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Low Prevalence of Expression of p53 Oncoprotein in Oral Carcinomas from Sri Lanka associated with Betel and Tobacco Chewing

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Overexpression of p53 oncoprotein has been demonstrated in a wide range of human malignancies. We have examined the p53 expression amongst 38 Sri Lankan subjects with histologically confirmed oral squamous cell carcinomas. The mean age of the subjects was 59.4 years and betel chewing with tobacco was the most common habit (84%) with a high percentage of patients smoking (63%). Buccal mucosa was the most frequently affected site (68%) with a high proportion (79%) of well differentiated carcinomas. p53 expression was examined by standard immuno-histochemical methods on frozen sections using monoclonal antibodies PAb 1801, 240 and 421. Only 4 (11%) carcinomas showed nuclear reactivity mostly in random clusters of basal neoplastic cells. The low frequency of p53 expression could be due to deletion of both alleles or to premature truncated protein products due to nonsense mutations resulting in loss of antibody recognition sites. Alternatively the much lower prevalence than reported by others could be due to differences in aetiological agents and/or genetic predisposition of this population.

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INTRODUCTION

ORAL CANCER accounts for 30–40% of all malignancies in South Asia [1, 2] including Sri Lanka [3]. Very often oral cancer in this population is preceded by a premalignant stage, frequently associated with betel quid chewing with or without tobacco [4] along with smoking [5, 6]. In Western populations tobacco consumption, predominantly smoked, is causally associated with oral [7], and lung cancers [8].

It is widely recognised that accumulation of genetic abnormalities, including those of oncogenes and oncosuppressor genes, play a key role in malignant transformation [9–11]. Benzo(α)pyrenes and nitrosamines present in tobacco [12] and nitrosamines derived from areca nut [13], are active carcinogens found in the betel quid. As these agents are known to cause genetic alterations in experimental carcinogenesis models [13, 14] it is possible that they may have a similar role in the pathogenesis of quid associated oral neoplasms.

Abberations of the p53 gene occur frequently in many types of neoplasm [15, 16]. The wild type p53 gene functions as an oncosuppressor gene, down-regulating cell growth [11, 18], while the mutant forms show dominant oncogenic properties favouring malignant transformation [19]. Tight evolutionary conserved regions observed in the p53 gene of different animal species [20] suggest the central role of this gene in growth regulation of cells. The involvement of p53 in controlling the cell cycle comes from observations of its expression in certain non-transformed cells, e.g. cells of 12–14 day old embryos [21] and NIH, 3T3 fibroblasts [22].

Expression of p53 in oral cancer has been assessed in Western populations [23, 24]. As the risk factors associated with oral carcinomas in south Asia are different to those of Western populations, the present study was undertaken to assess p53 expression in oral carcinomas from a group of patients in Sri Lanka.

PATIENTS AND METHODS

Patients

The study group was comprised of 33 histologically confirmed carcinomas and five clinically suspected premalignant lesions subsequently found to be malignant on histological examination. Most of the patients were selected from the Cancer Institute, Maharagama and a few from Teaching hospitals of Kandy and Peradeniya, Sri Lanka. Detailed histories related to betel chewing and smoking were recorded and clinical staging of the neoplasms [25] was performed in all but three clinically suspected cancers. An incisional biopsy specimen was taken from each patient encompassing clinical tumour and a portion of normal margin.

Tissue specimens

One third of each specimen was fixed in buffered formal saline and processed to paraffin for routine histology. The remaining piece was snap frozen in liquid nitrogen. Histological grading of carcinomas was performed according to Anneroth *et al.* [26] on a representative haematoxylin/eosin stained section.

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Table 1. *Tobacco habits of the patients with oral cancer* (n = 38)

	Male	Female	Total
Betel chewing			
With tobacco	27	5	32 (84%)
Without tobacco	1	2	3
Smoking			
Cigarettes	6	0	6
Cigars	2	0	2
Bedi	4	0	4
Mixed (smoking)			24 (63%)
Chewing + smoking			21 (55%)

Antibodies and immunohistochemistry

Three antibodies to p53 protein were used.

1. Monoclonal antibody (mouse) PAb 1801 (Oncogene Science), a human specific IgG_1 which recognises an epitope on p53 protein between amino acids 32–79 [27].
2. Monoclonal antibody (mouse) PAb 421 (a gift from the Imperial Cancer Research Fund), IgG_{2a} specific to mammalian p53, recognising an epitope located between amino acids 370–378 [28].
3. Monoclonal antibody (mouse) PAb 240 (Novocastra Laboratories), IgG_1 specific to mutant p53 which recognises a denaturation resistant epitope located between amino acids 156–330 [29].

A standard streptavidin–biotin complex protocol was employed as previously described from this laboratory [24] with all three antibodies, applied to 6–8 μm frozen sections. The following dilutions and conditions were used for each primary antibody: PAb 1801, 1:100 (60 min, at room temperature), PAb 421, 1:20 (overnight at 4°C) and PAb 240, 1:10 (overnight at 4°C). The positive control was a known positive case of colon carcinoma (courtesy of Dr A. Wyllie, Edinburgh) and for negative control the primary antibody was replaced by an irrelevant mouse monoclonal IgG_1 or IgG_{2a}.

RESULTS

The mean age of the group was 59.4, S.D. 12.7 (range 43–92) years; that of the males, 59.4, S.D. 13.6 ($n = 31$) and females 59.4, S.D. 12.7 ($n = 7$). Betel chewing with tobacco was the most common habit (84%) and a high percentage of patients (63%) also smoked. Both habits were present in more than half of the cases. None of the females smoked but all of them chewed betel with or without tobacco (Table 1). Carcinomas most frequently affected the buccal mucosa consistent with earlier descriptions of site distribution of oral carcinomas related to betel and/or tobacco chewing [3, 5]. An almost equal number of carcinomas distributed among stage 2, 3 and 4 (Table 2). Of the 38 neoplasms, 37 were squamous cell carcinomas, with a high proportion (79%) well differentiated: one was a spindle cell carcinoma (Table 2).

Table 2. *Clinical staging of patients and histological grading of cancers with reference to p53 expression*

		Clinical stage*				Histological grade		
	n	1	2	3	4	Well	Moderate	Poor
p53 + ve	4	1	0	0	2	1	2	1
P53 − ve	34	2	10	9	5	29	4	1

*Staging data were not available for 8 subjects.

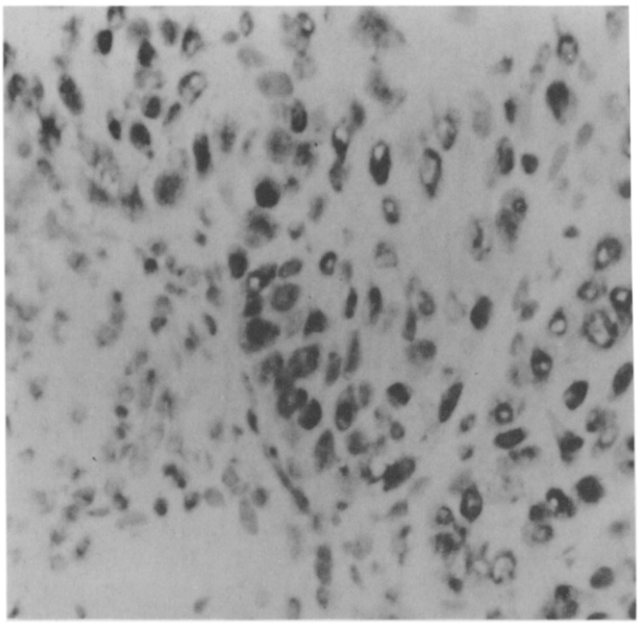

Fig. 1. Photomicrograph of a squamous cell carcinoma of the cheek showing a strong nuclear reaction against PAb 1801 (×100).

Only four (11%) carcinomas showed a positive nuclear reaction with all three antibodies (Fig. 1). Admixtures of positive and negative cells were present in focal areas and the staining intensities varied amongst the cells. The nuclear positivity was evident mostly in basal type cells and more differentiated cells stained negatively. A strong cytoplasmic staining was evident with PAb 421 in most specimens which were negative to the other two antibodies. However, one tumour showed a distinct nuclear reaction sparing the cytoplasm with PAb 421, whereas antibodies PAb 240 and 1801 gave negative results. PAb 1801 gave negative cytoplasmic staining with all but one specimen. The latter was a poorly differentiated carcinoma which gave a positive reaction both in the nucleus and cytoplasm.

A low proportion of well differentiated carcinomas (1/30, 3%) demonstrated p53 expression, with a higher proportion in moderately (2/6, 33%) and poorly differentiated carcinomas (1/2, 50%). No attempt was made, however, to assess the statistical significance of relationship to degree of differentiation as the number of lesions in the latter categories were too small.

DISCUSSION

In the present study 4/38 carcinomas showed nuclear positivity against all three antibodies. The staining profiles of PAb 1801 and PAb 240 were very similar. Cytoplasmic staining observed in some neoplasms could be due to p53 complexing with other cytoplasmic proteins such as heat shock proteins [30], and keratins [31].

The p53 expression was not related to age, sex or site of lesions. In the present series all the 4 patients who had enhanced p53 expression were heavy smokers or chewed betel with tobacco or had combined tobacco habits. Similar conclusions were reported in previous studies on tobacco related neoplasms of mouth [32] and carcinomas of the "head and neck" [33]. Nevertheless, there were many patients who chewed or smoked tobacco heavily but whose oral cancers failed to express p53 protein to detectable levels. With the

exception of one study [32], where a positive correlation has been observed between the intensity and distribution of p53 positive cells and STNMP score, the published data to date on oral [23, 24] and other "head and neck cancers" [33, 34] have not shown a positive relationship of p53 expression with either clinical staging or with pathological grading of the lesions. With such few positive samples in the present series it is not possible to address these questions.

The prevalence of p53 positive neoplasms in this study was low (11%) compared to that reported in previous studies carried out in the U.K., e.g. oral squamous cell carcinomas, 35% [24], 50% [23], 80% [32] and for "head and neck carcinomas" 35% [34] and 67% [35]. Similar disparities have been observed in relation to other neoplasms, e.g. basal cell carcinoma of skin, 0% [33] and 83% [35]. The use of different antibodies which do not show 100% concordance may account for some of the discrepancies. Furthermore the intensity of the p53 reaction product and the percentage of p53 positive cells in a specimen are critical parameters that determine the cut off point between p53 positive and negative cases.

It is important to address the question as to why p53 expression is infrequent in the present series. Firstly, allelic deletions of the p53 locus may account for under expression of the protein. A high frequency of allelic loss compared to overexpression of protein has been observed in certain carcinomas, e.g. oesophagus [36]. Secondly, the presence of nonsense mutations [15] and certain intronic mutations [37] may also result in underexpression of p53 protein. Finally, overexpression of p53 in certain neoplasms which do not show mutations has been documented [38, 39]. Such nonmutational p53 over expression may not be evident if these neoplasms contain human papilloma virus because the p53 protein is known to be susceptible to degradation by the E6 protein of the virus [40].

The p53 gene has oncogenic properties in its mutant form and functions as an oncosuppressor gene in its wild form [11]. Mutations which confer stability to p53 expression product provide an opportunity to visualise this protein using immunohistochemical methods, although as already noted, over expression without mutations [38, 39] has been reported. The limitations of immunohistochemical techniques in assessing p53 mutations are discussed elsewhere [41]. Even in the absence of mutations, if allelic deletions are common, it is possible that cells harbouring such deletions may have a growth advantage over their normal counterparts favouring malignant transformation. Therefore, the precise role of p53 oncosuppressor gene in betel quid related oral cancers can be determined only by assessing both mutations and allelic deletions.

In conclusion, proper evaluation of the role of p53 alterations in oral carcinogenesis, and certainly any diagnostic and prognostic implications of the findings, requires considerable further study. Many cases will need to be studied longitudinally recognising the different risk factors and natural histories in different population groups, the variability of techniques and the need for concurrent immunohistochemical and genetic studies.

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