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Differential expression of p53, p63 and p73 proteins in human buccal squamous-cell carcinomas

Y.K. CHEN, S.S. HUSE & L.M. LIN

Oral Pathology Department, School of Dentistry, Kaohsiung Medical University, Kaohsiung, Taiwan

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Abnormalities in the *p53* gene are regarded as the most consistent of the genetic abnormalities in oral squamous-cell carcinoma. Two new members of the *p53* gene family, *p73* and *p63*, have recently been identified, with the three sharing considerable sequence homology at the acidic N-terminal transactivation, central DNA-binding and C-terminal oligomerization domains, indicating possible functional and biological interactions. The differential expression of *p73*, *p63* and *p53* genes in human oral squamous-cell carcinoma does not yet appear to be completely understood, however. In this study, therefore, immunohistochemical analysis of protein expression was performed for 40 samples of well-differentiated human buccal squamous-cell carcinomas, with 10 specimens of normal buccal mucosa employed as controls. Differential expressions of p63, p73 and p53 proteins in the carcinoma samples were: p63+/p73+/p53 + ($n = 28$; 70%); p63+/p73+/p53– ($n = 4$; 10%); p63+/p73–/p53– ($n = 8$; 20%), respectively; and p63+/p73+/p53– for normal mucosa ($n = 10$; 100%). A significant correlation between p53, p63 and p73 immunoexpression was demonstrated for the buccal squamous-cell carcinoma samples ($P < 0.0001$; Fisher's exact test). Significance was not achieved for the correlation between p73 and p53 immunoexpression and clinicopathological parameters for buccal carcinomas ($P > 0.05$; Fisher's exact test). Our results indicate that both p73 and p63 may be involved in the development of human buccal squamous-cell carcinoma, perhaps in concert with p53.

Keywords *p53* *p63* *p73* *oral squamous-cell carcinoma*

The *p53* gene is one of those most frequently implicated in human cancer,[1] including oral variants of the disease.[2] Recently, two new *p53* homologues have been identified.[3,4] Each gene, containing 14/15 exons, is ~65 kb with a high degree of similarity in exon/intron organization. Very significant regions of *p53*-sequence identity have been demonstrated for *p73*, the first discovered *p53* homologue, which is located on chromosome 1p36, with extensive homologies in the transcriptional-activation, DNA-binding and oligomerization domains.[5] When over-produced, p73 can activate the transcription of *p53*-responsive genes and also induce apoptosis,[6] however, *p73* mutations are rare in human tumours, and altered levels of expression may occur.[7] Initially termed KET, the second *p53* homologue was identified by Schmale and Bamberger in 1997.[8] Other groups have independently identified this gene, which has been variously named, CUSP,[9] p40,[10] p73L,[11] p51[12] and p63.[13] Although the reported amino-acid sequences and molecular weights are different, it has been proven that these are isotypes derived from a single gene with at least three alternative splicing patterns (α, β and γ), two promoters and two 3′-end exons.[14] Further, it has been shown that this *p63* gene, located on human chromosome 3q, possesses p53 homology in the DNA-binding, transactivation and oligomerization domains.[13,14] It has also been reported that p63 can, at least when overproduced, activate the transcription of p53-responsive genes and inhibit cell growth by inducing apoptosis, similar to p53.[6] Unlike p53, however, both p73 and p63 have multiple splice variants (p73α, β, γ and δ; p63α, β and γ), which have different biological characteristics and cellular specificities.[15] As discussed above, both p73 and p63 share considerable sequence homology with p53,

Correspondence: L.M. Lin, Oral Pathology Department, School of Dentistry, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung, Taiwan (e-mail: k0285@ms22.hinet.net).

indicating possible functional and biological interactions. The differential expression of p73, p63 and p53 proteins in human oral squamous-cell carcinomas, however, does not yet appear to be completely understood. Therefore, the aim of this study was to investigate the immunoexpression of p73, p63 and p53 proteins in human buccal squamous-cell carcinomas in order to obtain more data for elaboration of their role in human oral squamous-cell carcinogenesis.

Materials and methods

STUDY POPULATION

Specimens of well-differentiated, primary squamous-cell carcinomas of the buccal mucosa were obtained from tissue samples of surgically resected tumours obtained from 40 men aged between 42 and 70 years (mean 52) treated at our institution. None of the patients had a medical history that included radiotherapy or chemotherapy, and all had undergone complete surgical excision of the tumour and elective neck dissection. All individuals had been exposed to risk factors such as betel-quid chewing, cigarette smoking or alcohol consumption. Maximum follow-up was 5 years, with survival regarded as the number of years from surgery to eventual death or to the final visit at our institution. Of the 26 patients who had expired during the follow-up period, mean survival time was ~2 years (range 0.8–4.8). Mean survival time for the remaining patients was 4.2 years (maximum 5). Tumours were graded according to the World Health Organization classification, with all of the tumours deemed well-differentiated. Staging of tumours was established according to the American Joint Committee on Center. The clinical characteristics of the patients, including tumour stage and recurrence, lymph-node involvement and survival time are summarized in Table 1. Samples of normal buccal-mucosa tissue taken from 10 healthy individuals aged between 36 and 62 years (mean age 47), none of whom had ever chewed betel-quid or smoked cigarettes, were used as controls. All tissues (including the normal specimens) were collected after informed consent had been obtained from the patient; the research was also approved by the Ethics Committee for Scientific Research on Human Beings, which supervises such investigations at our institution. The surgically resected buccal tissue was fixed in 10% neutral buffered formalin solution for about 24 h, dehydrated in graded alcohols, cleared in xylene and embedded in paraffin for immunohistochemical study.

Table 1. Correlations between p73 and p53 immunoexpression and clinicopathological parameters for buccal carcinomas

Variables	No. of cases	p73 (+)	*P*-value	p53 (+)	*P*-value
Stage					
I–II	8	5		4	
III–IV	32	27	0.1665	24	0.1675
Recurrence					
Yes	15	13		11	
No	25	19	0.4142	17	0.7216
Lymph-node involvement					
Yes	26	23		18	
No	14	9	0.0683	10	0.8850
Survival status					
Dead	26	22		16	
Alive	14	10	0.3200	12	0.1115

IMMUNOHISTOCHEMISTRY

Immunohistochemical staining was performed using the standard avidin–biotin–peroxidase complex (ABC; Dako, Santa Barbara, CA, USA) method.[16] The primary antibodies used were polyclonal antibody raised against p73 (Cat. No. sc-7957, 1 : 100 dilution; Santa Cruz Biotechnology, USA), monoclonal antibody for p63 (clone 4A4, 1 : 100 dilution; Santa Cruz Biotechnology, USA), and monoclonal antibody for p53 (DO-7, 1 : 100 dilution; Novocastra, Newcastle, UK). Rabbit polyclonal antibodies to p73 were raised against a recombinant protein corresponding to amino acids 1–80, mapping at the amino terminus of human-origin p73. The p63 antibody was raised against amino acids 1–205 mapping at the amino terminus of ΔNp63. According to the manufacturer's specifications, these antibodies of p73 and p63 react broadly with all the known p73 and p63 variants of human, rat and mouse origin, respectively, as determined by Western blotting and immunohistochemistry (including paraffin-embedded sections). The specificity of the anti-p63 antibody has been previously demonstrated in a variety of immunoblotting experiments, as well as in analogous immunohistochemical-staining studies of mouse tissues from which the *p63* gene had been deleted.[13,17] The p53 DO-7 antibodies detected both wild-type and mutant forms of *p53*.[18] Tissue sections were mounted on gelatin–chrome alum-coated slides. Subsequent to de-paraffinization in xylene (twice) and re-hydration with a descending ethanol series (absolute, 95%, 70% and 30% ethanol, and water), tissue sections were microwave-treated thrice (5 min each) in 10 mM citrate buffer (pH = 6.0) to retrieve antigenicity. The tissue sections were then treated in 0.3% H_2O_2–methanol and 10% normal goat serum (Dako; Santa Barbara, CA, USA). All sections were subsequently incubated with the primary antibodies (p73 and p63, room temperature for 60 min each; p53, 4 °C overnight). These sections were then incubated for a further 30 min at room temperature in the presence of biotinylated goat anti-rabbit IgG for p73, and biotinylated goat anti-mouse IgG for p63 and p53 (both 1 : 100; Vector, Burlingame, USA) and then for a final 30 min with ABC. The peroxidase-binding sites were visualized as brown reaction products of the benzidine reaction. The sections were subsequently counter-stained with

haematoxylin. Positive and negative controls were used for each experiment. As p73, p63 and p53 are nuclear proteins, only nuclear positivity was assessed. Immunohistochemical staining was classified as negative if no/positive staining was present for l0% of the cells or below or positive where more than 10% were positively stained.

Results

Using immunohistochemical techniques, nuclear staining of p73, p63 and p53 proteins was detected in a subset of buccal squamous-cell carcinomas (Tables 2 and 3) and normal buccal mucosa. Differential expressions of p63, p73 and p53 proteins in carcinomas were: p63+/p73+/p53+ ($n = 28$; 70%); p63+/p73+/p53– ($n = 4$; 10%); p63+/p73–/p53– ($n = 8$; 20%), respectively; and p63+/p73+/p53– for normal mucosa ($n = 10$; 100%).

Nuclear staining of p73 and p63 was noted in the basal layers of the normal buccal mucosa, while, by contrast, p53 expression was not noted in the normal epithelium. For the carcinoma samples, both p73 and p63 immunoreactivity were chiefly observed for the less-differentiated cells located at the periphery of carcinomatous clusters (Figs 1 and 2). For p53 staining, positive labelling was demonstrated for some cells in the upper layers of the tumour islands (Fig. 3). Staining for p73, p63 and p53 was not detected in the keratin-pearl areas (Figs 1–3).

Table 2. Differential expression of p73 and p53/p63 for human buccal carcinomas

	p73		
p53/p63	+	–	*P*-value
p53			
+	28	0	<0.0001
–	4	8	
p63			
+	32	8	<0.0001
–	0	0	
Total	32	8	

Table 3. Differential expression of p53 and p63 for human buccal carcinomas

	p53		
p63	+	–	*P*-value
+	28	12	<0.0001
–	0	0	
Total	28	12	

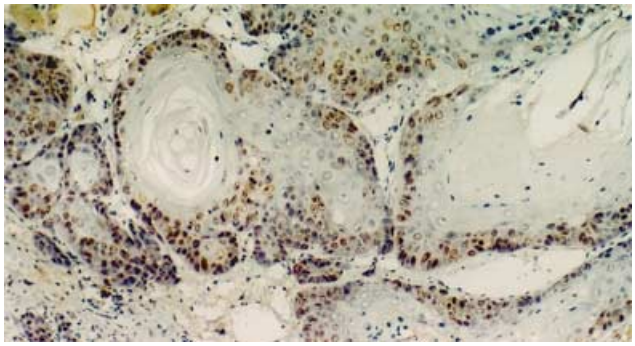

Figure 1. Representative section of a buccal-carcinoma specimen revealing p73 immunoreactivity, chiefly observed for the less-differentiated cells located at the periphery of tumour islands. Note the lack of staining in the keratin-pearl area (avidin–biotin–peroxidase complex (ABC) stain, 100×).

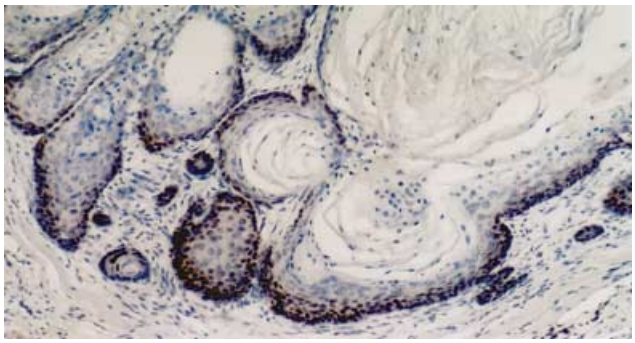

Figure 2. Representative section of a buccal-carcinoma specimen revealing p63 immunoreactivity was negative for the keratin-pearl area and mainly found for the less-differentiated cells situated at the periphery of tumour islands (ABC stain, 100×).

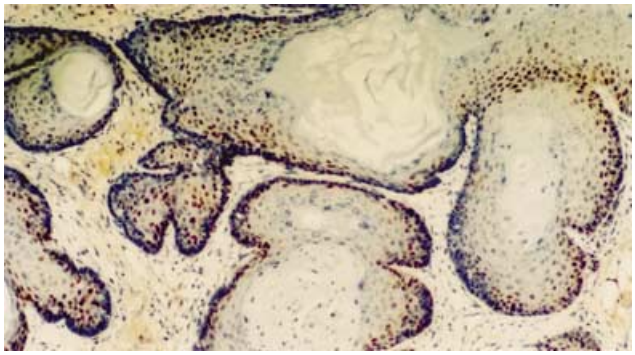

Figure 3. Representative section of a buccal-carcinoma specimen revealing p53 immunoreactivity, chiefly observed for the less-differentiated cells located at the periphery of tumour islands. Positive labelling is also demonstrated for some cells in the upper layers of the tumour islands. No staining was noted in the keratin-pearl area (ABC stain, 100×).

Statistically significant correlation was demonstrated between p53, p63 and p73 immunoexpression in the buccal squamous-cell carcinomas ($P < 0.0001$, Fisher's exact test; Tables 2 and 3). However correlation between p73 and p53 and clinicopathological parameters in buccal carcinomas was not significant ($P > 0.05$, Fisher's exact test; Table 1).

Discussion

Although the role of p73 and p63 proteins in the development of human carcinomas in different organs have been investigated in a number of studies,[19–24] only three of these have examined differential expression for all of the p53 homologues, namely, p73, p63 and p53 proteins, for head and neck squamous-cell carcinomas, including oral variants of the disease.[25–27] However conclusive results were not obtained from these three reports.[25–27] For instance, Choi *et al.* (2002) have reported up-regulation of p73 expression in head and neck tumorigenesis[27] while down-regulation of p73, possibly in concert with p53, was implicated in head-neck squamous epithelium carcinogenesis by Faridon-Laurens *et al.* (2001)[25] whereas, in the study of Weber *et al.* (2002) neither p73 nor p63 were associated with p53.[26] In our study, the differential expression of p73, p63 and p53 proteins was characterized for a subset of human oral primary squamous-cell carcinomas arising from the buccal mucosa, with further elucidation of the role for all of the p53 homologues in human oral squamous-cell carcinogenesis. In the present study, a significant correlation was demonstrated for the p53, p63 and p73 immunoexpression in buccal squamous-cell carcinomas, indicating that p73 and p63 may participate in oral carcinogenesis in concert with p53. Furthermore, the findings of the present study partly corroborate earlier work pertaining to the expression profiles of p73, p63 and p53 for head and neck squamous-cell carcinomas, with a significant correlation demonstrated between staining for p73 and p63, but not between p73/p63 and p53. As such differences may be because of the heterogeneity of the head and neck tumours enrolled in the previous studies;[25–27] a homogenous and well-characterized series of buccal squamous-cell carcinomas was utilized for our investigation. Further, the positive correlation demonstrated between p73/p63 and p53 has, in fact, been underestimated in previous research into head and neck squamous-cell carcinomas,[25–27] even though the examined specimens included oral variants of the disease.

Additionally, despite an intensive search of the available literature, mutation of the *p73* and *p63* genes has rarely been detected in human cancers.[6,14] Judging from these reported results, it remains possible that wild-type p73 and p63, but not the mutant forms, may have been expressed in the cancerous oral keratinocytes detected in the present study, alluding to the possibility that both p73 and p63 may play an oncogenic role in oral carcinogenesis through the expression of wild-type forms rather than as tumour suppressors via the mutant forms. Identifying the specific mechanism reflected in the correlation between p73, p63 and p53 protein expression in oral cancerous keratinocytes is critical if we are to elucidate the role of p73 and p63 in oral oncogenesis. This is possibly a result of the disruption of normal p53 function, resulting in a compensatory up-regulation of p73 and p63 expression, such that either the production mutant p53 or a reduction in $p21^{WAF/CIP1}$ may also trigger an increase in the expression of p73 and p63.[6]

In this study, p73 and p63 were frequently expressed simultaneously and were positively correlated with each other, suggesting a synergistic effect with respect to tumour development in the oral cavity. Our data is also consistent with previous observations for head and neck squamous-cell carcinomas[25–27] and other solid tumours.[19–24] Noteworthy, staining of either p73 and p63 (p63+/p73+/p53-; $n = 4$), or, p63 only (p63+/p73–/p53-; $n = 8$) was demonstrated for twelve p53-negative lesions, indicating that independent or complementary biological functions for these three genes may, despite possible modest contribution, also exist in human buccal squamous-cell carcinomas.

Our histochemical analysis study indicates that p73 and p63 proteins are chiefly restricted to undifferentiated cells situated in the basal layers of the normal, stratified squamous epithelium. Furthermore, p73 and p63 proteins are found in less-differentiated cells at the periphery of carcinomatous clusters of well-differentiated carcinomas, whereas negative staining is observed in areas of keratin-pearl formation. These observations suggest a relationship between p73 and p63 proteins and the differentiation of oral stratified squamous epithelia. These findings appear to be consistent with the results of Faridoni-Laurens *et al.* (2001)[25] and Choi *et al.* (2002).[27]

To the best of our knowledge, no studies have examined the effects, if any, of betel-quid chewing on p73 and p63 expression for various types of cancer. All patients in the current study were betel-quid chewers. It may be of interest, therefore, to test the association between betel-quid chewing and p73/p63 expression. A more corroborative conclusion could be achieved by comparing p73 and p63 activity between oral-cancer patients who are not habitual betel-quid chewers and those who do chew betel-quid but exhibit no disease in their buccal mucosa. On the other hand, as p63 protein expression was universal in this study, clinical correlation with immunoexpression was deemed moot. No correlation was determined between the immunoexpression of p73 and p53 and the clinicopathological parameters for buccal carcinomas examined in this study; however, we believe that our findings will be statistically validated using larger sample sizes in future studies.

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