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High Prevalence of Expression of p53 Oncoprotein in Oral Carcinomas from India Associated with Betel and Tobacco Chewing

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A recent study reported a low prevalence of p53 expression (11%) in oral squamous cell carcinomas (SCCs) from South Asia, in contrast to a high prevalence (averaging 52%) in other studies. It was proposed that the different aetiologies for oral SCCs in the South Asia population, i.e. betel and tobacco chewing in combination with smoking and alcohol consumption as compared to smoking and alcohol consumption alone in other populations, may account for the low prevalence of p53 expression. To confirm this hypothesis, we examined p53 expression immunohistochemically in 23 cases of oral SCC from patients in Southern India. Thirteen of the 23 SCCs (56.5%) demonstrated nuclear p53 staining. The expression of *p53* was strongly correlated with the number of tobacco-containing quids chewed per day ($r=0.8$). These data support the hypothesis that carcinogens derived from tobacco and betel chewing may induce p53 mutations, which in turn are involved in the development of oral cancer.

Keywords: oral cancer, p53, smoking, betel quid

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INTRODUCTION

ORAL SQUAMOUS cell carcinomas (SCCs) account for about 2–4% of all cancers in Western populations, and are attributed mainly to smoking and alcohol consumption [1]. In contrast, oral SCC is one of the leading cancers in Southeast Asia, accounting for 30–40% of all cancers. These cancers are frequently associated with the chewing of tobacco-containing betel quids, in addition to smoking and alcohol consumption [2].

The *p53* gene is currently one of the most studied genes in cancer research. While the mutant form of *p53* acts as an oncogene, wild-type *p53* acts as a tumour suppressor gene and negatively regulates cell growth. Mutations or increased expression of the p53 protein have been identified in over 50% of diverse malignancies, including cancers of the lung, breast, nasopharynx and oesophagus [3]. As summarised in Table 1, a high prevalence (average 52%) of p53 expression has been reported in oral SCCs [4–17]. These studies focused on populations with Western lifestyles. However, a recent study by Ranasinghe and associates [18] on oral SCCs from Sri Lanka, a part of the Indian subcontinent, has shown a low prevalence of p53 expression with only 11% of the samples positive for p53 expression. The authors suggested two possible explanations for the low prevalence: the presence of different aetiologic factors or alternatively, the genetic and

Table 1. Immunohistochemical analysis of p53 oncoprotein in human oral squamous cell carcinomas

Authors	No. of SSC cases	No. of p53+ cases (%)
Field *et al.*, 1991 [4]*	73	49 (67%)
Gusterson *et al.*, 1991 [5]*	47	16 (34%)
Ogden *et al.*, 1992 [6]	27	14 (52%)
Langdon and Partridge, 1992 [7]	15	12 (80%)
Warnakulasuriya and Johnson, 1992 [8]	27	13 (35%)
Watling *et al.*, 1992 [9]	19	8 (42%)
Zhang *et al.*, 1993 [10]	10	7 (70%)
Nishioka *et al.*, 1993 [11]	40	21 (52%)
Caamano *et al.*, 1993 [12]	17	7 (41%)
Girod *et al.*, 1993 [13]	85	46 (54%)
Burns *et al.*, 1993 [14]	19	7 (37%)
Mathews *et al.*, 1993 [15]	40	12 (30%)
Field *et al.*, 1994 [16]*	93	63 (68%)
Shin *et al.*, 1994 [17]*	33	15 (45%)
Total (average %)	555	290 (52%)

*Study performed on head and neck SCC including oral SCC.

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Table 2. *Patients' data and p53 staining results*

Case no.	Age (years)/ gender	Chewing (quid per day)	Smoking (bidis* per day)	Alcohol (ounces per day)	Contents of quid†	p53 staining intensity	p53 staining pattern
1	40/F	5–9	0	0	BNTL	+	Scattered
2	45/F	10–15	0	0	BNTL	+ +	Diffuse
3	50/F	10–15	0	0	BNTL	+ + +	Diffuse
4	52/M	0	0	10+	0	—	
5	52/F	NA	NA	NA	NA	—	
6	55/M	5–9	20+	5–9	BNTL	+ + +	Diffuse
7	55/M	<5	5–9‡	0	BNT	—	
8	57/M	10–15	20+	5–9	BNTL	+ + +	Diffuse
9	57/M	5–9	<5	<5	BNTL	+ +	Diffuse
10	59/F	5–9	0	0	BNTL	—	
11	60/F	5–9	0	0	BNTL	—	
12	60/F	NA	NA	NA	NA	—	
13	60/M	<5	0	0	BNT	—	
14	60/M	5–9	5–9	0	BNTL	+ +	Diffuse
15	60/F	10–15	0	0	BNTL	+ +	Scattered
16	61/F	<5	0	0	BNTL	—	
17	62/M	<5	<5	0	BNTL	—	
18	62/F	5–9	0	0	BNTL	+ +	Diffuse
19	64/M	10–15	20+	5–9	BNTL	+ + +	Diffuse
20	64/M	10–15	5–20	0	BNTL	+ +	Diffuse
21	65/M	5–9	5–20	0	BNTL	+ +	Diffuse
22	66/F	10–15	0	0	BNTL	+ + +	Diffuse
23	75/F	<5	0	0	BNTL	—	

*Bidis; local cigarettes made by wrapping less than 0.5 g of coarse tobacco in a dry temburni leaf.
†Abbreviations: B = betel leaf; N = areca nut; T = tobacco; L = lime; NA = information not available.
‡Smoking cigarettes instead of bidis.

ethnic differences in the population. This study was designed to test this hypothesis by looking at the expression of p53 oncoprotein in a tobacco-betel chewing population.

PATIENTS AND METHOD

Case selection

Twenty-three formalin-fixed and paraffin-embedded oral SCC specimens (mostly well-differentiated SCC) dating from 1990 to 1993 were selected from the archives at the Division of Oral Pathology, Government Dental College, University of Kerala, Trivandrum, Kerala, India. Trivandrum is about 300 miles from Kandy and Peradeniya, Sri Lanka where Ranasinghe *et al.* [18] obtained their cases. The patients were Indian ethnic and mostly (90.5%) from low socio-economic classes, primarily manual labourers in fisheries and farming, and non-professional office workers. The four main ingredients of the quid in Kerala are: areca nut (fresh), tobacco (dried and processed), slaked lime (from seashells) and betel leaf (fresh). Information on habits was available for 21/23 patients (Table 2). 20 of the 21 patients were quid chewers and all of them had chewing tobacco included in their quid. In addition to quid chewing, 9/21 (43%) patients smoked bidi (a local form of cigarette made by wrapping approximately 0.5 g coarse tobacco in a dry temburni leaf) and 5/21 (24%) patients consumed home-brewed liquor (Arrack/Toddy) which contains about 40–50% ethanol. All but 2 patients used lime in the quids. The mean age of the patients was 58 years, including 12 females and 11 males.

Immunohistochemical staining

Staining was performed on paraffin sections with monoclonal anti-p53 antibody (DO-7, Dimension Lab, Ontario, Canada), using a standard avidin–biotin–peroxidase complex (ABC) method similar to our previous experiment [10]. The intensity of p53 staining was classfied into: negative (−); weakly positive (+); moderately positive (+ +); and strongly positive (+ + +). The pattern of positive staining was divided into diffuse when the staining was generalised or scattered when only occasional tumour cells were positive.

RESULTS

The expression of p53 protein was detected in 13 of the 23 (56.5%) SCCs in this study (Table 2). All p53 staining was located exclusively in cell nuclei. The majority of the cases showed moderate to strong diffuse p53 staining (Fig. 1).

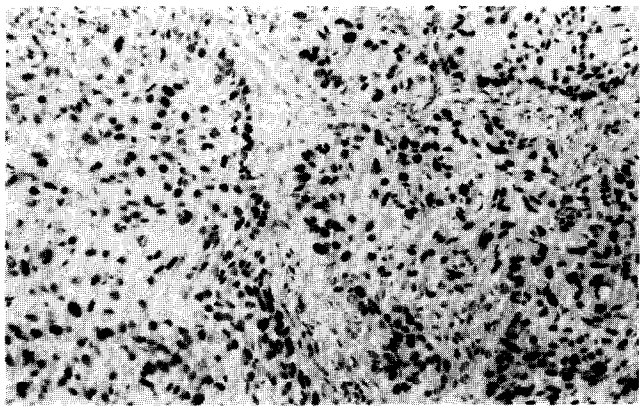

Fig. 1. Photomicrograph of a well-differentiated oral squamous cell carcinoma demonstrating diffuse strong nuclear p53 staining. Original magnification ×80.

Table 3. The relationship between quid chewing and the expression of p53

	Number of patients	
	Chewing ≥5 quids/day	Chewing <5 quids/day or no quid
p53 negative	2	6
p53 positive	13	0

Table 4. The relationship between p53 staining intensity and the amount of tobacco used

Category	p53 staining			
	− or ±	+	++	+++
No quid, or <5 quids/day with or without smoking	6	0	0	0
5–9 quids/day without smoking	2	1	1	0
5–9 quids/day with smoking	0	0	3	1
10–15 quids/day with or without smoking	0	0	3	4

There was a strong correlation between the number of tobacco-containing quid chewed daily and the expression of p53 ($r=0.8$, Table 2). The cancer from the 1 patient who did not chew quid was p53 negative and all five SCCs from patients chewing less than five quids per day were p53 negative, while 13/15 (94%) SCCs from patients chewing five or more quids per day were p53 positive (Table 3). The difference was highly significant ($P=0.0005$, χ^2). Additionally, there seemed to be a tendency towards higher intensity of p53 staining in SCCs from patients consuming higher amounts of tobacco, regardless of the forms (Table 4).

DISCUSSION

It is well known that *p53* mutation and over-expression can be influenced by the cancer aetiology. Smoking has been found to be positively correlated with p53 expression in tumours of a number of tissues, including the oral mucosa [16, 19]. Most of the *p53* mutations found in the smoking-related cancers have been G:C to T:A transversion and it is believed that benzo[a]pyrene derived from cigarette smoking is responsible for this as it can induce such substitution.

Carcinogens in tobacco-betel chewing are different from cigarette smoking and are more complex. Most of the quid components generate carcinogens, such as specific nitrosamines and alkaloids from chewing tobacco and areca nut, phenolic compounds from areca nut, and reactive oxygen species from lime, while betel leaf is considered anticarcinogenic [20]. It seems conceivable that the low prevalence of p53 expression in oral SCCs from a tobacco-betel chewing population, as shown in the study by Ranasinghe *et al.* [18], may be a reflection of different pathways of oral carcinogenesis caused by different carcinogens in tobacco-betel chewing as compared with smoking. Our results, however, revealed a high prevalence of p53 expression in a similar tobacco-betel chewing East Indian population. The finding of a strong correlation between p53 expression and the number of tobacco-containing quids chewed per day in the present study is also supportive of the finding of high prevalence of p53 expression in a population of habitual tobacco-betel chewers.

What caused the differences in the expression of p53 protein in oral cancers from two populations that have been exposed to apparently similar carcinogens remains speculative. It is possible that the local genetic composition of the patients plays a role. The population in Sri Lanka is more homogeneous than that of Kerala and mainly consists of Sinhalese (70%) and also offsprings of two castes of agricultural labourers migrated from Tamil Nadu in Southern India [21, 22], although the populations in both regions are mostly descendants of Dravidians and Indo-Aryans. People from the suburban coastal Kerala area are less likely to have malnutrition than low-income tea-estate labourers from the rural mountainous regions of Kandy [22]. However, there has been no report demonstrating that nutrition could affect p53 expression, even though malnutrition may contribute to cancer through the loss of protection by nutrients.

Differences in experimental method may also contribute to the differences in p53 prevalence. Many factors in experimental methodology could affect the experimental results. Of particular importance are: the type of antibody used; the length and method of tissue fixation and paraffin processing; the use of any epitope unmasking procedure; and the criteria used for designating positivity and negativity. Since Ranasinghe *et al.* [18] used frozen tissue, and there was no fixation, tissue processing and antigen retrieval, the possible variations in experimental method seem focused on the types of antibody used and the criteria on positivity designation. Other investigators who have studied frozen tissues using the same monoclonal p53 antibodies as Ranasinghe *et al.* [18], however, found a high p53 prevalence in oral SCCs [7, 8].

The most likely explanation should lie in the quid, as it has been shown that even minor variations in the components of quid, or the pattern of quid chewing, could affect the genetic damages to the oral mucosa. For example, pH levels in saliva of quid chewers have been found to correlate well with genotoxic damages in the oral mucosa [23]. Lime is responsible for the temporary pH elevation. Variation in the types of lime used (with different pH), the amount of lime used, the duration of use each time and daily frequency of use will all affect genotoxic damages to the oral mucosa. Naturally we have looked hard for differences in either the ingredients or the pattern of chewing of quid between the two regions. We could find no documented differences in the ingredients of quid between the two regions in the literature.

There is some evidence suggesting that quid chewing in Kerala is more frequently daily and that the quid is kept in the mouth for a longer period of time at each chewing, or sometimes even kept overnight when sleeping [24], as compared to that in Sri Lanka [21]. Studies have shown that daily frequency of quid chewing is the strongest predictor for oral cancer [25]. The fact that Kerala has the highest incidence of oral cancer in India [26] also suggests that people in Kerala chew more daily. Since our results showed that the duration of daily quid exposure was critical for p53 expression, the low prevalence of p53 in Sri Lanka may be attributed to a shorter daily quid exposure.

One striking difference does exist between the two regions. Kerala has the highest radiation level in India as one of the five major naturally radioactive zones in the world with a background radiation at least three times the maximum

permissible (threshold) dose for a population in general [26]. The radioactivity arises mainly from the presence of monazite in the sand, and the principle radionuclides in monazite are from the Th^{232} series although some radionuclides from U^{238} are also present [27]. A study of regions of India without high natural radiation showed the presence of radionuclides in all four main components of quid [28]. The highest concentration of radionuclides was found in slaked lime from seashells and its concentration in lime was about 50–100 times that in tobacco leaves. Betel leaves also contained high concentrations of radionuclides as compared to that in other food and plant families. It is possible that tobacco, betel leaves and areca nuts that are grown in soils with a high radionuclide content and seashells formed from sand with a high radionuclide content in Kerala may contain a higher amount of radionuclides than those in Sri Lanka. When used frequently and in large amounts, as in the habitual tobacco-betel quid chewers, the quid with a possibly higher concentration of radionuclides in Kerala may be partially responsible for the *p53* mutation in oral cancer from Kerala, as radiation has been reported to cause *p53* mutation [29]. Studies are needed to confirm this hypothesis.

Two reports have recently been published on p53 expression in quid chewing populations. Similar to our study, a study from Northern India [30] demonstrated a high prevalence of p53 expression (75%) in oral SCC from a tobacco-betel chewing population. In contrast, another study from Papua New Guinea [31] showed a low frequency of *p53* mutation (10%) in oral SCC from a population chewing betel quid containing no tobacco. It would seem that tobacco used in chewing mixtures, similar to tobacco used in smoking, is a critical factor in *p53* mutation.

In summary, our study demonstrated a high prevalence of p53 expression in oral SCC from a habitual tobacco-betel chewing population. The differences between our results and those of Ranasinghe *et al.* [18] may be attributed to differences in the quid, i.e. the ingredients of quid, and patterns of chewing. Oral cancer is the most common cancer in Southeast Asia and some other parts of the world, and chewing a tobacco-containing mixture is considered the most important risk factor for oral cancer in these regions. Worldwide, more than 600 million individuals chew carcinogenic tobacco-containing mixtures with great variations in the ingredients of these mixtures and patterns of chewing [23]. Since these variations could affect the genetic pathways of oral cancer development, future studies on the genetics of oral cancer should document these populations well, particularly the ingredients and pattern of chewing. With this detailed information, we should be able to better understand the molecular mechanisms of oral cancer development.

1. Boring C, Squires T, Tong T. Cancer statistics 1992. *CA, Cancer J Clin* 1992, **42**, 19–38.
2. Kuriakose M, Sankaranarayanan R, Nair MK, *et al.* Comparison of oral squamous cell carcinoma in younger and older patients in India. *Oral Oncol, Eur J Cancer* 1992, **28B**, 113–120.
3. Soussi T, Legros Y, Lubin R, Ory K, Schlichtholz B. Multifactorial analysis of p53 alteration in human cancer: A review. *Int J Cancer* 1994, **57**, 1–9.
4. Field JK, Spandidos DA, Malliri A, Gosney JR, Yiagnisis M, Stell PM. Elevated p53 expression correlates with a history of heavy smoking in squamous cell carcinoma of head and neck. *Br J Cancer* 1991, **64**, 573–577.
5. Gusterson BA, Anbazhagan R, Warren W, *et al.* Expression of p53 in premalignant and malignant squamous epithelium. *Oncogene* 1991, **6**, 1785–1789.
6. Ogden GR, Kiddie RA, Lunny DP, Lane DP. Assessment of p53 protein expression in normal, benign and malignant oral lesions. *J Pathol* 1992, **166**, 389–394.
7. Langdon JD, Partridge M. Expression of tumor suppressor gene p53 in oral cancer. *Br J Oral Maxillofac Surg* 1992, **30**, 214–220.
8. Warnakulasuriya KAAS, Johnson NW. Expression of p53 mutant nuclear phosphoprotein in oral carcinoma and potentially malignant oral lesions. *J Oral Pathol Med* 1992, **21**, 404–408.
9. Watling DL, Gown AM, Coltrera MD. Over-expression of p53 in head and neck cancer. *Head Neck* 1992, **14**, 437–444.
10. Zhang L, Rosin MP, Priddy R, Xiao Y. p53 expression during multi-stage human oral carcinogenesis. *Int J Oncol* 1993, **3**, 735–739.
11. Nishioka H, Hiasa Y, Hayashi I, Kitahori Y, Konishi N, Sugimura M. Immunohistochemical detection of p53 oncoprotein in human oral squamous cell carcinomas and leukoplakias: comparison with proliferating nuclear antigen staining and correlation with clinicopathological findings. *Oncology* 1993, **50**, 426–429.
12. Caamano J, Zhang SY, Rosvold EA, Bauer M, Klein-Szanto AJ. p53 mutations in human squamous cell carcinomas and carcinoma cell lines. *Am J Pathol* 1993, **142**, 1131–1139.
13. Girod SC, Krueger G, Pape H-D. p53 and Ki-67 expression in preneoplastic and neoplastic lesions of the oral mucosa. *Int J Oral Maxillofac Surg* 1993, **22**, 285–288.
14. Burns JE, Baird MC, Clark LJ, *et al.* Gene mutations and increased levels of p53 protein in human squamous cell carcinomas and their cell lines. *Br J Cancer* 1993, **67**, 1274–1284.
15. Mathews JB, Scully C, Jovanovic A, Van der Waal I, Yeudall WA, Prime SS. Relationship of tobacco/alcohol use to p53 exposure in patients with lingual squamous cell carcinomas. *Oral Oncol, Eur J Cancer* 1993, **29B**, 285–289.
16. Field JK, Zoumpourlis V, Spandidos D, Jones AS. p53 expression and mutations in squamous cell carcinoma of the head and neck: expression correlates with the patients' use of tobacco and alcohol. *Cancer Detect Prev* 1994, **18**, 197–208.
17. Shin DM, Kim J, Ro JY, *et al.* Activation of p53 gene expression in malignant lesions during head and neck tumorigenesis. *Cancer Res* 1994, **54**, 321–326.
18. Ranasinghe AW, Warnakulasuriya KAAS, Johnson NW. Low prevalence of expression of p53 oncoprotein in oral carcinomas from Sri Lanka with betel and tobacco chewing. *Oral Oncol, Eur J Cancer* 1993, **29B**, 147–150.
19. Zhang Z-F, Sarkis AS, Cordon-Cardo C, *et al.* Tobacco smoking, occupation, and p53 nuclear over-expression in early bladder cancer. *Cancer Epidemiol Biomark Prev* 1994, **3**, 19–24.
20. IARC. International Agency for Research on Cancer. Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans. *Tobacco Habits other than Smoking; Betel Quid and Areca Nut Chewing and some Related Nitrosamines.* Lyon, IARC Press, 1985, Vol. 37.
21. Senewiratne B, Uragoda CG. Betel chewing in Ceylon. *Am J Trop Med Hyg* 1973, **22**, 418–422.
22. Gajanayake I, Caldwell JC, Caldwell P. Why health is relatively poor on Sri Lanka's tea estates? *Soc Sci Med* 1991, **32**, 793–804.
23. Stich HF, Rosin MP. Towards a more comprehensive evaluation of a genotoxic hazard in man. *Mut Res* 1985, **150**, 43–50.
24. Sankaranarayanan R. Oral cancer in India: an epidemiologic and clinical review. *Oral Surg Med Pathol* 1990, **69**, 325–330.
25. Sankaranarayanan R, Mohideen MN, Nair MK, Padmanabhan TK. Aetiology of oral cancer in patients less than or equal to 30 years of age. *Br J Cancer* 1989, **59**, 439–440.
26. Rajendran R, Raju GK, Nair SM, Balasubramaniam G. Prevalence of oral submucous fibrosis in the high natural radiation belt of Kerala, South India. *Bullet WHO* 1992, **70**, 783–789.
27. Radhakrishna AP, Somashekarappa HM, Narayana, Y, Siddappa K. A new natural background radiation area on the southwest coast of India. *Health Phys* 1993, **65**, 390–395.
28. Chakravarti SK, Dhiman J, Nagpaul KK. Uranium trace analysis of a chewable betel-leaf preparation and tea leaves. *Health Phys* 1981, **40**, 78–81.
29. Vähäkangas KH, Samet JM, Metcalf RA, *et al.* Mutations of *p53* and *ras* genes in radon-associated lung cancer from uranium miners. *Lancet* 1992, **339**, 576–580.

30. Kaur JA, Srivastava A, Ralhan R. Over-expression of p53 protein in betel- and tobacco-related human oral dysplasia and squamous cell carcinoma in India. *Int J Cancer* 1994, **58**, 340–345.
31. Thomas S, Brennan J, Martel G, *et al.* Mutations in the conserved regions of p53 are infrequent in betel-associated oral cancers from Papua New Guinea. *Cancer Res* 1994, **54**, 3588–3593.

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