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Expression of p63 protein and mRNA in oral epithelial dysplasia

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BACKGROUND: Abnormalities in the *TP53* are regarded as the most consistent findings in oral squamous cell carcinoma. Two related members of the *TP53* family, *p73* and *p63*, have shown remarkable structural similarity to *TP53*, indicating possible functional and biological interactions. The aim of the present study was to investigate the expression of p63 protein and mRNA in oral epithelial dysplasia.

METHODS: Immunohistochemical p63 staining was compared for samples from 90 male patients with buccal epithelial dysplasias and 15 healthy individuals with normal buccal mucosa and 15 subjects with reactive epithelial hyperplasia of the oral mucosa secondary to traumatic insult. The buccal lesions consisted of mild, moderate and severe epithelial dysplasias (30 samples in each category). The mRNA expression using reverse transcription polymerase chain reaction (RT–PCR) was also included for a subset of available fresh tissue specimens (four samples in each category of mild and moderate epithelial dysplasia; five samples in severe epithelial dysplasia; five samples in each of normal and reactive epithelial hyperplasia).

RESULTS: Nuclear p63 staining was demonstrated predominantly in the basal layers of the epithelium of the normal buccal mucosa and reactive epithelial hyperplasia specimens. For epithelial dysplasia lesions, however, staining was not restricted to the basal layers, extending to the middle spinous layer for samples in the mild category, with p63 immunoexpression observed across almost the full thickness of the dysplastic epithelium for analogous moderate and severe specimens. Compared with normal/reactive hyperplastic mucosa, p63 staining in the dysplastic mucosa was significantly increased. The severity of dysplasia was increased with the increase of p63 staining. Furthermore, ΔNp63mRNA was identified in all of the fresh tissue samples whereas expression of transactivation (TA) isotype was not detected. A subset of moderate epithelial dysplasia and severe variant showing p63-positive staining has undergone malignant transformation to squamous cell carcinomas in about 5 years follow-up.

CONCLUSION: Our results indicate that impaired p63 immunoexpression (predominantly ΔN isoform) is associated with the severity of oral epithelial dysplasias and up-regulation of p63 may play a role in the early stage of human oral tumorigenesis.

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Introduction

About two decades after the discovery of the *p53* tumour-suppressor gene (*TP53*), two related genes (*p73* and *p63*) have been cloned giving rise to the notion of a *TP53* family (1–4). Because of the significant structural similarity of these two genes with *TP53*, it was expected that their function would be similar to *TP53* in terms of tumour suppression, induction of apoptosis, and cell cycle control. In this study, the investigative emphasis was on p63.

Structurally, *TP53* has a single promoter with three conserved domains, namely, transactivation (TA) domain, the DNA-binding domain and the oligomerization domain (5). By contrast, *p63* has two promoters, resulting in two different types of protein products: those containing the TA domain (TAp63) and those lacking the TA domain (ΔNp63) (2). Furthermore, p63 mRNA undergoes alternative splicing at the COOH-terminus, giving rise to three isoforms (α, β and γ) (4–6). These various isoforms have been reported to possess either similar or opposite functions to those of p53-related transcription factors, depending on which particular isoforms are expressed (7). In general, the TAp63 isoform might behave like p53 because they transactivate various p53 downstream targets, induce apoptosis, and mediate cell cycle control, whereas, the ΔNp63 isoform has been shown to display opposing functions of the TAp63 isoform, including acting as oncoproteins

(8–10); however, a recent study (11) has indicated that this may not be absolutely true. Some ΔNp63 isoforms have shown to be as effective, or even more effective than TAp63 isoforms regarding TA and growth suppression (11). Most recently, Thurfjell et al. (12) has showed that the high levels of ΔN isoforms present in basal layers were similar to those seen in tumour tissues of the head and neck.

Further, it has been shown that *p63* knock-out mice manifest profound defects in limb and craniofacial development and in the differentiation of tissues, such as the skin, oral cavity and oesophagus (13), with the stratified epithelia, suggesting that p63 is essential for ectodermal differentiation during embryogenesis. Additionally, p63 has been identified in keratinocyte stem cells (14). This may be of practical importance for studies of epithelial tumorigenesis because it is thought that stem cells are involved in the formation of malignant tumours (15, 16). Because of the almost restricted expression of p63 in epithelial cells, as well as the functional similarity to p53, it is suggested that p63 may play a role in the regulation of proliferation and differentiation in pre-malignant and malignant lesions of epithelial origin. Although a few studies have examined p63 in head and neck (17–20) and oral (21) squamous cell carcinoma, the involvement of p63 in human oral pre-malignant lesions remains largely unelaborated. Thus, the aim of the present study was to investigate the expression of the p63 protein and mRNA in oral epithelial dysplasia.

Materials and methods

Patients

Specimens of epithelial dysplasia of the buccal mucosa were obtained from tissue samples derived from 90 male patients aged between 34 and 75 years (mean: 54), who had visited our institution. All of the patients were betel-quid chewers. All cases of dysplasia included in this study were subjected only to biopsy and standard follow-up for at least 5 years. All cases selected for this investigation consisted of single oral epithelial dysplasia without multifocal lesions at time of diagnosis. The diseased buccal mucosa involved in this study comprised mild, moderate and severe epithelial dysplasia (30 samples in each category). The histopathological characteristics of epithelial dysplasia include: (i) basal layer hyperplasia; (ii) nuclear enlargement and hyperchromatism; (iii) loss of intercellular adhesion and normal polarization; (iv) abnormal mitoses above the basal cell layer; (v) individual cell keratinization within the spinous layer; (vi) cellular pleomorphism; (vii) drop-shaped epithelial ridges; (viii) irregular stratification; and (ix) altered nuclear-cytoplasmic ratio (22). Among these histological changes, the presence of basal cell hyperplasia, nuclear enlargement and hyperchromatism and drop-shaped rete-ridges are regarded as the minimal criteria for the histological diagnosis of epithelial dysplasia (23). Diagnosis was successfully achieved and the degrees of dysplasia were graded with reference to the following criteria (24): (i) mild epithelial dysplasia – dysplastic alterations confined to the lower third of the buccal epithelium; (ii) moderate epithelial dysplasia – dysplastic changes observed for up to two-thirds of the thickness of the buccal epithelium; and (iii) severe dysplasia – more than two-thirds but less than the whole thickness of the buccal epithelium contains the dysplastic cells. The lesions were independently graded by two broad certified oral pathologists. If there was disagreement, the haematoxylin and eosin slides were evaluated again by the two pathologists until a consensus was reached. Additionally, normal buccal mucosal tissue was taken from 15 healthy individuals (mean age: 47 years; range: 22–65) using punch biopsy. Reactive hyperplastic oral mucosal tissue secondary to traumatic insult was obtained from other 15 subjects (mean age: 54 years; range: 43–67), none of whom chewed betel-quid or smoked cigarettes, for inclusion as control samples. All tissues (including control samples) were conformed to an informed consent protocol, approved by the Ethics Committee for Scientific Research on human beings, which reviews research proposals at this institution. The biopsied 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. Twenty-three fresh tissues samples (four samples in each category of mild and moderate epithelial dysplasia; five samples in severe epithelial dysplasia; five samples in each of normal and reactive epithelial hyperplasia) were available for mRNA analysis.

Immunohistochemistry

Staining was performed using a standard avidin-biotin peroxidase complex (ABC) (25). The p63 protein expression was examined using p63 antibody raised against amino acids 1–205, mapping at the amino-terminus of ΔNp63 (Clone 4A4; Santa Cruz® Biotechnology, Santa Cruz, CA, USA). According to the manufacturer's specifications, this antibody reacts with tissues of human, rat and mouse origin, as determined from Western blotting and immunohistochemistry (including paraffin-embedded sections). The specificity of the anti-p63 antibody has been previously demonstrated using a variety of immunoblotting experiments, as well as analogous studies of immunohistochemical staining of mouse tissues from which the *p63* gene has been deleted (4, 13). Subsequent to deparaffinization in xylene and ethanol, the tissue sections were treated in 0.3% H_2O_2-methanol and 10% normal goat serum (Dako, Santa Barbara, CA, USA). All sections were subsequently incubated at room temperature with the primary antibody (1:200) for 30 min, then for a further 30 min with biotin-conjugated goat antirabbit immunoglobulin G (IgG) (1:100; Vector, Burlingame, CA, USA), and finally for 30 min using ABC (Dako). The sites of peroxidase-binding were visualized as brown products of the diaminobenzidine reaction. The sections were then counterstained with haematoxylin. Each set of experiments included a human buccal squamous cell carcinoma specimen known to express p63, which served as a positive control and ensured the reproducibility of

the staining process. A negative-control, in which the primary antibody had been omitted, was also included in each set of experiments. Tissue sections, where the primary antibody was substituted for one of the same IgG subclass but different antigen specificity, served as additional negative staining controls.

At least three representative areas were viewed per section, depending on size. The area of staining was evaluated according to the extent of positively stained tissue within the microscopic fields as follows: 0, $<10\%$; 1+, 10–20%; 2+, 20–40%; 3+, 40–60%; 4+, 60–80%; 5+, $>80\%$.

Reverse transcription polymerase chain reaction

Total RNA was extracted by homogenizing the 23 available fresh tissue specimens in guanidium isothiocyanate followed by ultracentrifugation in caesium chloride, as described previously (26). The RNA concentration was determined by way of the sample's optical density at a wavelength of 260 nm (by using an OD_{260} unit equivalent to 40 μg/ml of RNA).

Isolated total RNA (1 μg) was reverse-transcribed to cDNA in a reaction mixture (with a final volume of 20 μl) containing 4 μl of $MgCl_2$ (5 mM), 2 μl of 10X reverse transcription buffer [10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton® X-100], 2 μl of dNTP mixture (1 mM each), 0.5 μl of recombinant RNasin® ribonuclease inhibitor (1 μ/μl), 15 units of avian-myeloblastosis virus (AMV) reverse transcriptase (high concentration) (15 μ/μg), 0.5 μg of oligo$(dT)_{15}$ primer (catalogue no. A3500; Promega, Madison, WI, USA). The reaction mixture was incubated for 15 min at 42°C. The AMV reverse transcriptase was inactivated by heating for 5 min at 99°C and then incubated at 0–5°C for a further 5 min.

All oligonucleotide primers were purchased from Genset Corp. (La Jolla, CA, USA). The primer pairs were chosen from the published cDNA sequences of p63 (20) (TA and ΔN isoforms; GenBank accession number NM-003722) and β-actin (27) (GenBank accession no. X-00351). Oligonucleotide primers used for polymerase chain (PCR) reactions are as indicated in Table 1. The 20 μl first-strand cDNA synthesis reaction product obtained from the reverse transcriptase reaction was diluted to 100 μl with nuclease-free water. The PCR amplification reaction mixture (with a final volume of 100 μl) contained diluted, first-strand cDNA reaction product (20 μl; <10 ng/μl), cDNA reaction dNTPs (2 μl; 200 μM each), $MgCl_2$ (4 μl; 2 mM), 10X reverse transcription buffer (8 μl; 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton® X-100), upstream primer (50 pmol), downstream primer (50 pmol) and *Taq* DNA polymerase (2.5 units, catalogue no. M7660; Promega).

Table 1 Oligonucleotide primers used to amplify ΔNp63, TAp63 and β-actin cDNAs

Oligonucleotide primers	*Sequences*	*PCR products (bp)*
ΔNp63 sense	5′-TTAGTGAGCCACAGTACACG-3′	681
ΔNp63 antisense	5′-GCCCATCTCTGGTTTCCAG-3′	
ΔTAp63 sense	5′-ATTCCCAGAGCACACAG-3′	600
ΔTAp63 antisense	5′-AGCTCATGGTTGGGGCAC-3′	
β-actin sense	5′-AACCGCGAGAAGATGACCCA GATCATGTTT-3′	350
β-actin antisense	5′-AGCAGCCGTGGCCATCTCTT GCTCGAAGTC-3′	

PCR, polymerase chain reaction.

The PCR steps were carried out on a DNA thermal cycler (TaKaRa MP, Tokyo, Japan). Thermocycling conditions included denaturing at 94°C for 1 min (one cycle), then denaturing at 94°C (1 min), annealing at 52°C (1 min) for both ΔNp63 and TAp63, or at 60°C (1 min) for β-actin, and extending at 72°C (1 min) for 30 cycles and a final extension at 72°C for 7 min. The β-actin primers were utilized as positive controls. Negative-controls, i.e. those conducted in the absence of RNA and reverse transcriptase, were also performed. Amplification products were analysed by electrophoresis in a 2% agarose gel along with the relevant DNA molecular weight marker (Boehringer Mannheim, Mannheim, Germany) and stained with ethidium bromide. The PCR products were visualized as bands with a UV transilluminator. Photographs were taken with a Polaroid DS-300 camera (Applied Biosystems Taiwan, Taipei, Taiwan). The PCR products were then sequenced to confirm their identities using a T7 Sequenase version 2.0 kit (Amersham International, Little Chalfont, UK).

Results

Immunohistochemistry

For the normal buccal mucosa specimens, p63 nuclear staining was predominantly detected in the basal layers of the epithelium and only focally in parabasal cells layers (Fig. 1a). For lesions of hyperplastic oral epithelium, nuclear p63 positivity was also chiefly noted in the basal cells and occasionally in cells a few layers above basal layers (Fig. 1b). For lesions from mild epithelial dysplasias, however, nuclear staining of p63 was no longer restricted to the basal layers and was detected obviously in the middle spinous layer (Fig. 1c). Further, p63 immunoexpression could be observed across almost the full thickness of the dysplastic epithelium for the moderate (Fig. 1d) and severe analogues (Fig. 1e; p63 immunoexpression is summarized in Table 2). The severity of dysplasia was increased with the increase of p63 staining (Table 2). The staining scores were analysed statistically using Statistical Parameter for Social Sciences (SPSS) for Window 9.0 Software. The Kolmogorov–Smirnov non-parametric two-sample test was employed to compare dysplasia and control samples. The level of significance for all tests was set at $P < 0.05$. The increase in p63 staining for dysplasia specimens compared with control samples was significant ($P < 0.05$). Furthermore, in about 5 years follow-up, five of 30 cases (16.7%) of moderate epithelial dysplasia and nine of the 30 cases (30%) of severe variant showing p63-positive staining have undergone malignant transformation to develop squamous cell carcinomas (Table 2); however, the case number was not large enough for statistical analysis.

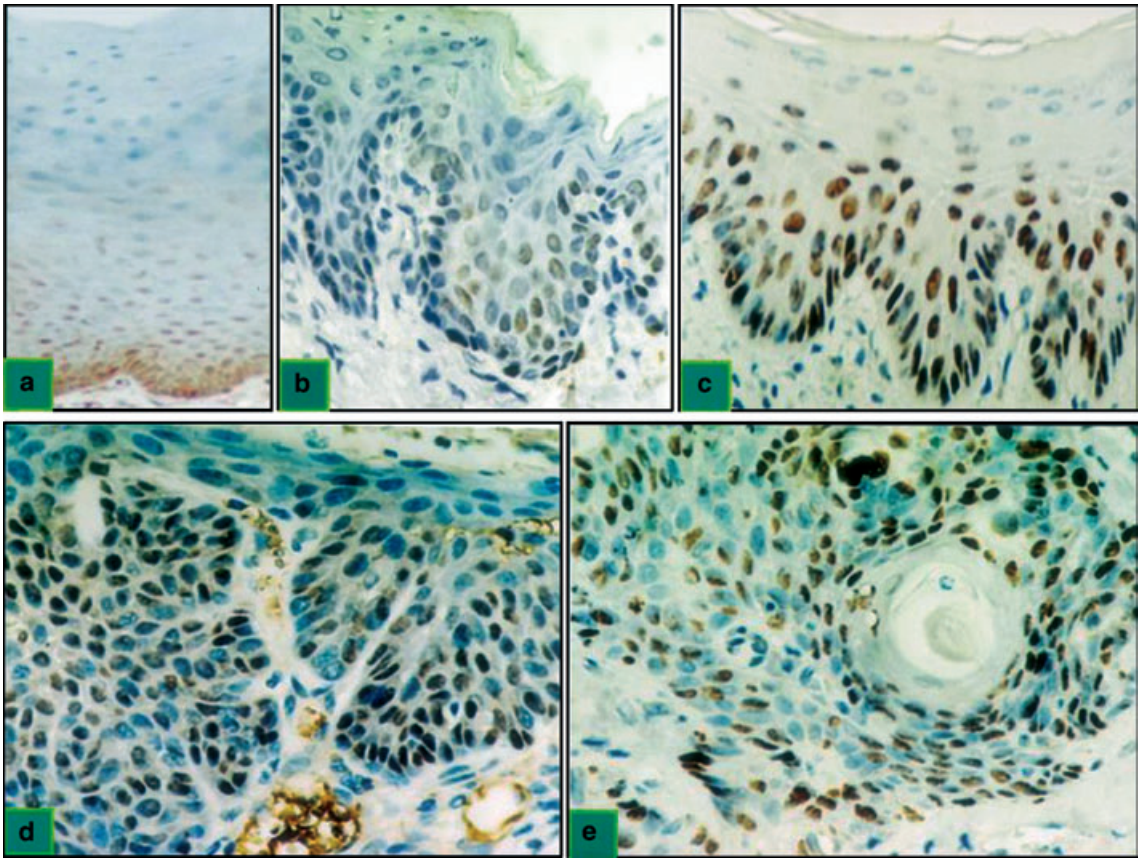

Figure 1 Representative sections of (a) normal buccal mucosa specimen revealing nuclear p63 positivity predominantly in basal layers and only focally in parabasal cells layers; (b) reactive epithelial hyperplastic oral mucosa specimen revealing nuclear p63 positivity was noted in some basal cells, and with occasionally in cells a few layers above basal layers; (c) mild epithelial dysplasia specimen showing positive p63 staining in the middle spinous layer; (d) moderate epithelial dysplasia and (e) severe epithelial dysplasia specimens showing p63 positivity through nearly the whole mucosal layer (avidin-biotin peroxidase complex, ABC stain ×100).

Table 2 p63 immunoexpression in samples of normal/reactive hyperplastic mucosa and human buccal epithelial dysplasia

	Staining scores					
Tissue/dysplasia type	*0*	*1+*	*2+*	*3+*	*4+*	*5+*
Normal mucosa	0[a]	15	0	0	0	0
Reactive epithelial hyperplasia	0	15	0	0	0	0
Mild	0	3	27	0	0	0
Moderate	0	0	2	26 (1)	2 (2)	0
Severe	0	0	0	0	3 (1)	27 (8)

Data in parenthesis indicating the number of cases undergo malignant transformation to squamous cell carcinomas.
Positive-staining scores, proportion of cells in microscopic fields: 0, < 10%; 1+, 10–20%; 2+, 20–40%; 3+, 40–60%; 4+, 60–80%; 5+, > 80%.
[a]Number of cases.

Reverse transcription polymerase chain reaction

Upon reverse transcription (RT)–PCR, ΔNp63mRNA was detectable as a band corresponding to a 681-bp PCR product for all of the fresh tissue specimens of buccal epithelial dysplasia as well as for the normal mucosa and reactive epithelial hyperplastic specimens whereas expression of TA isotype was not detected in all of the tissue specimens (Fig. 2). All samples apart from the negative-control sample reveal bands of β-actin (350-bp) (Fig. 2). The expression of ΔNp63mRNA for the fresh tissue specimens was noted to be consistent with the findings of corresponding specimens using immunohistochemistry.

Discussion

Reviewing the English language medical literature, most reports are focused on p63 expression in head and neck squamous cell carcinoma (17–21) and there is only one published immunohistochemistry-based report of p63

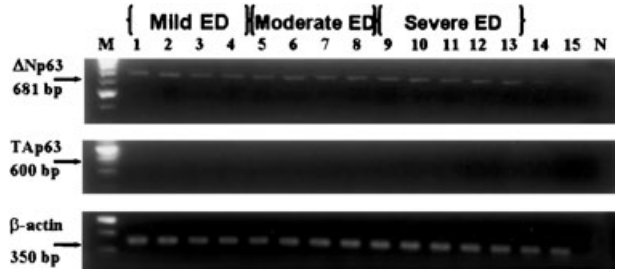

Figure 2 Expression of p63 [ΔN and transactivation (TA) isotypes] mRNA in human buccal epithelial dysplasia (ED) using reverse transcription polymerase chain reaction (RT–PCR). A band of a 681-bp PCR product corresponding to ΔN isotype is observed for all the fresh tissue specimens of oral epithelial dysplasia (lanes 1–13) as well as for the normal mucosa (lane 14), and reactive epithelial hyperplastic (lane 15) specimens whereas no specimens reveal a 600-bp PCR product corresponding to TAp63mRNA (lanes 1–15). All samples (lanes 1–15) apart from the negative-control sample (lane N) reveal bands of β-actin (350-bp). Lane M is the DNA molecular weight marker.

expression in samples of epithelial dysplasia lesions taken from the head and neck (17). Our results are in agreement with those of this small study of 16 epithelial dysplastic lesions (five, mild; six, moderate; five, severe). Choi et al. (17) demonstrated that all samples expressed p63 protein; however, no attempt was made to grade degree of staining with respect to the severity of the dysplastic lesions (17). As far as we have been able to determine, this is the first investigation of the relationship between p63 protein expression and grade of oral epithelial dysplasia.

The *p63* gene can be expressed into at least six protein isotypes, which are divided into two groups, those containing the TA domain (TA isotypes) and those that do not (ΔN isotypes) (2). Immunohistochemistry using the 4A4 antibody, however, was not able to absolutely confirm which isotypes (TA or ΔN) were implicated in oral carcinogenesis. The presence of TAp63mRNA within skeletal muscle tissue, in the absence of staining with the 4A4 antibody, has been previously reported (28), indicating that this antibody may not necessarily identify all isotypes of p63 using immunohistochemical techniques. In order to elucidate which isotypes of p63 were expressed in the current study, RT–PCR was performed using isotype-specific primers. The ΔNp63mRNA was identified within all dysplastic buccal tissue specimens as well as the normal and reactive epithelial hyperplastic specimens, whereas expression of TAp63 was not able to be detected in any of these tissue specimens. No variation of ΔNp63mRNA at the expression level was recognized between normal and dysplastic buccal tissue. On the basis of this finding, it was able to conclude that ΔNp63 is the major isotype of p63 in this study. A similar result has been reported in squamous cell carcinomas for both human (29) and hamster (30) buccal tissues but, this may be, to our knowledge, the first study to demonstrate the presence of ΔNp63mRNA in human oral epithelial dysplastic lesions.

In the present study, it was observed that the number of p63-positive epithelial cells increased as severity increased (from normal/reactive epithelial hyperplasia specimens through to mild, moderate and severe epithelial dysplastic buccal lesions). Further, this increased penetration is reflected in an upward extension of p63-stained keratinocytes, from the basal/parabasal layers in normal buccal mucosa/reactive hyperplastic oral mucosa to the middle spinous layer in mild epithelial dysplasia, and spanning almost the entire epithelial layer in the moderate and severe variants. These results suggest that the extent of up-regulation of p63 expression may be correlated with the severity of epithelial dysplasia. It is important to consider the potential role of p63 protein in lesions of oral epithelial dysplasia.

A dual role of p63 protein has been reported (31): (i) during embryogenesis, p63 may be the molecular switch required for initiation of epithelial stratification because, if lacking p63, epithelium remains single-layered; (ii) for mature epithelium, p63 needs to be switched off for terminal differentiation to take place; otherwise, p63 may maintain the proliferative potential of basal keratinocytes preventing stratification to occur. Based on our immunohistochemical data, p63 protein is expressed in the proliferative layer of cells near the basement membrane of the normal oral mucosa, where it likely serves to prevent basal cells from differentiating and thereby helps to maintain their basal cell status. Then, upon the maturation of normal oral stratified squamous epithelium, the expression of p63 protein should have been down-regulated and p63 protein has rarely been detected in upper layers of the epithelium. However, upon dysplastic change (i.e. transition from normal oral mucosa to epithelial dysplasia), dysplastic keratinocytes above the basal layers may shift to a status similar to the embryogenesis condition and are still able to express p63 protein producing an antidifferentiation effect as well as a proliferative capacity of dysplastic cells in oral dysplastic mucosa. Under this presumption, it can explain why there is an upward extension of p63 protein as the dysplastic lesions progress from mild to moderate and severe variants.

Sniezek et al. (32) reported that p63 protein is under expressed in cases of oral lichen planus, a mucosal lesion that seldom progresses to malignancy and is characterized by hyperdifferentiation and apoptosis. A similar phenomenon has also been observed for lesions of reactive epithelial hyperplasia secondary to traumatic insult in our study. Could the lymphocytic infiltrates of these two kinds of lesions responsible for the release of cytokines that result in decreased p63 expression? Surely, further study is required. Then, in summary, if our hypothesis is correct, there are strong grounds for speculation that for circumstances of malignant transformation of oral dysplastic lesion to squamous cell carcinoma, the overexpressed p63 proteins might exert an alternative mechanism to overcome p53 tumour-suppressor function and hence induce clonal expansion of the dysplastic kerationcytes. Therefore, it appears reasonable to suggest that the p63-stained keratinocytes could be important in neoplastic transformation of the squamous cell, favouring neoplastic proliferation and antidifferentiation effect,

which cannot be ascertained by conventional haematoxylin and eosin staining.

Furthermore, it has been shown that ΔNp63 isoforms were predominated in squamous cell carcinomas of the head and neck (12, 33) as well as of the oral cavity (29). Similarly, in this study, we demonstrated that ΔN isoforms were also dominated in the basal layer from which carcinomas are thought to arise, and the expression of p63 increased with the severity of dysplaisa. Therefore, the enhanced expression of these N-terminal truncated proteins could have a negative impact in the differentiation of the epithelial cells in lesions of dysplasia, and might contribute to subsequent cancer formation.

It is interested to note that a similar immunoexpression pattern has been reported for keratin-19 for human oral carcinogenesis (34) when compared with our results for p63 protein. Both keratin-19 and p63 protein have been regarded as a novel basal cell immunohistochemical marker (35). Indeed, an association of keratin-19 and p63 expression has been found for human oesophageal dysplasia and carcinoma (36). Whether there is a similar correlation between keratin-19 and p63 protein for human oral carcinogenesis is warranted for further study.

Paralleling recent findings for pulmonary (37), cutaneous (38), nasopharyngeal (39), oesophageal (40) and laryngeal (41) squamous cell carcinomas, in our study p63 immunoexpression was determined for all of the buccal epithelial dysplasias. Importantly, p63 is located in the 3q27–29 chromosomal region, the most frequently overexpressed genomic locus for head and neck carcinoma (42), and amplification of the p40/p51/p63 locus has been demonstrated for most such specimens (6–9). Taken together, these findings suggest that abnormal status and expression of the *p63* gene may be associated with the early phase of human oral squamous cell carcinogenesis. A similar finding has recently been reported with respect to the expression of p63 in dysplastic laryngeal (41) and oesophageal (40) mucosa.

Both p63 and p73 up-regulation have already been demonstrated in the early stages of 7,12-dimethylbenzanthrance (DMBA)-induced hamster buccal-pouch squamous cell carcinogenesis (43, 44). An upward extension of the p63 protein from the basal/parabasal layers to the entire epithelial layer was noted with prolonged DMBA exposure suggesting that elevated p63 expression is consistent with the progression of dysplasias in DMBA-induced hamster buccal-pouch carcinogenesis (43, 44). The results of the current human study are compatible with the aforementioned findings of the animal experiment (43). Therefore, in human oral carcinogenesis, it may be expected that expression of p63 (perhaps in concert with p73) will block the growth-inhibition and apoptosis-induction activities of p53 (45) and, thus, may help to maintain the proliferative capacity of progenitor cells in human oral dysplastic mucosa.

To study tumour development, it is preferred to follow a tissue that has developed into a tumour. In order to evaluate the potential predictive value and biological implication of p63 expression in oral carcinogenesis, it is better to employ control samples from subjects with exposure to risk factors similar to the affected subjects because this would reduce the likelihood of identifying alterations only indicative of exposure but not transformation. As not all patients chewing betel-quid have dysplasia, it should been, if possible, to include also betel-quid chewers with clinically normal oral mucosa as controls. In fact, that is necessary before confirmative conclusions in the role of p63 in dyspastic development can be drawn. The effects of another risk factor, long time heavy smoking, on the p63 expression in clinically normal oral mucosa has been studied by Thurfjill et al. (12) who found that tobacco usage had no effect on p63 expression in oral epithelium. However, such kinds of controls (with exposure to betel-quid but without disease), to our experience in Taiwan, are very difficult to collect as without any oral mucosal abnormalities, the possibility for them to visit a dental clinic is very low. Therefore, non-betel-quid users have been selected for normal control mucosa in the present study. In about 5 years follow-up, a subset of moderate epithelial dysplasia (16.7%) and severe variant (30%) showing p63-positive staining have undergone malignant transformation to develop squamous cell carcinomas. It also appears that the higher the staining scores, the greater the likelihood of malignant transformation, but the number of cases is not large enough for statistical analysis. Nevertheless, to our knowledge, this may be the first report to provide follow-up data of p63 expression for lesions of oral epithelial dysplasia. These observations suggest that impaired p63 expression has an early role in oral tumorigenesis and deserves additional evaluation as a biomarker for oral cancer progression.

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