)	—	1	2	2
Oral cavity ($n=4$)	—	1	2†	1*
Oropharynx ($n=2$)	1	1	—	—
Hypopharynx ($n=1$)	—	—	1*	—
Total ($n=12$)	1	3	5	3

*Patient with more than two tunours. †Included 1 patient with synchronous tumours.

Table 3. p53 expression in multiple primary tumours and normal epithelium from upper aerodigestive tract in 12 HNCP with multiple malignancies

Patient no.	Site and stage* of tumours First primary	Second primary	p53 immunoreactivity Primary tumour	Secondary tumour	FOM	Tonsil	BOT	HYP	EPI	VCL	ESO	Smoking history
1	Larynx (T1aN0)	Larynx (T2N0)	+	−	−	−	+	−	−	−	−	Non-smoker
2	Oral cavity (T3N1)	Oral cavity (T1N0)	+	+	+	+	+	−	−	−	+	Heavy smoker
3	Oropharynx (T2N0)	Oesophagus (T2N0)	+	−	−	+	+	−	+	+	+	Heavy smoker
4	Oral cavity (T2N0)	Oral cavity (T1N0)	−	+	+	+	+	−	+	−	−	Moderate smoker
5	Oral cavity (T3N0)	Hypopharynx (T3N0)	+	+	+	+	+	+	+	+	−	Heavy smoker
6	Larynx (T1N0)	Oral cavity (T2N1)	+	+	+	+	+	−	+	−	+	Heavy smoker
7	Hypopharynx (T2N1)	Oral cavity (T1N0)	+	+	+	+	−	+	−	−	+	Moderate smoker
8	Larynx (T3N0)	Oropharynx (T2N0)	+	−	−	+	+	+	+	+	−	Heavy smoker
9	Larynx (T1N0)	Hypopharynx (T1N0)	−	+	+	−	+	+	−	−	+	Moderate smoker
10	Oral cavity (T2N0)	Oral cavity (T1N0)	−	−	−	−	−	−	−	−	−	Non-smoker
11	Larynx (T2N0)	Oral cavity (T2N0)	+	+	+	+	+	−	+	+	−	Heavy smoker
12	Oropharynx (T1N0)	Larynx (T2N1)	+	+	−	+	+	+	−	+	−	Heavy smoker

*TNM(UICC, 1987). †Patient with synchronous tumours. FOM, floor of mouth; BOT, base of tongue; HYP, hypopharynx; EPI, epiglottis; VCL, vocal cord larynx; ESO, oesophagus.

In this group of HNCP with multiple cancers, 6 patients showed positive staining of both first primary and second primary cancers, whereas 3 had positive labelling of the first primary cancer but not of the subsequent second primary, 2 patients showed no p53 expression in the first primary but did have positive staining in subsequent second cancers, and finally only 1 patient showed no immunoreactivity for p53 in neither first nor second primary carcinomas (Table 3). The analysis of p53 immunoreactivity in the biopsy specimens from macroscopically uninvolved epithelium in HNCP is shown in Table 3. Forty-seven of 84 (56%) of the biopsies analysed were p53 immunoreactive. The percentage of p53-immunoreactive biopsies was highest in oropharynx (83% for the base of tongue and 75% for the tonsil) and in oral cavity (58% for the floor of mouth), and lower in larynx (50% for epiglottis and 42% for vocal cord), hypopharynx (42% for pyryform sinus) and finally oesophagus (42%) (Figure 1a, b). In 4 cases, biopsy specimens from larynx (epiglottis in 2 cases and vocal cord in 1 case), and from base of tongue (1 case) at the histological examination showed clear signs of dysplasia with p53-positivity (Figure 1c). Moreover, a p53-positive *in situ* carcinoma of the vocal cord was observed in patient no. 11, who experienced a supraglottic laryngeal carcinoma as first primary tumour (Figure 1d).

Biopsy specimens of analogous areas of the upper aerodigestive tract from control group were p53-positive in three samples out of 35 (8.5%) analysed from 2 of 5 heavy smokers studied. The p53-positive specimens were obtained from the vocal cord and epiglottis in 1 case and from the vocal cord in the other, both patients showing signs of chronic laryngeal inflammation at the direct laryngoscopy. In addition, p53 immunoreactivity in these specimens was restricted to a few epithelial cell nuclei.

Thus, the overall rate of p53 immunoreactivity in apparently unaffected normal epithelium was 56% in HNCP in comparison with 8.5% in non-neoplastic heavy smokers ($P<0.001$).

The analysis of smoking history and of p53 expression in the 12 patients examined showed that patients with p53-positive tumours consumed significantly greater numbers of cigarettes

(mean 50.3 ± 7.5 pack-years) than patients with negative p53 tumours (mean 17.8 ± 3.2) ($P < 0.001$). In addition, in our series the only 2 non-smoker patients had few or absent p53 immunoreactivity in their neoplastic and non-neoplastic specimens analysed, whereas, 5 out of 6 patients, who showed p53 immunoreactivity in both cancers and in several tumour-distant biopsies, were heavy smokers.

DISCUSSION

Approximately 10–30% of patients with head and neck cancer develop a subsequent second primary in the same area, the early staged patients being at particularly high risk [13–15]. As most of them died of their second primary, it would be useful to improve our understanding of the molecular basis of field cancerisation theory, since identification of genetic alterations and subsequently altered expression of regulatory gene products leading to cellular proliferation and transformation may enable early identification of subjects and/or sites at highest risk, for preventive and therapeutic approaches [16].

The aim of this study was to validate p53 oncoprotein expression as a potential biomarker able to identify the process of field cancerisation in the upper aerodigestive tract. Our results, showing p53-positive staining in 71% of multiple squamous cell carcinomas analysed and in 56% of biopsy specimens from macroscopically uninvolved epithelium from tumour-adjacent and -distant sites of the upper aerodigestive tract from HNCP with multiple malignancies, strongly support Slaughter's original hypothesis [1]. In fact, the detection of altered p53 expression in a very large number of macroscopically unaffected mucosal biopsies of the upper aerodigestive tract, mostly but not exclusively from patients with both first and second primary p53-immunoreactive tumours, in contrast to sporadic immunoreactivity observed in specimens from heavy smoker subjects as controls, indicate that p53 expression might be an early event in head and neck carcinogenesis. In addition, over 30% of macroscopically uninvolved epithelium from HNCP studied showed, at the histological examination, signs of dysplasia, and in one case an *in situ* carcinoma of the true vocal cord. All these specimens showed a high percentage of p53-positive dysplastic cells. These data indicate that altered p53 expression occurs in normal epithelium, in non-invasive premalignant lesions and

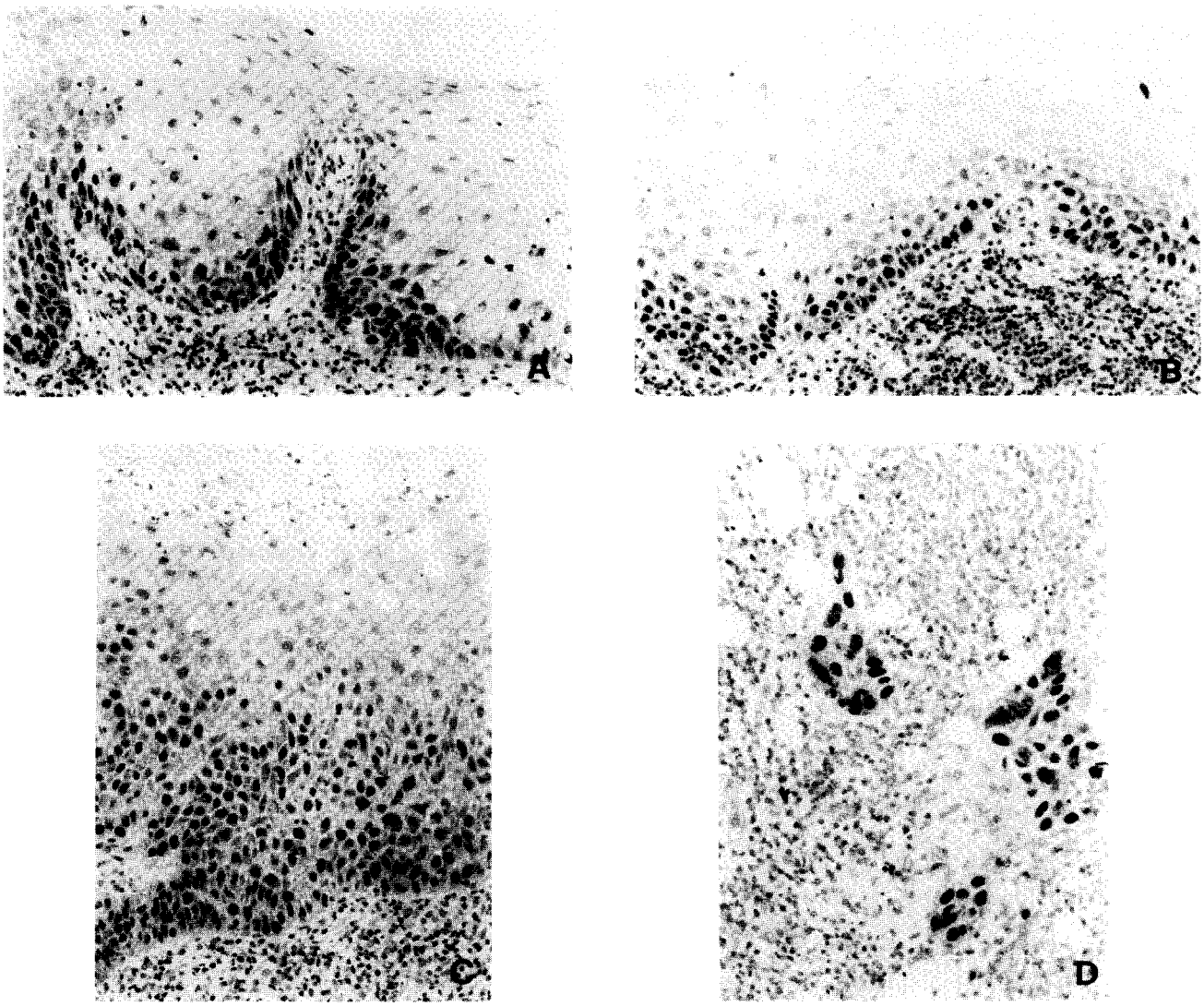

Fig. 1. p53 nuclear staining in normal mucosae from base of tongue (a) and vocal cord (b), in dysplastic laryngeal epithelium (c), and in squamous cell carcinoma (d) (original magnification 200 ×).

continues to increase in frequency in invasive carcinomas from HNCP with multiple malignancies. This strongly suggests that histologically normal epithelium already exposed to tobacco and/or alcohol has a variable degree of p53 expression and this expression, in several areas of the entire aerodigestive tract, can precede the development of second primary malignancies in HNCP.

Our data of 71% of carcinomas being p53-positive at immunohistochemical evaluation is in keeping with 67% of p53 gene mutations in multiple cancers by HNCP recently reported by Chung *et al.* [17]. They shared the occurrence of discordant p53 gene mutations in primary head and neck cancers and corresponding second primary cancers of the upper aerodigestive tract, providing the first demonstration of a molecular basis for field cancerisation effects in cancer of the head and neck. Our results extend these original observations showing that the p53 aberration also occurs in high frequency in tumour-distant epithelium in HNCP with multiple malignancies, particularly in those with both first primary and second primaries p53-immunoreactive. Recently, Nees *et al.* [18] published an interesting paper on the same issue, showing p53 gene mutations in tumour-distant epithelia of 15 HNCP with single cancer, 5 of whom developed secondary or recurrent tumours during follow-up. However, our results support more strongly the central role of the p53 tumour suppressor gene in multiple carcinogenesis of the upper aerodigestive tract, particularly because our results refer only to HNCP with well-documented multiple primary cancers. In addition, it is noteworthy to mention that p53-negative cases detected in our study may include carcinomas which arose independently of the p53 gene, but also may result from either deletion of both alleles or an alteration in transcription of the p53 gene. A further possibility is that, in such negative cases, point mutations of the p53 gene may result in the production of an unstable product undetectable immunocytochemically. Thus, our data could underestimate the actual role of the p53 gene mutations in negative specimens.

In accordance with several recent reports, we confirm the linkage existing between p53 expression and cigarette smoking [19, 20]. In fact, although most of our HNCP with multiple malignancies were moderate to severe smokers (84%), a lower p53 expression in both neoplastic and non-neoplastic specimens was observed in moderate or non-smokers than in heavy smokers. However, these data are limited by the low number of patients studied, of whom there were only two non-smokers.

In conclusion, our observations, as well as those of others [10, 18] indicate that p53 oncoprotein may be a potential biomarker of intermediate endpoints of multiple carcinogenesis. However, while p53 tumour suppressor gene expression seems to correlate with stage of carcinogenesis [9, 10], its validity should be closely related to the differential expression in normal and high-risk sites. However, our results showing that p53 expression in normal and dysplastic epithelium exposed to tobacco and/or alcohol is higher in HNCP with multiple malignancies than in healthy controls might indicate that this epithelium is at increased risk of transformation to carcinoma. Thus, the analysis of p53 expression may be a useful biomarker for assessing the potential high-risk sites for development of multiple cancers in HNCP.

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