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Experimental Oral Pathology

Immunohistochemical demonstration of p63 in DMBA-induced hamster buccal pouch squamous cell carcinogenesis

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OBJECTIVES: Abnormalities in the p53 gene are regarded as the most consistent genetic abnormalities detected in head and neck squamous cell carcinogenesis. Two new members of the p53 gene family, p73 at the 1p36 region and p63 at the 3q27-29 region, have recently been identified. They share considerable sequence homology with p53 in the transactivation, DNA binding, and oligomerization domains, indicating possible involvement in carcinogenesis. To our knowledge, however, p63 expression in experimental oral carcinogenesis has not been studied.

MATERIALS AND METHODS: Immunohistochemical analysis of p63 protein expression was performed in 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch squamous cell carcinogenesis. Fifty outbred, young (6 weeks), male, Syrian golden hamsters (*Mesocricatus auratus*) were randomly divided into three experimental groups (each consisting of 10 3-, 9- and 15-week DMBA treated animals), and two control groups (with 10 animals in each). The pouches of the three experimental groups were painted bilaterally with a 0.5% DMBA solution three times a week. The treatment protocol for animals in one of the control groups was identical with only mineral oil applied, while the other control group remained untreated throughout the experiment.

RESULTS: In all of the untreated and mineral oil-treated pouch mucosa, nuclear positivity for p63 was mainly observed in the basal/parabasal cell layers. The p63 nuclear positivity extended from the basal/parabasal layers to the whole epithelial layers in the 3- and 9-week DMBA-treated pouch mucosa. Furthermore, the positive nuclear-stain cells were randomly distributed throughout the entire epithelial layers in the 3- and 9-week DMBA-treated pouch-mucosa specimens. In carcinomas from 15-week DMBA-treated pouch specimens, p63 staining was more uniform and homogeneous for the less-differentiated tumor areas. By contrast, p63 expression was noted mainly in the peripheral cells of tumor nests in the well-differentiated tumor areas.

CONCLUSIONS: The results of this study are consistent with those from previous analyses of p63 expression in human oral mucosa, suggesting that p63 may be associated with the regulation of epithelial differentiation and proliferation in DMBA-induced hamster buccal pouch squamous cell carcinogenesis. Further study is required to investigate which p63 isoform(s) is/are involved in hamster buccal pouch carcinogenesis.

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Introduction

The p53 gene is one of those most frequently involved in human cancer (Donehower and Bradley, 1993), including the oral variants of the disease (Rowley *et al*, 1998). Encoding by a gene that lacks any close relatives has been considered probable, with the existence of a p53 homolog long regarded unlikely. However, this point of view has recently shifted with the identification of two new genes homologous to p53 which encoded proteins, termed p73 and p63, which are structurally and functionally similar to p53, suggesting the existence of a family of p53-like proteins (Kaelin, 1999; Hall *et al*, 2000).

Very significant regions of p53-sequence identity have been demonstrated for p73, the first p53 homolog, which is located at chromosome 1p36, with extensive homologies in the transcriptional-activation, DNA-binding and oligomerization domains. However, there is an additional C-terminal extension not present in p53 (Kaghad *et al*, 1997). When over-produced, p73 can activate the transcription of p53-responsive genes and also induce apoptosis (Jost *et al*, 1997). However,

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p73 mutations are rare in human tumors and altered levels of expression may occur (Han *et al*, 1999).

A second p53 homolog, initially termed KET, was identified by Schmale and Bamberger (1997). Other groups independently identified this gene, which has been named, variously, as CUSP (Augustin *et al*, 1998), p40 (Trink *et al*, 1998), p73L (Senoo *et al*, 2001), p51 (Osada *et al*, 1998), and p63 (Yang *et al*, 1998). Although the amino acid sequences and the molecular weights reported are different, it is a proven fact that there are at least six different proteins which are derived from a single gene (located on human chromosome 3q) (Hagiwara *et al*, 1999). Two variants with different N-termini also exist, one of which, called transcriptionactivation (TA), contains a transactivation domain with 22% homology to the transactivation domain of p53 (Meredith and Kaelin, 2001) whereas the other variant, designated as ΔN, is devoid of this N-terminal domain (Yang *et al*, 1998). The six protein isomers referred to earlier are represented by 3TA and 3ΔN versions.

Further, it has been reported that, similar to p53, p63 can, at least when overproduced, activate the transcription of p53-responsive genes and inhibit cell growth by inducing apoptosis (Jost *et al*, 1997). Targeted disruption of the murine p63 gene results in neonatal death (Mills *et al*, 1999). Homozygotes show profound defects in limb and craniofacial development and in the differentiation of tissues with stratified epithelium, such as the skin, oral cavity, and esophagus (Yang *et al*, 1999). For example, the skin of p63-/- mice lacks stratification and the expression of differentiation markers, with these mice also lacking teeth, hair follicles and mammary, lacrymal and salivary glands (Yang *et al*, 1999). Thus, it has been demonstrated that p63 is essential for ectodermal differentiation during embryogenesis. Furthermore, p63 has been identified in keratinocyte stem cells (Pellegrini *et al*, 2001). 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 tumors (Miller *et al*, 1993; Morris *et al*, 1997). Due to the almost restricted expression of p63 in epithelial cells, as well as its functional similarity to p53, it is suggested that p63 may play a role in the regulation of proliferation and differentiation in premalignant and malignant lesions of epithelial origin.

The hamster buccal pouch mucosa constitutes one of the most widely accepted experimental models for oral carcinogenesis (Gimenez-Conti and Slaga, 1993). Despite anatomic and histologic variations between hamster pouch mucosa and human buccal tissue, experimental carcinogenesis protocols for the former induce premalignant changes and carcinomas that resemble those that take place during analogous development in human oral mucosa (Morris, 1961).

As the expression of the p53 homolog, p63, in experimental oral carcinogenesis is not completely understood, this study was designed to investigate p63-protein expression in 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch squamous cell carcinogenesis.

Materials and methods

Outbred, young (6 weeks), male, Syrian golden hamsters (*Mesocricatus auratus*; 50 animals, purchased from the National Science Council Animal Breeding Center, Taipei, ROC), weighing approximately 100 g at the beginning of the experiment, were randomly divided into three experimental and two control groups (10 animals per group). The animals were housed under constant conditions (22°C, and a 12-h light/dark cycle) and supplied with tap water and standard Purina laboratory chow *ad libitum*. Appropriate animal care and an approved experimental protocol ensured humane treatment, and all procedures were conducted in accordance with the *NIH Guide for the Care and Use of Animals*. After allowing the animals 1 week of acclimatization to their new surroundings, both pouches from all of the animals of the experimental groups were painted with a 0.5% DMBA solution at 9 AM on Monday, Wednesday and Friday of each week, using a no. 4 sable-hair brush. Bilateral pouches from each animal of one of the control groups were similarly treated with mineral oil. Approximately 0.2 ml of the appropriate solution was applied topically to the medial walls of both pouches at each painting. Another control group of 10 animals remained untreated throughout the experiment.

At the end of 3 weeks (3 days after the last treatment), all of the animals from one of the experimental groups were simultaneously killed at 9 AM, by administration of a lethal dose of diethyl ether, to avoid any influence of diurnal variation (Lin and Chen, 1997). Their pouches were exposed by dissection and examined grossly. Both pouches were then excised and placed on cardboard to prevent distortion of the pouch tissues. Nine weeks later, the 10 animals from one of the two remaining experimental groups were killed in a similar manner. Then, at 15 weeks, all of the animals from the last experimental group and those from the two control groups were killed, using the same procedure.

The pouch-mucosa specimens were routinely processed for light microscopy by fixation in 10% neutral-buffered formalin solution, with dehydration in ascending concentrations of alcohol solution, clearing in xylene, and finally paraffin embedding. Two serial sections of each specimen were prepared, at a thickness of 4 μm. One of the sections was prepared for p63-immunohistochemistry study while another was used for hematoxylin–eosin staining.

Staining was performed using a standard avidin–biotin peroxidase complex (ABC) method (Hsu *et al*, 1981). 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). With reference to the manufacturer's specifications, this antibody reacts broadly with all known p63 variants of human, rat and mouse origin by Western blotting and immunohistochemistry (including paraffin-embedded sections). The specificity of the p63 antibody has been previously demonstrated in a variety of immunoblotting

experiments, and in experiments that used immunohistochemical staining of tissues from mice that contain deletion of the p63 gene (Yang *et al*, 1998, 1999). 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 with the primary antibody (1:100) at room temperature for 60 min. These sections were then incubated for a further 30 min at room temperature with biotin-conjugated goat anti-rabbit IgG (1:100; Vector, Burlingame, CA, USA), and then for a final 30 min using ABC (Dako). The sites of peroxidase binding were visualized as brown reaction products from diaminobenzidine reaction. The sections were then counterstained with hematoxylin. 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 was omitted, was also included in each set of experiments. Tissue section in which the primary antibody was substituted for one of the same IgG subclass, but different antigenic specificity, served as additional negative staining controls.

Results

Upon gross examination, there were no apparent changes for any of the mineral oil-treated or untreated pouches. Thickened mucosa, with a rough surface and of whitish granular appearance, were noted for the 3- and 9-week DMBA-treated pouches, with 100% tumor incidence apparent for all of the 15-week analogs.

No significant histologic changes were observed for any of the mineral oil-treated or untreated pouches. However, hyperkeratosis was noted for the 3-week DMBA-treated pouches, and areas of epithelial dysplasia were observed in the 9-week DMBA-treated pouches. Furthermore, squamous cell carcinomas were detected in the 15-week DMBA-treated mucosa.

For all of the untreated and mineral oil-treated pouch mucosa, nuclear positivity for p63 was mainly observed in basal/parabasal cell layers (Figure 1). Generally, strong cell-nuclei immunoreactivity was observed within all of the DMBA-treated pouch-mucosa specimens. With a few exceptions, the cytoplasm appeared to reveal negative immunoreactivity. Faint intracytoplasmic immunoreactivity was observed for only a very small number of the DMBA-treated pouch-mucosa specimens, with strong nuclear staining. The positive nuclear-staining cells observed were randomly distributed throughout the whole epithelial layer of the 3-week (Figure 2) and 9-week DMBA-treated pouch-mucosa specimens (Figure 3). For carcinomas in 15-week DMBA-treated pouch specimens, p63 staining was consistently more uniform and homogeneous in less differentiated tumor areas (Figure 4). By contrast, p63 expression was noted mainly in the peripheral cells of tumor nests in well differentiated tumor areas

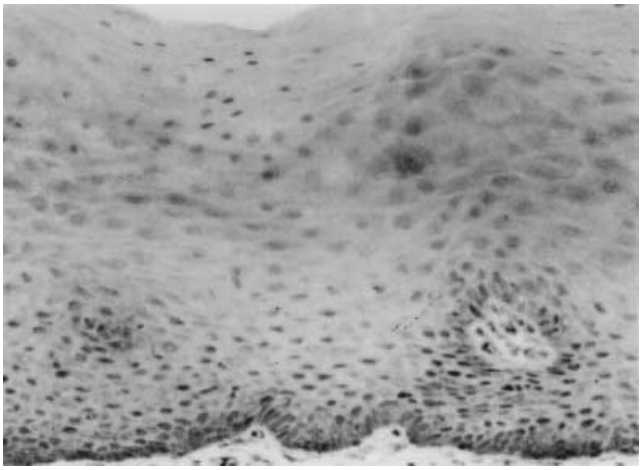

Figure 1 DMBA-untreated pouch mucosa specimen revealing nuclear positivity for p63 was mainly observed in basal cell layers and only focally in parabasal cell layers (ABC stain ×100)

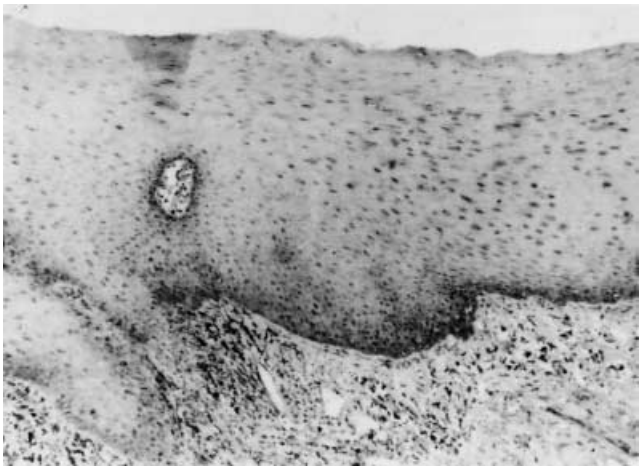

Figure 2 p63 nuclear positivity was extended from basal/parabasal layers to spinous layer in a section of a 3-week DMBA-treated pouch mucosa (ABC stain ×100)

(Figure 4). No definitive staining was demonstrated in negative control sections while definite, positive p63-protein immunostaining was revealed in positive control sections.

Discussion

Although p63 expression has been reported in human studies of head and neck squamous cell carcinomas (Nylander, Coates and Hall, 2000; Faridoni-Laurens *et al*, 2001; Choi *et al*, 2002; Weber *et al*, 2002), its expression in experimental oral carcinogenesis had not been studied. Therefore, to our knowledge, this may be the first study to document the expression of p63 protein in DMBA-induced hamster buccal pouch squamous cell carcinogenesis.

Although some background and faint cytoplasmic staining have been noted which will likely be eliminated

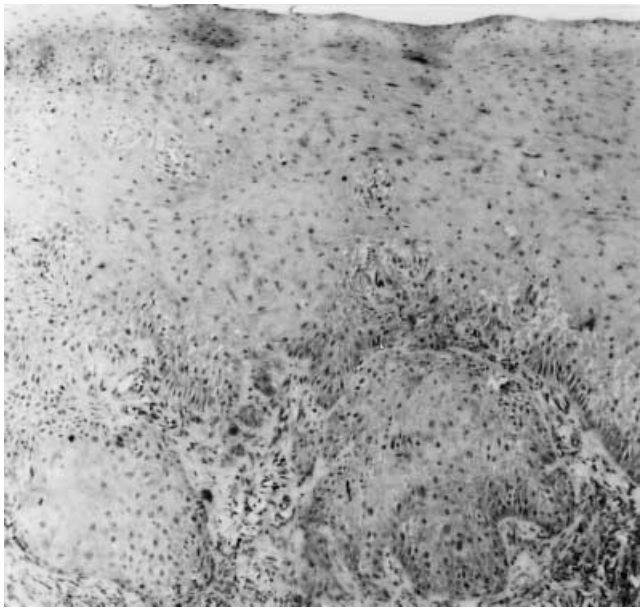

Figure 3 Positive nuclear staining cells were randomly distributed within the whole layer of a representative section of a 9-week DMBA-treated pouch mucosa specimen (ABC stain ×100)

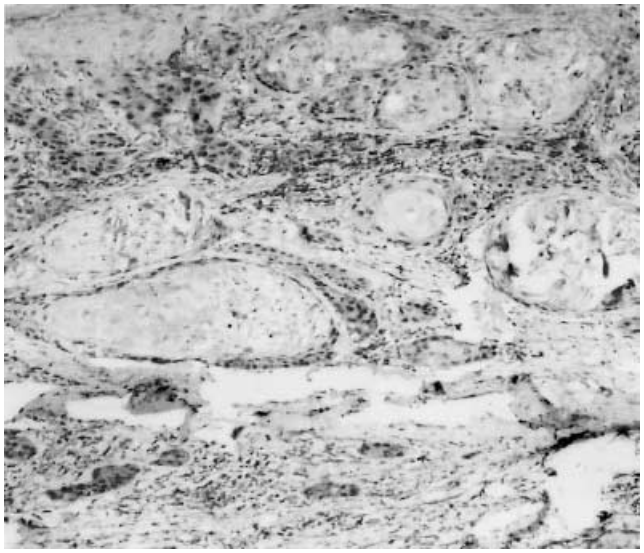

Figure 4 Fifteen-week DMBA-treated pouch specimens, staining for p63 was more uniform and homogeneous in less-differentiated tumor areas whereas p63 was noted mainly in the peripheral cells of well-differentiated tumor nests (ABC stain ×100)

with the dilution of the antibody, strong p63 nuclear staining is mainly restricted to the basal and parabasal cell layers of the untreated and mineral oil-treated pouch mucosa, which correspond to the histologically normal pouch mucosa. This finding is in agreement with the results of previous studies for human oral mucosa (Nylander *et al*, 2000; Faridoni-Laurens *et al*, 2001; Choi *et al*, 2002; Weber *et al*, 2002), and supports the proposition that p63 expression is associated with basal cells and the actively proliferating variants.

In this study, an upward extension of the p63 protein from the basal/parabasal layers to the entire epithelial layer was noted with prolonged DMBA exposure. This suggests that elevated p63 expression is consistent with the progression of dysplasias and carcinomas in DMBA-induced hamster buccal pouch carcinogenesis. Our results are compatible with the findings of a previous study of human esophageal dysplasia and carcinoma (Glickman *et al*, 2001). As p63 is constitutively expressed in normal, pouch-epithelium progenitor (basal) cells, its overexpression in squamous dysplasia and neoplasia may reflect immaturity of the tumor cell lineage.

In this study it was observed that p63 staining was more uniform and homogeneous in less differentiated tumor areas. However, p63 expression was mainly noted in the peripheral cells of tumor nests in well differentiated tumor areas. This is consistent with the finding of Sniezek *et al* (2002), who reported that p63 expression has been reduced in the hyperdifferentiated state of lichen planus, supporting the hypothesis that p63 may play an antidifferentiation role in oral mucosa. Furthermore, a high level of p63 expression has been reported in undifferentiated nasopharyngeal carcinoma (Crook *et al*, 2000). Therefore, it seems 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 proves to be effective, positive p63 immunostaining in poorly differentiated metastatic carcinomas may be helpful for prediction of primary tumors of squamous epithelial origin.

As a consequence of similarities between p63 and p53 in terms of transcriptional and apoptotic function (Jost *et al*, 1997; Yang *et al*, 1998; Hagiwara *et al*, 1999), it has been suggested that tumor suppressor function may also be comparable. Frequent overexpression (Parsa *et al*, 1999; Quade *et al*, 2001; Tannapfel *et al*, 2001) and rare genetic alterations have been reported for p63 in different tumors (Osada *et al*, 1998; Hagiwara *et al*, 1999; Tani *et al*, 1999). Further, it is important to note that the p63 locus, chromosome 3q27-ter, is not commonly lost in human carcinomas. However, it is very frequently amplified in head and neck squamous cell carcinomas (Bockmuhl *et al*, 1996), and squamous cell variants from other sites, such as the skin (Reis-Filho *et al*, 2002), lung (Bjorkqvist *et al*, 1998), and esophagus (Taniere *et al*, 2001). Therefore, p63 appears to play an oncogenic role in the development of human cancer. Our finding of DMBA-induced pouch carcinogenesis is also compatible with this view, suggesting an oncogenic function for p63 in the hamster buccal pouch model.

As mentioned before, the p63 gene can be expressed in at least six protein isoforms, which are divided into two groups – those containing the TA domain (TA isoform) and those that do not (ΔN isoform) (Little and Jochemsen, 2002). These various p63 isoforms have been reported to possess either similar or opposite functions to those of p53-related transcription factors, giving rise to the possibility that p63 could act either as a

p53-like tumor suppressor gene, or as a dominant oncogene, depending on which particular isoforms are expressed (Jost *et al*, 1997). The immunohistochemical results of this study using the 4A4 antibody have confirmed the finding of p63 overexpression which was observed in squamous cell carcinomas of the oral cavity and esophagus (Nylander *et al*, 2000; Glickman *et al*, 2001). However, the use of 4A4 antibody could not absolutely confirm whether TA or ΔN isoform has been involved in DMBA-induced hamster pouch carcinogenesis (Nylander *et al*, 2002). The presence of TAp63 mRNA in skeletal muscle, in the absence of staining with 4A4 antibody, has been reported, indicating that this antibody may not identify all p63 isoforms by immunohistochemistry (Di Como *et al*, 2002). Therefore, in order to elucidate the existence and distribution of p63 isoforms, further studies using specific p63 antisera, which are specific for the TA and ΔN isoforms or reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, can identify which particular p63 isoform(s) is/are involved in DMBA-induced hamster buccal pouch carcinogenesis. Our preliminary data using RT-PCR has revealed that ΔNp63 mRNA has been detected in all the tumors under analysis whereas the TA variant appears to be down-regulated in hamster pouch carcinogenesis (data not shown).

Up-regulation of p73 has already been reported in DMBA-induced hamster buccal pouch squamous cell carcinogenesis (Chen, Hsue and Lin, 2002). Therefore, it may be expected that p63 expression (perhaps in concert with p73) in DMBA-induced pouch carcinogenesis will block the growth-inhibition and apoptosis-induction activities of p53 or the signals that are modulated by it and, thus, may help maintain the proliferative capacity of progenitor cells in hamster buccal pouch mucosa.

In conclusion, the results of this study are consistent with those from previous analyses of p63 expression in human oral tissues (Nylander *et al*, 2000; Faridoni-Laurens *et al*, 2001; Choi *et al*, 2002; Weber *et al*, 2002), suggesting that p63 may be associated with the regulation of epithelial differentiation and proliferation in DMBA-induced hamster buccal pouch squamous cell carcinogenesis.

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