PERGAMON

Archives of Oral Biology 47 (2002) 695–699

Archives of Oral Biology

www.elsevier.com/locate/archoralbio

Short communication

Immunohistochemical demonstration of p73 protein in the early stages of DMBA-induced squamous-cell carcinogenesis in hamster buccal pouch

Yuk-Kwan Chen, Shui-Sang Hsue, Li-Min Lin*

Oral Pathology Department, School of Dentistry, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung, Taiwan

Accepted 26 April 2002

Abstract

The identification of a new protein, p73, with structural and functional similarities to p53 protein suggests that a family of p53-like proteins is likely to exist. This study investigated the status of p73 protein in the early stages of 7,12-dimethyl benz[*a*]anthracene (DMBA)-induced carcinogenesis. Outbred young (6-week-old) male Syrian golden hamsters (*Mesocricatus auratus*; 40 animals) were randomly divided into four equal groups: a 3-week DMBA-treated experimental group, a 6-week DMBA-treated experimental group, a mineral oil-treated control group, and a non-treated control group. Following this, a total of 80 specimens of pouch mucosa were obtained from the 40 animals in the four groups. Positive nuclear staining for p73 protein was randomly distributed throughout the whole epithelial layer of the DMBA-treated specimens and was absent in controls. Positive p73 staining was observed in 8 of the 20 (40%) 3-week, and 14 of the 20 (70%) 6-week DMBA-treated specimens. None of the 3-week DMBA-treated specimens revealed more than 25% p73-positive keratinocytes, but, in 12 (60%) of the 6-week-treated specimens, more than 25% of the keratinocytes examined were p73-positive. This suggests that the longer the DMBA painting period, the higher the proportion of p73-stained pouch keratinocytes. Furthermore, a p73-dependent mechanism may be associated with the early stages of oral carcinogenesis. Such a mechanism could be very important to an understanding of the participation of p73 in the development of oral squamous-cell carcinomas.

Keywords: p73; DMBA carcinogenesis; Hamster

It was thought that p53 was encoded by a gene lacking any close relatives and the existence of a p53 homologue had long been considered unlikely (Kaghad et al., 1997). In 1997, however, this viewpoint changed with the identification of a human homologue of p53, which was termed p73 (Kaghad et al., 1997). The *p73* gene, located on chromosome 1p36.33, encodes a nuclear protein with significant homology to p53 (Kaghad et al., 1997). New evidence has now indicated that there exists a *p53* gene family (Kaelin, 1999) and that *p73* is one of its members. The p73 protein has substantial structural similarity to p53 in all three domains. The NH_2-terminal *trans*-activation domain (29% identity with p53 amino acids), the sequence-specific DNA-binding region (63% identity with p53) and the oligomerization region (38% identity with p53) are remarkably conserved between the two proteins (Kaghad et al., 1997).

The p73 can, at least when overproduced, activate the transcription of p53-responsive genes and inhibit cell growth in a p53-like manner by inducing apoptosis (Jost et al., 1997), but unlike p53, no mutant p73 has been found amongst human tumours (Nomoto et al., 1998; Yokozaki et al., 1999). In contrast, mutations in the *p53* gene are detected in more than 50% of many types of cancers (Harris and Hollstein, 1993), and in 69% of human head-and-neck squamous-cell carcinomas (Ahomadegbe et al., 1995).

Immunohistochemical expression of p73 protein has recently been reported in human oral squamous-cell carcinoma (Faridoni-Laurens et al., 2001), suggesting that this

Abbreviations: ABC, avidin–biotin peroxidase complex; DMBA, 7,12-dimethyl benz[*a*]anthracene

* Corresponding author. Tel.: +886-7-3121101x7007; fax: +886-7-3210637.

E-mail address: limin@cc.kmu.edu.tw (L.-M. Lin).

0003-9969/02/$ – see front matter
PII: S0003-9969(02)00054-7

p53-related protein may be associated with the late stages of oral cancer. Thus, we have now investigated whether or not the p73 protein could also participate in the early stages of oral carcinogenesis.

The hamster buccal pouch mucosa is one of the most widely accepted experimental models of oral carcinogenesis (Gimenez-Conti and Slaga, 1993). Despite anatomical and histological differences between pouch mucosa and human buccal tissue, experimental carcinogenesis in the hamster cheek pouch produces premalignant changes and carcinomas that resemble the development of premalignancy and malignancy in human oral mucosa (Morris, 1961). To understand better the status of *p73* in oral carcinogenesis, we have studied the immunohistochemical expression of p73 protein in the early stages of DMBA-induced squamous-cell carcinogenesis in the hamster buccal pouch.

Outbred young (6-week-old) male Syrian golden hamsters (*Mesocricatus auratus*) (40 animals; purchased from the National Science Council Animal Breeding Centre, Taipei, ROC), weighing approximately 100 g at the beginning of the experiment, were randomly divided into two experimental groups (each containing 10 animals) and two control groups (each containing 10 animals). The animals were housed under constant conditions (22 °C; 12 h light/dark cycle) and fed with tap water and standard Purina laboratory chow ad libitum. Appropriate care and an approved experimental protocol ensured humane treatment of the test animals; all procedures were conducted in accordance with the *NIH Guide for the Care and Use of Animals*.

After allowing the animals a week of acclimatisation to their new surroundings, both pouches in all animals of the experimental groups were painted with a 0.5% DMBA solution at 9 a.m. on Monday, Wednesday and Friday of each week, using a number four sable-hair brush. Both pouches from each animal of one of the control groups were similarly treated with mineral oil. Approximately, 0.2 ml of each solution was applied topically to the medial walls of both pouches at each painting. The other control group was untreated throughout.

At the end of 3 weeks (3 days after the last treatment), all the animals in one of the experimental groups were killed simultaneously with a lethal dose of diethyl ether at 9 a.m. to avoid the influence of diurnal variation (Lin and Chen, 1997). Their pouches were exposed by dissection, cut from their oral opening to their caudal ends along the middle of their lateral walls, and examined grossly. Both pouches were then excised and placed on cardboard to prevent tissue distortion. Six weeks later, all the animals in the other experimental group and the two control groups were killed in a similar manner. A total of 80 specimens of pouch mucosa were obtained from the 40 animals in the two experimental and control groups.

The specimens were routinely processed for light microscopy by fixation in 10% neutral-buffered formalin solution, dehydration in ascending concentrations of alcohol solutions, clearing in xylene and then embedding in paraffin. Two serial sections of each specimen were cut at 4 μm thickness. One of the sections was prepared for the p73 immunohistochemical study; the other was used for haematoxylin–eosin staining.

Immunohistochemical staining was done by a standard ABC method (Hsu et al., 1981). Rabbit polyclonal antibodies to p73, obtained from Santa Cruz Biotechnology Inc. (cat. no. sc-7957), had been raised against a recombinant protein corresponding to amino acids 1–80 mapping at the amino terminus of human-origin p73. According to the manufacturer, this antibody recognises all p73 isoforms of human, rat and mouse origin by Western blotting, immunoprecipitation and immunohistochemistry. After deparaffinization in xylene and ethanol, the sections were treated with 0.3% H_2O_2–methanol and 10% normal goat serum (Dako, Santa Barbara, CA, USA). All sections were subsequently incubated with primary antibody (1:200) at room temperature for 30 min. They were then incubated for a further 30 min at room temperature with biotin-conjugated goat antirabbit IgG (1:100; Vector, Burlingame, CA, USA) and then for a final 30 min with ABC (Dako). The sites of peroxidase binding were visualised as brown reaction products by the diaminobenzidine reaction. The sections were counterstained with haematoxylin. Each set of experiments included a specimen of human buccal squamous-cell carcinoma known to express p73 to serve as a positive control and ensure the reproducibility of the staining process. A negative control omitting the primary antibody was also included in each set of experiments.

Because p73 is a nuclear protein, only nuclear positivity was assessed quantitatively; cells expressing only cytoplasmic staining were excluded. Therefore, only the proportion of cells demonstrating nuclear expression of p73, not the associated intensity of histochemical staining (Hall and Lane, 1994), was quantified. The proportion of positively stained cells in the whole layer of epithelium was determined by observing the entire section. The scoring system used was as follows: 3—more than 25%; 2—10–25%; 1—<10% of epithelial cells scoring positive; 0—no epithelial cells stained. Pouches were considered positive when more than 10% of the epithelial cells proved to be positive (scores 2 and 3).

Gross examination of all of the mineral oil-treated and untreated pouches revealed no apparent changes. Thickened mucosa with a rough surface and whitish granular appearance was observed in the 3- and 6-week DMBA-treated pouches. Occasional erythematous areas were also observed in the DMBA-treated mucosa.

No significant histological changes were observed in any of the mineral oil-treated and untreated pouches (Fig. 1), whereas hyperkeratosis and epithelial dysplasia were found in the 3- and 6-week DMBA-treated pouches (Fig. 2A and B).

The results of the immunostaining are summarised in Table 1. The presence of p73 as indicated by positive staining was not revealed in all of the keratinocytes of the DMBA-treated specimens. Within those specimens, strong

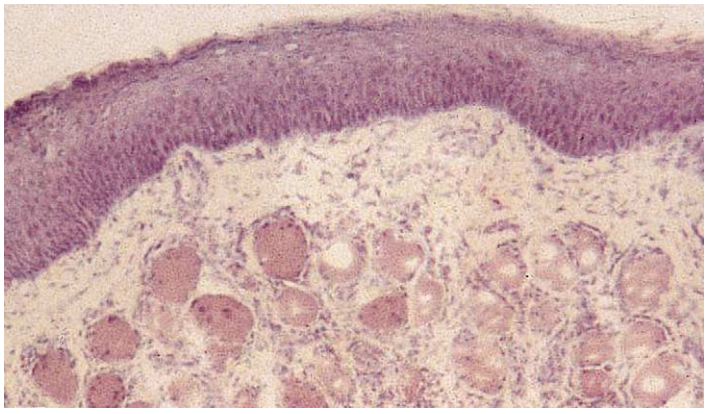

Fig. 1. Representative section of untreated pouch mucosa; negative for p73 nuclear staining. ABC stain; 40×.

nuclear immunoreactivity was generally observed; with a few exceptions, the cytoplasm was not immunoreactive. Only in a very small number of DMBA-treated specimens that had strong nuclear staining was a faint intracytoplasmic immunoreactivity observed. Furthermore, cells exhibiting positive nuclear staining were randomly distributed throughout the whole layer of pouch mucosa (Fig. 2A and B). Positive p73 staining was observed in 8 of the 20 (40%) 3-week (Fig. 2A) and 14 of the 20 (70%) 6-week (Fig. 2B) DMBA-treated specimens. None of the 3-week specimens

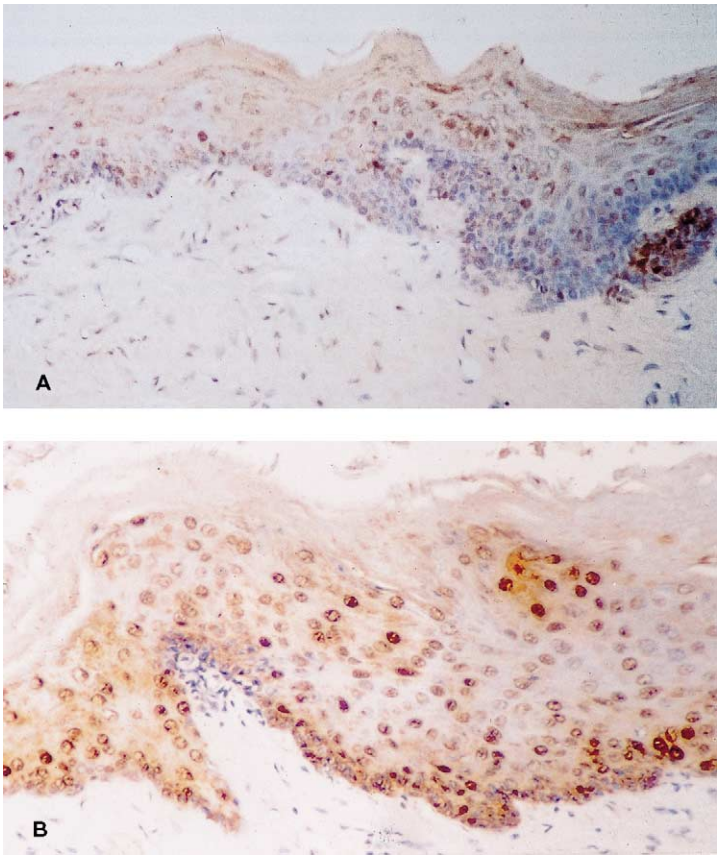

Fig. 2. Nuclear positivity for p73 located randomly within the entire epithelium in representative sections of (A) 3-week and (B) 6-week DMBA-treated pouch mucosa. ABC stain; 100×.

Table 1
Immunostaining of p73 in the early stage of DMBA-induced hamster buccal pouch squamous-cell carcinogenesis

DMBA painting period	Total number of pouch mucosa specimens	Scores for p73 staining (number of cases)				Positive rate (%)
		Score 0	Score 1	Score 2	Score 3	
3 weeks	20	0	12	8	0	40
6 weeks	20	0	6	2	12	70
No painting	20	20	0	0	0	0
Mineral oil painting	20	20	0	0	0	0

Score 3: >25%; score 2: 10–25%; score 1: <10% of the positive epithelial cells; score 0: no epithelial cells stained.

exhibited more than 25% p73-positive keratinocytes, but in 12 (60%) of the 6-week specimens, more than 25% of the keratinocytes were p73 positive. Neither mineral oil-treated nor untreated specimens exhibited p73 positivity. The negative control omitting the primary anti-p73 antibody demonstrated no definitive staining; the positive control revealed distinct positive immunostaining for the p73 protein.

The p73 protein has recently been demonstrated in a subset of head-and-neck squamous-cell carcinomas, including oral carcinomas (Faridoni-Laurens et al., 2001). In the current study, using the hamster buccal pouch model, we detected the immunoexpression of p73 protein in the early stages of DMBA-induced experimental oral carcinogenesis. The longer the DMBA painting period, the higher the proportion of p73-stained pouch keratinocytes (Table 1). To the best of our knowledge, this is the first study describing the expression of p73 protein in the early stages of experimental oral carcinogenesis. Furthermore, despite an intensive search of the available literature, no mutation of the p73 protein has been found in human cancers (Kaghad et al., 1997; Mai et al., 1998; Nomoto et al., 1998; Takahashi et al., 1998; Yokozaki et al., 1999), including oral carcinomas (Faridoni-Laurens et al., 2001). Judging from these results, it seems likely that wild-type, not mutant, p73 was overexpressed in the present study, suggesting that *p73* could play an important part in oral carcinogenesis through the overexpression of the wild type rather than as a tumour suppressor.

On the other hand, due to the remarkable homology between p73 and p53, together with the ability of p73 to induce the expression of $p21^{WAF/CIP1}$ (Clurman and Groudine, 1997; Jost et al., 1997), the (potential) participation of p73 in oral carcinogenesis in concert with p53 and/or $p21^{WAF/CIP1}$ should not be overlooked: the disruption of normal p53 function may result in a compensatory upregulation of p73 expression, and, either mutant p53 or the reduction of $p21^{WAF/CIP1}$ may also trigger an increase in p73 expression. Further study of the association between the p53 mutation and p73 overexpression is required to comprehend fully the role of p73 in the early stages of oral carcinogenesis.

In conclusion, regardless of which mechanism is implicated, the outcome of the current study suggests that a p73-dependent mechanism may be associated with the early stages of oral carcinogenesis. Such a mechanism could be very important in the overall understanding of the participation of p73 in the development of oral squamous-cell carcinomas.

Acknowledgements

We wish to acknowledge the technical assistance of Ms. N.Y. Dai. This research was supported by a grant from the National Science Council, ROC (N.S.C. 89-2314-B-037-123).

References

Ahomadegbe, J.C., Barrois, M., Fogel, S., LeBihan, M.L., DoucRasy, S., Duvillard, P., Armand, J.P., Riou, G., 1995. Frequent incidence of p53 alterations (mutation, deletion, overexpression) in head-and-neck primary tumor and metastasis; absence of correlation with clinical outcome. Frequent protein overexpression in normal epithelium and in early non-invasive lesions. Oncogene 10, 1217–1227.

Clurman, B., Groudine, M., 1997. Killer in search of a motive? Nature 389, 122–123.

Faridoni-Laurens, L., Bosq, J., Janot, F., Vayssade, M., Bihan, M.-L., Kaghad, M., Caput, D., Benard, J., Ahomadegbe, J.-C., 2001. p73 expression in basal layers of head-and-neck squamous-cell epithelium: a role in differentiation and carcinogenesis in concert with p53 and p63? Oncogene 20, 5302–5312.

Gimenez-Conti, I.B., Slaga, T.J., 1993. The hamster cheek pouch carcinogenesis model. J. Cell. Biochem. 17F (Suppl.), 83–90.

Hall, P.A., Lane, D.P., 1994. p53 in tumor pathology: can we trust immunohistochemistry? Revisited. J. Pathol. 172, 1–4.

Harris, C.C., Hollstein, M., 1993. Clinical implications of the *p53* tumor-supressor gene. N. Engl. J. Med. 329, 1318–1327.

Hsu, S.M., Raine, L., Fanger, H., 1981. Use of avidin–biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. J. Histochem. Cytochem. 29, 577–580.

Jost, C.A., Marin, M.C., Kaelin, W.G., 1997. p73 is a human p53-related protein that can induce apoptosis. Nature 389, 191–194.

Kaelin Jr., W.G., 1999. The p53 family. Oncogene 18, 7701–7705.

Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J.C., Valent, A., Minty, A., Chalon, P., Lelias, J.M., Dumont, X., Ferrara, P., McKeon, F., Caput, D., 1997. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 90, 809–819.

Lin, L.M., Chen, Y.K., 1997. Diurnal variation of γ-glutamyl transpeptidase activity during DMBA-induced hamster buccal pouch carcinogenesis. Oral Diseases 3, 153–156.

Mai, M., Yokomizo, A., Qian, C., Yang, P., Tindall, D.J., Smith, D.I., Liu, W., 1998. Activation of p73 silent allele in lung cancer. Cancer Res. 58, 2347–2349.

Morris, A.L., 1961. Factors influencing experimental carcinogenesis in the hamster cheek pouch. J. Dent. Res. 40, 3–15.

Nomoto, S., Haruki, N., Kondo, N., Konishi, H., Takahashi, T., 1998. Search for mutations and examination of allelic expression of allelic expression of the *p73* gene at 1p36.33 in human lung cancers. Cancer Res. 58, 1380–1383.

Takahashi, H., Ichimiya, S., Nimura, Y., Watanabe, M., Furusato, M., Wakui, S., Yatani, R., Nakagawara, A., 1998. Mutation, allelotyping, and transcription analysis of the *p73* gene in prostatic carcinoma. Cancer Res. 58, 2076–2077.

Yokozaki, H., Shitara, Y., Fujimoto, J., Hiyama, T., Yasui, W., Tahara, E., 1999. Alterations of p73 preferentially occur in gastric adenocarcinomas with foveolar epithelial phenotype. Int. J. Cancer 83, 192–196.