Research and Emerging Technologies
Head and Neck Oncology

Expression of p63 (TA and ▲N isoforms) in human primary well differentiated buccal carcinomas

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Abstract. Abnormalities in the p53 gene have been regarded as the most consistent genetic abnormalities detected in head and neck squamous cell carcinogenesis. Two new members of the p53 gene family, p73 and p63, have recently been identified. We investigated the expression of the two N-terminal p63 isoforms (TA and ▲N isoforms) in human primary well-differentiated buccal squamous cell carcinoma. Both TAp63 and ▲Np63 isoforms were detected in the basal/suprabasal layers of all of the five specimens of normal buccal mucosa. The ▲Np63 isoform was found in all of the 23 specimens of human primary well-differentiated buccal carcinoma whereas TAp63 isoform was absent in 18 (78.3%) of the 23 specimens. The immunostaining patterns of both TAp63 and ▲Np63 isoforms were similar in that the p63 positivity was noted mainly in the peripheral cells of tumor nests whereas negative staining was observed in the areas with keratin pearl formation. A higher number of T3–T4 patients and patients with recurrence showed negative staining of TAp63 than T1–T2 patients and patients without recurrence but the difference was not statistically significant. These results suggested that specific p63 isoforms were associated with human oral squamous cell carcinogenesis. The ▲Np63 isoforms might be involved in epithelial differentiation and proliferation in human oral carcinogenesis whereas there was evidence for a possible role of TAp63 under-expression in human oral tumorigenesis.

The p53 tumor suppressor gene has been one of those most frequently involved in human cancer[7], including the oral variants of the disease[23]. Data has emerged about p53 homologues, such as p73 and p63[4–6]. p63, which mapped to 3q27–29, had different protein isoforms due to alternative splicing (α, β, γ) and also due to the use of different promoters which resulted in retention of the transcription-activation domain (TA isoforms) and those that did not (▲N

isoforms)[13,27,32]. These p63 isoforms were referred to as TAp63α, β, γ and ▲Np63α, β, γ. All p63 isoforms contained DNA binding and hetero-oligomerization domains. The ▲Np63 isoforms lacked the NH_2-terminal trans-activation domain but could act in a dominant-negative fashion to counteract the transactivation isoforms of not only p63 but also p53 by competition for DNA binding sites to prevent p53 or TAp63 from binding DNA.

The TAp63γ and TAp63β transactivated promoters at levels comparable with wild-type p53, but TA63α did not have this property[33]. TAp63 could activate *in vitro* p53 responsive promoters such as p21, GADD45, Bax, and mdm2[12,25]. TAp63γ and TAp63β induced apoptosis in transient transfection experiments in contrast to TAp63α[1,17,33,32]. The ▲N isoforms blocked the functions of p53. This might be attributable to competition for DNA binding sites to

prevent p53 or TAp63 from binding DNA. It was conceivable that p53 or TAp63 might be sequestered by ▲Np63 through the oligomerization domain or another domain[6]. The p63 gene appeared to produce a range of proteins with either similar or opposite functions, giving rise to the possibility that p63 could either act as p53-like tumor suppressor gene, or as a dominant oncogene, depending on which particular isoforms were expressed[11].

A number of studies have investigated the role of p63 in human squamous cell carcinomas from different organs[5,9,19]. Only a few studies have examined p63 in head and neck squamous cell carcinoma including oral variants of the disease[4,8,15,30], indicating that p63 may function as oncogene in the development of these tumors. The expression of particular isoforms of p63 was not investigated in most of these studies. In this study, we characterized the expression of p63 (TA and ▲N isoforms) in human primary buccal squamous cell carcinomas.

Materials and methods

Study population

Specimens of primary well-differentiated squamous cell carcinoma of the buccal mucosa were obtained from tissue samples of surgically-resected tumors from 23 male patients aged between 35 and 70 years (mean age 52), who were treated at the Oral Pathology Department at Kaohsiung Medical University Hospital. All of the patients had clinically negative neck and had no history of previous radiotherapy or chemotherapy. They underwent complete surgical excision of the tumor by means of elective neck dissection. Eleven of the 23 patients had histologically confirmed cervical lymph-node involvement. All of the patients had been exposed to risk factors such as betel-quid chewing, cigarette smoking, and alcohol drinking. 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 12 patients who had expired during the follow-up period, mean survival time was approximately 2 years (range 0.7–4.6). Mean survival time for the remaining patients was 4.1 years (maximum 5). The characteristics of the patients including clinical tumor extension, histological lymph-node involvement, recurrence and survival were summarized in Table 1. Normal buccal mucosal tissue as a control was taken from five healthy individuals between 30 and 62 years (mean age: 49), none of whom chewed betel-quid or smoked cigarettes. All tissues (including the normal tissues) were collected after informed consent was obtained from the patient and the research was approved by the Ethics Committee for Scientific Research on Human Beings of this 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 immunohistochemistry study.

Immunohistochemistry of TAp63 and Np63 proteins

Paraffin-embedded, 4-μm thick tissue sections were stained for TAp63 protein using a primary goat polyclonal anti-p63 antibody (D-20) (catalogue no.: sc-8608) obtained from Santa Cruz Biotechnology Inc. This antibody was reactive with TAp63α, β, and γ and exhibited no cross-reactivity with other p63 isoforms (information from manufacturer). ▲Np63 protein was recognized using a primary goat polyclonal anti-p63 antibody (N-16) (catalogue no.: sc-8609) purchased from Santa Cruz Biotechnology Incorporated. This antibody was reactive with ▲Np63α, β, and γ and exhibited no cross-reactivity with other p63 isoforms (information from manufacturer). Both antibodies recognized p63 protein in humans, rats and mice.

Deparaffinization of all sections was performed through a series of xylene baths, and rehydration was performed through graded alcohols. To retrieve the antigenicity, tissue sections were treated three times with microwave radiation in a 10 mM citrate buffer (pH 6.0) for 5 min each. The sections were then immersed in methanol containing 0.3% hydrogen peroxidase for 45 min to block the endogenous peroxidase activity and were incubated in normal goat serum to reduce non-specific binding. Sections were incubated for 60 min at room temperature with primary anti-p63 antibodies [TAp63 (D-20); ▲Np63 (N-16); both at 1:200]. The sections were then processed using standard avidin–biotin immunohistochemistry in accordance with the manufacturer's recommendations (Vector Laboratories, Burlingame, CA)[10]. Diaminobenzidine was used as a chromogen, and commercial hematoxylin was used for counterstaining. Each set of experiments included a human laryngeal squamous cell carcinoma specimen known to express both TAp63 and ▲Np63 isoforms, which served as a positive control and ensured the reproducibility of the staining process. Immunohistochemical staining was classified as negative if no staining was found or positive staining was present in <10% of the cells, or positive if >10% of the cells stained positively. Negative controls were included following the same procedure, but with omission of the primary antibody and using an irrelevant block peptide as recommended by the manufacturer (sc-8608P for TAp63 isoform; sc-8609P for ▲Np63 isoform).

Results

In all of the normal buccal mucosa, nuclear immunoreactivity for both TAp63 (Fig. 1) and ▲Np63 isoforms (Fig. 2) was detectable in the basal and

Table 1. Comparison between p63 immunostaining results for specimens with clinical tumor extension, histologically confirmed cervical lymph node involvement, recurrence and survival

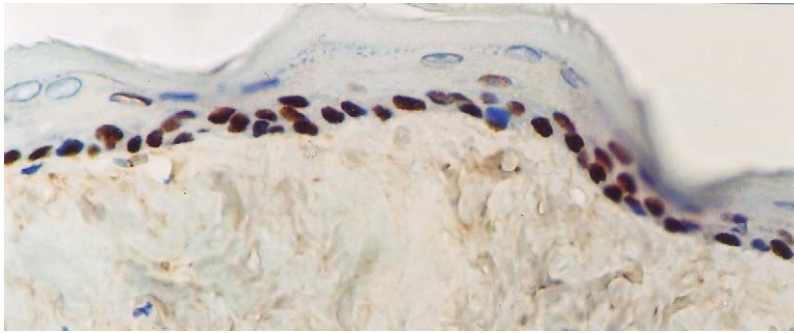|	TA63 protein (+)	TA63 protein (−)	▲Np63 protein (+)	▲Np63 protein (−)
Tumor extension				
T1–T2	4	7	11	0
T3–T4	1	11	12	0
Lymph node				
N0	3	9	12	0
N+	2	9	11	0
Recurrence				
Yes	2	10	13	0
No	3	8	10	0
Survival				
Dead	3	9	12	0
Alive	2	9	11	0

N0: negative lymph nodes; N+: metastatic lymph nodes.

Fig. 1. Representative section of a normal buccal mucosa specimen reveals nuclear positivity for ▲Np63 isoforms in basal/parabasal cell layers (ABC stain 100×).
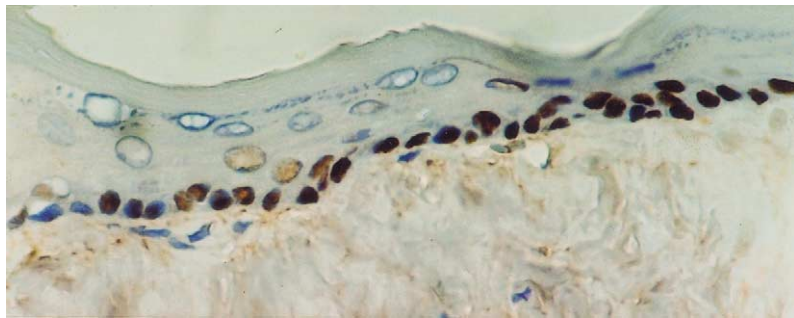

Fig. 2. Basal/parabasal layers in a representative section of a normal buccal mucosa specimen showing TAp63 isoform nuclear positivity (ABC stain 100×).

suprabasal layers of the epithelium. ▲Np63 immunostaining was detected in all of the specimens of buccal squamous cell carcinomas (Fig. 3). TAp63 staining was absent in 18 specimens of squamous cell carcinomas (Fig. 4). The immunostaining patterns of both TAp63 and ▲Np63 isoforms were similar in that p63 positivity was noted mainly in the peripheral cells of tumor nests whereas negative staining was observed in areas of keratin pearl formation (Fig. 3). A comparison of immunostaining for both TAp63 and ▲Np63 isoforms in specimens with clinical tumor extension, histological lymph-node involvement, recurrence and survival were summarized in Table 1. A higher number of T3–T4 patients showed negative staining of TAp63 than T1–T2 patients although this difference was not significant (Table 1). A larger number of patients with recurrence showed negative TAp63 st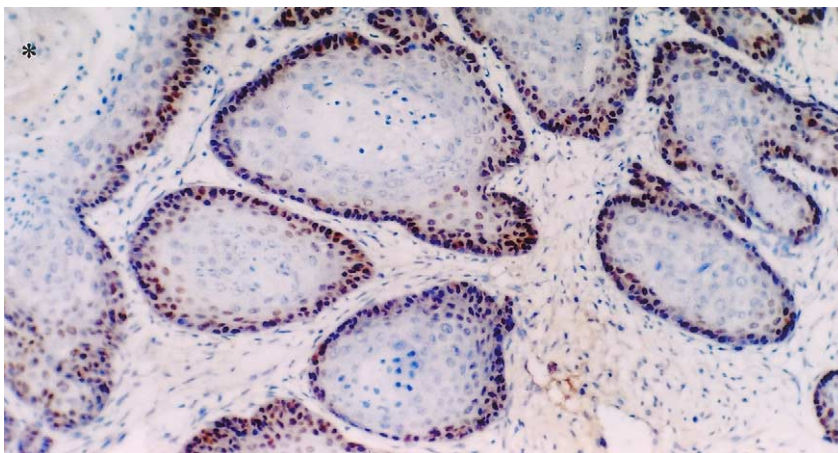aining than those without recurrence but the difference was insignificant (Table 1). No definitive staining was found in the negative-control sections while definite, positive p63-protein immunostaining was revealed in the positive-control sections.

Discussion

Four previously reported studies have examined p63 protein expression in head and neck squamous cell carcinoma including oral variants of the disease[4,8,15,30]. The expression of particular isoforms of p63 was not investigated in three of them[4,8,30], while one of them reported the immunoexpression of p63α/▲Np63α on oral squamous cell carcinomas[15], but did not investigate the TAp63 isoform. In the current study, we investigated TAp63 and ▲Np63 expression in a homogenous and well-characterized series of patients with buccal squamous cell carcinomas.

The p63 played an important role in ectodermal differentiation, as indicated by the finding that p63 knockout mice have major defects in their limb, craniofacial, and epithelial development[14,34]. In this study, we found TAp63 and ▲Np63 proteins in all of the samples of normal buccal mucosa, where it was restricted to the basal and suprabasal epithelial cell layers. Similar findings have been recently reported in laryngeal[21] and esophageal[28] epithelia. Because transactivating and truncated p63 isoforms possess contradictory effects in regulating proapoptotic and differentiating genes, such as p21 and Bax[26], these results indicated that the process of epithelial differentiation at these anatomic sites depended on a dynamic balance between TAp63 and ▲Np63 isoforms.

In keeping with recent findings in pulmonary[20], cutaneous[22], nasopharyngeal[5], bladder[29], and laryngeal[21] squamous cell carcinomas, we found ▲Np63 immunoexpression in all of the buccal carcinoma in the present study. The 3q27–29 chromosomal region, where p63 was located, was the most frequently over-expressed genomic locus in head and neck carcinoma[26], and most of the head and neck carcinomas showed amplification of the p40/p51/p63 locus[9,31]. Taken together, these data suggested that an abnormal status and expression of p63 gene might be associated with multiple stages of human oral squamous cell carcinogenesis, although data concerning p63 aberrations in oral premalignant lesions were still lacking. Further study
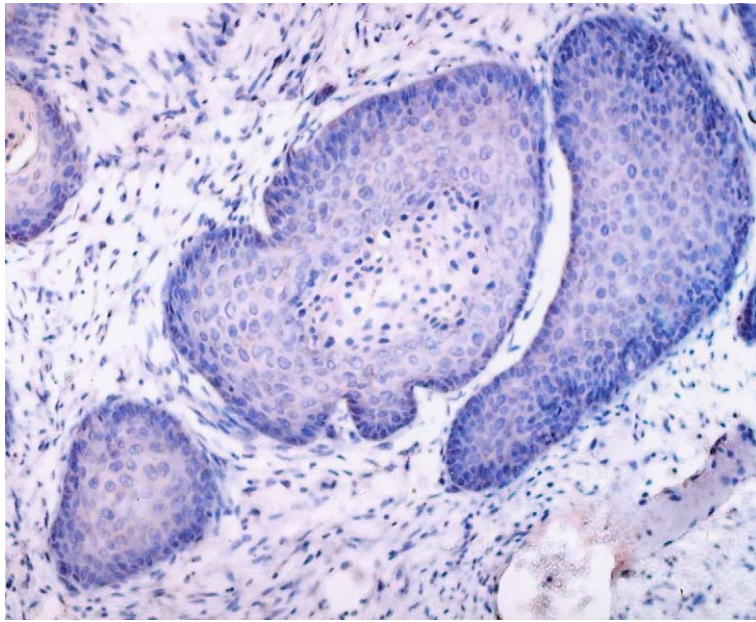

Fig. 3. Representative section of squamous cell carcinoma specimen shows staining for ▲Np63 mainly in the peripheral cells of well-differentiated tumor nests and lack of staining in areas of keratin pearl formation (*). (ABC stain 100×.)

Fig. 4. Absence of TAp63 staining in a representative section of a squamous cell carcinoma specimen (ABC stain 100×).

of p63 expression in human oral precancerous lesions might help to elucidate whether p63 gene abnormalities were involved in the early stages of oral squamous cell carcinoma development in a similar manner to p53.

In 2002, Nylander et al.[16] suggested that p63 had biologic effects on the differential expression of either transactivating or truncated isoforms. In this study, we found detectable levels of ▲Np63 immunoexpression in all of the buccal carcinomas examined. By contrast, approximately 78.3% (18/23) of the specimens analyzed showed absence of TAp63 immunoexpression, similar to primary bladder[18] and laryngeal carcinomas[21]. The ▲Np63 isoforms acted as a dominant-negative factor on G1-cell-cycle arrest and apoptosis[5]. Accordingly, their over-expression might confer a growth advantage to the tumor cells. Under-expressed TAp63 immunoexpression has been associated with a higher risk of tumor progression[29] and recurrence[18] of bladder carcinomas. In this study, the number of specimens with negative immunostaining of TAp63 in T3–T4 and recurrence tumors was higher than that of T1–T2 and non-recurrence tumors but the difference did not reach statistical significance. A larger number of cases would need to be analyzed in order to attain a significant correlation between TAp63 expression and advanced tumor stage and tumor recurrence.

A number of molecular markers for oral carcinomas, including p53, have been excellently reviewed by Schliephake[24]. p63, being a close relative to p53, has not been mentioned in this review[24]. In the current study, expression of both TAp63 and ▲Np63 isoforms was mainly noted in the peripheral cells of tumor nests in tumor areas whereas negative staining was observed in areas of keratin pearl formation. A high level of p63 expression has been reported in undifferentiated nasopharyngeal carcinoma[5]. It seemed reasonable to suggest that the efficacy of p63 protein as an immunohistochemical marker for the differential diagnosis of poorly differentiated and undifferentiated squamous cell carcinomas merits further study. If the protein has shown to be an effective marker, positive p63 immunostaining in poorly differentiated metastatic carcinomas might be helpful for prediction of primary tumors of squamous epithelial origin. An association of p53 with inducible nitric oxide synthase (iNOS) in oral carcinomas has been reported by Brennan et al.[2]. Furthermore, iNOS was reported to be over-expressed in a number of oral premalignant epithelial lesions and verrucous carcinoma[3]. Whether there has been a similar correlation between p63 and iNOS in both oral premalignant lesions and carcinomas remained to be elucidated.

In conclusion, our results indicated specific p63 isoforms might be associated with human oral squamous cell carcinogenesis. The ▲Np63 isoforms could be involved in the epithelial differentiation and proliferation in human oral squamous cell carcinogenesis whereas TAp63 might play a role in under-expression in human oral tumorigenesis.

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