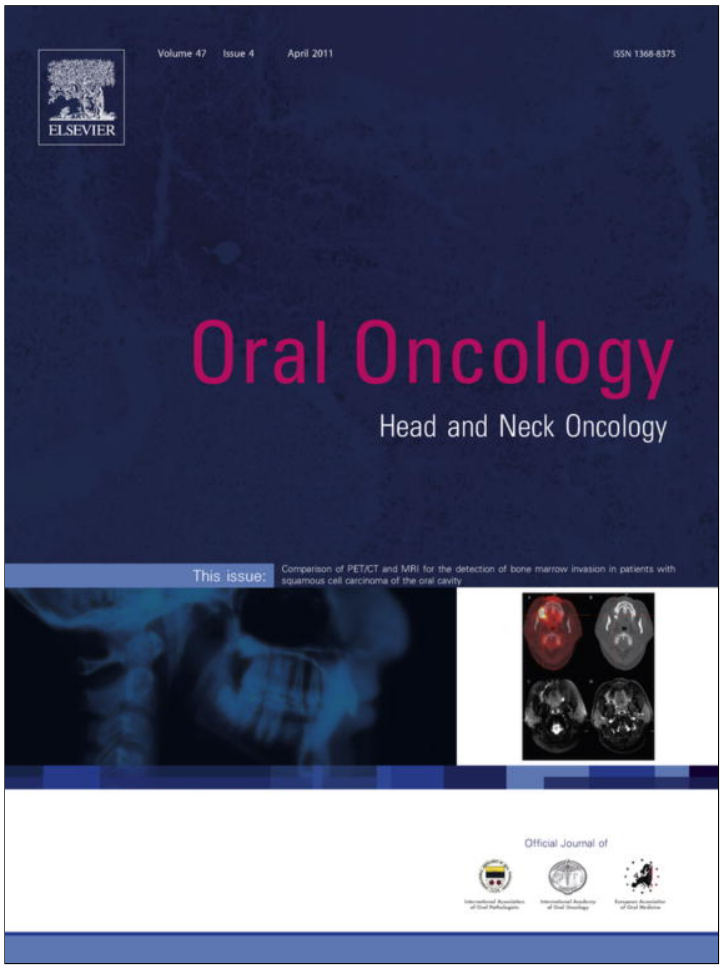

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Aberrant expression in multiple components of the transforming growth factor-β1-induced Smad signaling pathway during 7,12-dimethylbenz[a]anthracene-induced hamster buccal-pouch squamous-cell carcinogenesis

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SUMMARY

Transforming growth factor (TGF)-β1 signaling controls a plethora of cellular processes including tumorigenesis. The TGF-β1 ligand initiates signaling by binding to TGF-βreceptor II (TβRII) and allowing heterodimerization with TGF-βreceptor I (TβRI); thus, TβRI is phosphorylated by TβRII. After phosphorylation, Smad2 and Smad3 heterodimerize with Smad4, and this complex migrates to the nucleus to regulate the expression of specific target genes. However, Smad7 interrupts above signal transduction by preventing phosphorylation of Smad2 or Smad3. The objective of this study was to examine the TGF-β1-induced Smad signaling pathway during 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch squamous-cell carcinogenesis.

Fifty 6-week-old male Syrian golden hamsters were divided into three experimental and two control groups (10 animals in each). Both pouches of each animal in the experimental groups were painted with 0.5% DMBA solution, and both pouches of each animal of one of the control groups were similarly treated with mineral oil; the other control group remained untreated throughout the experiment. Animals from three experimental groups were sacrificed at the end of 3rd, 9th, and 14th-weeks after DMBA treatment, respectively, and animals from two control groups were all sacrificed at 14th-weeks after the treatment. Immunohistochemical staining for TGF-β1, TβRI, TβRII, Smad2–4 and Smad7 were performed. *Results*: A significant increase in the expression of Smad7 and significant decreases in the expression of TβRII, Smad 2, Smad3 and Smad4 were noted during hamster buccal-pouch carcinogenesis induced by DMBA.

Our findings indicate that a disruption in TGF-β1-induced Smad signaling occurs as a result of aberrant expression of multiple components in the TGF-β1 signaling pathway during DMBA-induced hamster buccal-pouch carcinogenesis, leading to loss of TGF-β1 growth-suppressive effects on transformed pouch keratinocytes.

Introduction

The development of oral squamous-cell carcinoma (SCC) is a multistage process comprising 'initiation', 'promotion', and 'progression' phases, which are reflected by accumulated genetic changes inducing malignant transformation of normal mucosa.[1] In recent years, several molecular mechanisms including the transforming growth factor (TGF)-β1 pathway have been identified as being involved in the development of oral SCC[2]; nevertheless, a complete understanding of the molecular pathogenesis of oral SCC formation is still lacking.

TGF-β1, a multifunctional growth factor, regulates the growth and differentiation in many types of cells, including epithelial cells.[3] TGF-β1 propagates the signals of these cells via a signal transduction network involving two types of transmembrane receptor serine/threonine kinases (TGF-βreceptor I, TβRI; TGF-βreceptor II, TβRII) and a set of signal-transducing proteins collectively referred to as "Smad" proteins.[4,5] The name Smad is derived from an abbreviation of the names of the TGF-βligand-signaling intermediates first identified in *Drosophila* (Mad) and *Caenorhabditis elegans* (Sma).[6–8] There are eight Smads, which are grouped into three classes: five receptor-activated Smads (Smad1–3, Smad5 and Smad8), one common mediator Smad (Smad4), and two inhibitory Smads (Smad6–7).[9–11] In TGF-β1-induced Smad (TGF-β1/Smad) signaling, binding of the TGF-β1 ligand to TβRII allows for its het-

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erodimerization with TβRI, and thereby TβRI is activated/phosphorylated by TβRII. After activation/phosphorylation, Smad2 and Smad3 heterodimerize with Smad4, and this complex translocates to the nucleus to regulate the expression of specific target genes. Smad7 can disrupt signal transduction by preventing phosphorylation of Smad2 or Smad3.[6]

The hamster buccal-pouch mucosa provides one of the most widely-accepted experimental models of oral carcinogenesis. Despite the anatomical and histological differences between (hamster) pouch mucosa and human buccal tissue, experimental carcinogenesis protocols for the former induce premalignant changes and carcinomas that are similar to the development of premalignancy and malignancy in human oral mucosa.[12] The objective of this study was to investigate the TGF-β1/Smad signaling pathway in 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch cancer model (HBPCM) in order to determine at what point abnormalities develop in hamster buccal-pouch squamous-cell carcinogenesis induced by DMBA.

Materials and methods

Animals

Fifty out-bred, 6-week-old, male Syrian golden hamsters (*Mesocricatus auratus*; purchased from the National Science Council Animal Breeding Center, Taipei, Taiwan), weighing about 100 g each at the commencement of the experiment, were randomly divided into three experimental and two control groups (ten animals in each). The animals were housed under constant conditions (22 °C; 12 h light/dark cycle) and fed tap water and standard Purina laboratory chow ad libitum. The animal-handling protocol ensured that humane practices were adhered to throughout the experimental process. Subsequent to 1-week of acclimatization to their new surroundings, both pouches of each animal from the experimental groups were painted with a 0.5% DMBA solution (Sigma, St. Louis, MO, USA; purity, ~95%) dissolved in mineral oil (Sigma; purity, 100%) at 9 AM on Monday, Wednesday and Friday of each week using a No. 4 sable-hair brush. Both pouches of each animal in one of the control groups were similarly treated with mineral oil (Sigma; purity, 100%). Approximately 0.2 ml of the respective solution was applied topically to the medial walls of both pouches at each painting session. The remaining control group of ten animals remained untreated throughout the experiment.

After 3-week (3-day after the last treatment), all of the animals from one of the experimental groups were simultaneously killed by administration of a lethal dose of diethyl ether, at 9 AM to avoid any influence of diurnal variation.[13] Their pouches were exposed, examined grossly and then excised. Nine-week later, the ten animals from one of the two remaining experimental groups were killed in a similar manner. Then, at the end of the 14-week, 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 was fixed in 10% neutral-buffered formalin solution, with dehydration in ascending concentrations of alcohol solution, clearing in xylene, and finally embedding in paraffin. Serial sections of each specimen were prepared at 4 μm thickness. One section was prepared for hematoxylin-eosin staining, while the other sections were used for immunohistochemical staining for TGF-β1 (1:100; Cat. No.: ab64715; Abchem Corporation, Cambridge, UK; mouse/monoclonal), TβRI (1:100; Cat. No.: ab31013; Abchem Corporation; mouse/monoclonal), TβRII (1:100; Cat. No.: ab28382; Abchem Corporation; mouse/monoclonal), Smad2 (1:100; Cat. No.: 3122; Cell Signaling Technology®, Danvers, MA, USA; rabbit/monoclonal), Smad3 (1:100; Cat. No.: #9523; Cell Signaling Technology®; rabbit/monoclonal), Smad4 (1:100; Cat. No.: #9515; Cell Signaling Technology®; rabbit/monoclonal) and Smad7 (1:100; Cat. No.: #H4092-M09; Abnova Corporation, Walnut, CA, USA; mouse/monoclonal) proteins.

Immunohistochemistry

Staining was performed using the standard avidin–biotin peroxidase complex method[14] subsequent to deparafinization in xylene and ethanol; the tissue sections were treated with 0.3% H_2O_2–methanol to block endogenous peroxidase activity. The antigenicity was subsequently unmasked by microwave heating for a period of 3 min in a 10 mM solution of sodium citrate. Following this, a 10% solution of normal goat serum was applied to reduce non-specific staining for all tissue sections stained for Smad2–4 proteins, while a blocking solution of 2% dry milk in phosphate-buffered saline (PBS) was applied to those sections stained for TGF-β1 and Smad7 proteins. All sections were subsequently incubated with primary antibodies at 4 °C overnight. Following rinsing with PBS, those sections intended for Smad2–4 staining were incubated for 30 min at room temperature in the presence of biotin-conjugated goat anti-rabbit IgG (Vector, Burlingame, CA, USA; 1:100). In contrast, the sections intended for TGF-β1 and Smad7 staining were treated with biotinylated anti-mouse IgG antibody (Vector; 1:100) for 30 min, following which all sections were again washed with PBS and incubated with avidin–biotin complex conjugated to horseradish peroxidase (Dako, Santa Barbara, CA, USA) for a further 30 min. Subsequent to rinsing with PBS, the sites of peroxidase binding were visualized as brown reaction products via a benzidine reaction. The sections were then counterstained with hematoxylin. The percentage of positive immunostaining (P) was scored as follows: 0 (<1%); 1 (1–24%); 2 (25–49%); 3 (50–74%); and 4 (75–100%), whereas the intensity of staining (I) was scored as 0, no staining; 1, light yellow color (weak staining); 2, brown color (moderate–strong staining); and 3, dark brown color (strong staining). The immunoscore (IS) was designated as P × I for each section. Each set of experiments included a human buccal SCC specimen, which served as a positive control and ensured the reproducibility of the staining process. A negative control, in which the primary antibody step was omitted, was also included in each set of experiments.

Statistical analysis

Statistical analyses were performed using JUMP 7.0 software (SAS, Cary, NC, USA). $p < 0.05$ was considered as significant.

Results

Gross and histopathological findings

Upon gross examination, there were no apparent changes for any of the untreated/mineral oil-treated pouches. Thickened mucosa with a rough surface and of whitish granular appearance was observed in the 3-week and 9-week DMBA-treated pouches, with 100% tumor incidence apparent for all of the 14-week analogs.

No significant histologic changes were noted for any of the untreated/mineral oil-treated pouches. Hyperkeratosis was noted in the 3-week DMBA-treated pouches, and areas of epithelial dysplasia were observed in the 9-week DMBA-treated pouches. Furthermore, SCCs were detected in the 14-week DMBA-treated mucosa.

Immunohistochemistry

No definitive staining was apparent for any of the negative control sections, while definite positive immunostaining was evident

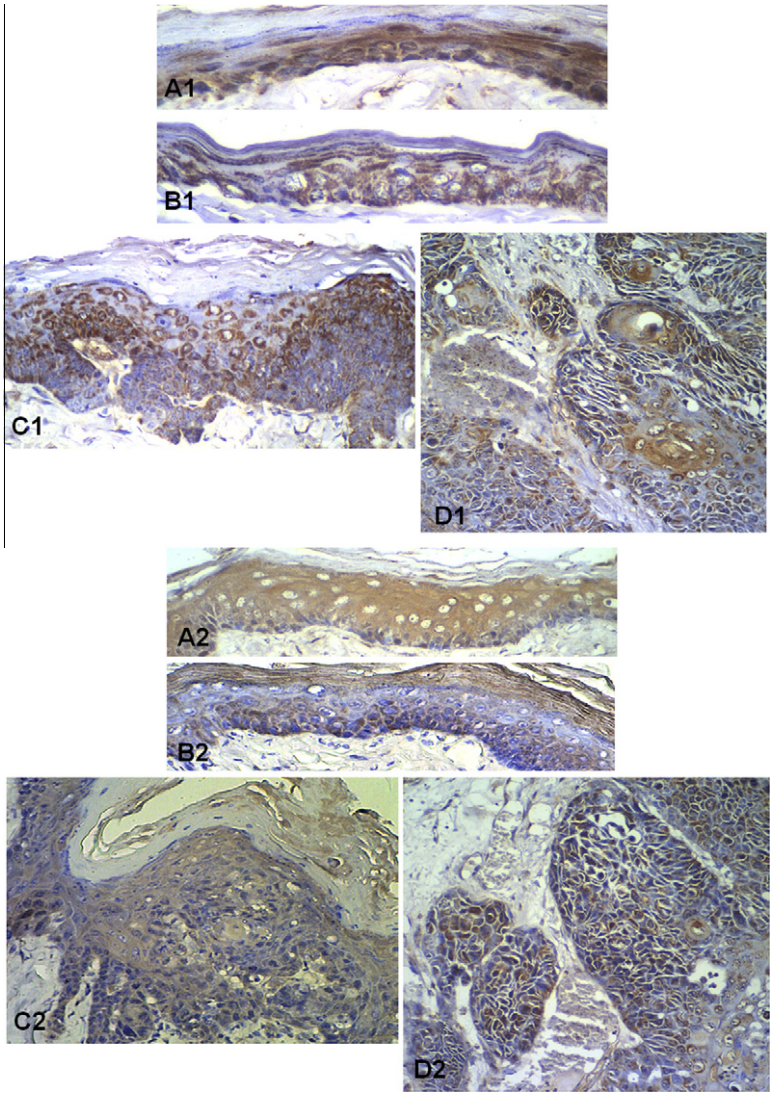

Figure 1 Representative immunohistochemical staining of TGF-β1 (A1–D1, ×200) and TβRII (A2–D2, ×200) during DMBA-induced hamster buccal-pouch squamous-cell carcinogenesis. A1, A2: untreated/mineral oil-treated group; B1, B2: 3-week DMBA-treated group; C1, C2: 9-week DMBA-treated group; D1, D2: 14-week DMBA-treated group. Similar staining pattern was observed for TβRI staining.

for all of the positive control sections. Representative immunohistochemical stainings for TGF-βI, TβRI, TβRII, Smad2–4 and Smad7 are shown in Figs. 1 and 2, and the ISs are summarized in Table 1. Cytoplasmic stainings were observed for TGF-βI, TβRI, TβRII, Smad2 and Smad3 whilst nuclear stainings were noted for Smad4 and Smad7.

No significant changes in IS for TGF-β1 and TβRI were observed in 9-week/14-week DMBA-treated groups as compared with the untreated/mineral oil-treated/3-week DMBA-treated groups. On the other hand, significant decreases in IS for TβRII and Smad2–4 were noted in 9-week/14-week DMBA-treated groups (ANOVA, $p < 0.001$) as compared with the untreated/mineral oil-treated/3-week DMBA-treated groups, while significant increases in the IS for Smad7 were observed in the 9-week/14-week DMBA-treated groups in comparison with the untreated/mineral oil-treated/3-week DMBA-treated groups (ANOVA, $p < 0.001$).

Discussion

Multiple molecular pathways such as the TGF-β1 pathway have been recognized as being involved in head and neck SCC formation,[2,15] and the TGF-β1 pathway has also been demonstrated to play a significant role in embryogenesis, organogenesis and tumor formation[16]; however, the role of TGF-β1 in tumorigenesis is complex. Currently, it is believed that TGF-β1 functions as a tumor suppressor in early tumorigenesis when epithelial cell responsiveness to TGF-β1 is still normal. Later in tumorigenesis, TGF-β1 functions predominantly as an oncogene, promoting progression to aggressive metastatic disease.[17,18]

Reductions in TGF-βreceptors and/or downstream signaling molecules (Smad) have been observed in human esophageal, head and neck cancers[19–21]; however, little is known about the expression of the TGF-β1/Smad signaling pathway in DMBA-induced HBPCM. Thus, the present study, to our knowledge, may be the first to demonstrate aberrant expression in multiple components of the TGF-β1/Smad signaling pathway during DMBA-induced hamster buccal-pouch carcinogenesis, including upregulation Smad7 as well as downregulation of TβRII and Smad2–4. These results may imply that dysregulated TGF-β1/Smad signaling occurs as a result of a variety of defects in the TGF-β1 signaling pathway upon the accumulated carcinogenic effect of DMBA on hamster buccal-pouch mucosa. Without DMBA treatment, the proliferation-inhibitory effect of TGF-β1 is active for the untreated/mineral

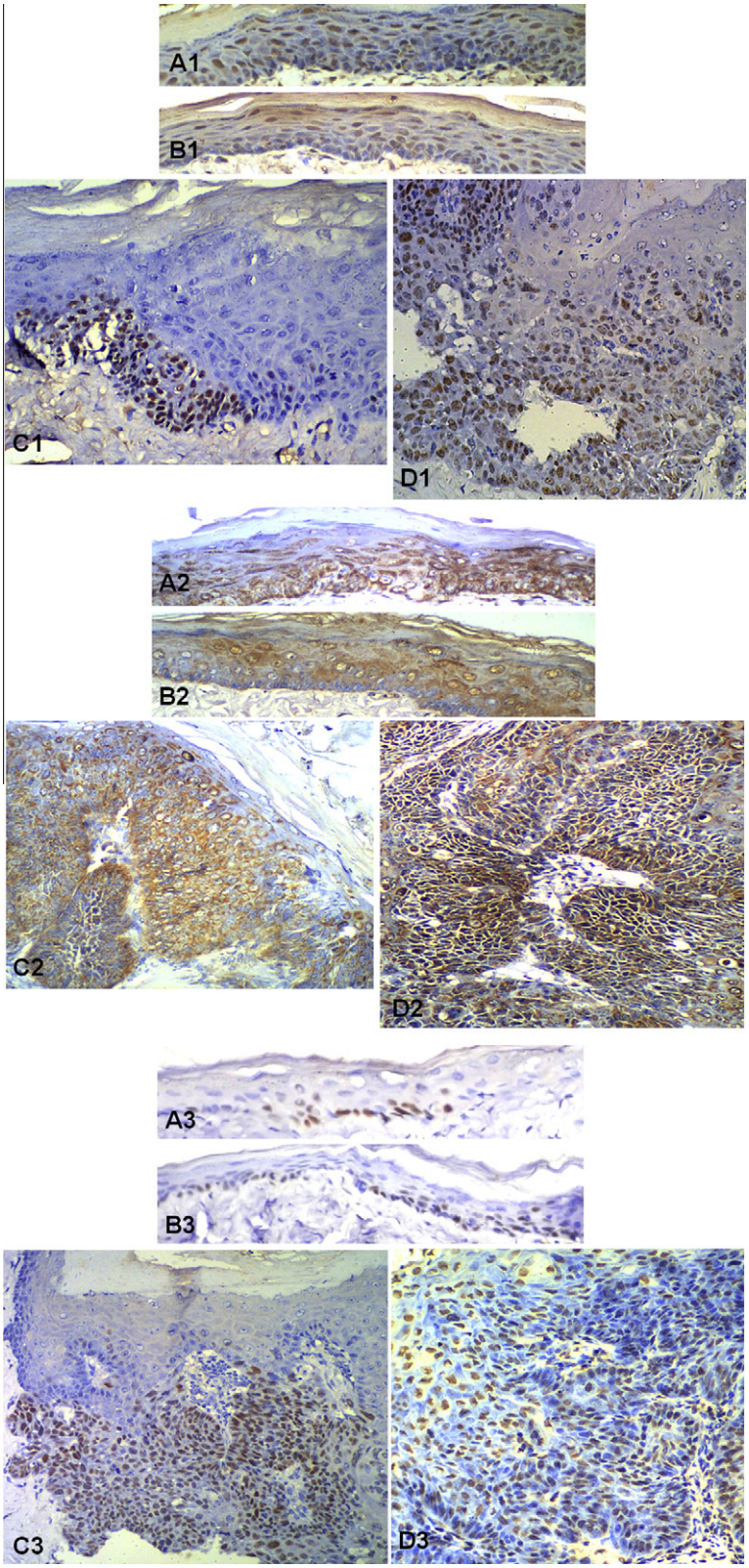

Figure 2 Representative immunohistochemical staining of Smad2 (A1-D1, ×200), Smad4 (A2–D2, ×200) & Smad7 (A3–D3, ×200) during DMBA-induced hamster buccal-pouch squamous-cell carcinogenesis. A1–A3: untreated/mineral oil-treated group; B1-B3: 3-week DMBA-treated group; C1–C3: 9-week DMBA-treated group; D1–D3: 14-week DMBA-treated group. Similar staining patterns were observed for Smad3 staining.

oil-treated pouch keratinocytes. Then, after 3-weeks of DMBA-treatment, the TGF-β1/Smad signaling pathway is still intact for the pouch mucosa. However, with longer periods of DMBA treatment, the TGF-β1/Smad signaling pathway becomes defective, leading to loss of response to the original proliferation-inhibitory effect of TGF-β1, and shifting to an oncogenic effect of tumor pro-

Table 1
Immunoscores (mean ± standard deviation) for TGF-β1, TβRI, TβRII, Smad2–4 and Smad7 during 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal-pouch squamous-cell carcinogenesis.

	Untreated group	Mineral oil-treated group	3-week DMBA-treated group	9-week DMBA-treated group	14-week DMBA-treated group
TGF-β1	11.2 ± 1.7	10.8 ± 1.7	10.9 ± 1.5	8.1 ± 3.8	9.8 ± 2.7
TβRI	11.3 ± 1.5	10.7 ± 1.5	9.6 ± 2.5	8.4 ± 2.6	10.6 ± 1.5
TβRII	11.4 ± 1.4[a]	10.8 ± 1.4[a]	9.6 ± 1.8[a]	7.0 ± 1.4[a1]	6.6 ± 1.4[a2]
Smad2	11.1 ± 1.5[b]	10.5 ± 1.4[b]	9.7 ± 1.6[b]	6.7 ± 1.2[b1]	6.7 ± 1.2[b2]
Smad3	11.0 ± 1.6[c]	10.6 ± 1.6[c]	10.6 ± 1.8[c]	6.3 ± 1.0[c1]	6.7 ± 1.2[c2]
Smad4	10.5 ± 1.7[d]	10.1 ± 1.5[d]	9.9 ± 1.9[d]	6.4 ± 0.8[d1]	6.8 ± 1.0[d2]
Smad7	3.2 ± 0.8[e]	3.4 ± 1.0[e]	4.1 ± 1.2[e]	7.6 ± 3.0[e1]	10.0 ± 2.1[e2]

Statistical decrease as compared with a and a1/a2; b and b1/b2; c and c1/c2; d and d1/d2 ($p < 0.001$, ANOVA); Statistical increase as compared with e and e1/e2 ($p < 0.001$, ANOVA). TGF: transforming growth factor; TβR: transforming growth factor- receptor; Smad: an abbreviation of the names of TGF- ligand-signaling intermediates first identified in *Drosophila* (Mad) and *Caenorhabditis elegans* (Sma).

motion occurred in the 9-week and 14-week DMBA-treated hamster buccal-pouch mucosa. Thus, we found that the defective TGF-β1/Smad signaling pathway occurs as early as the premalignant stage of DMBA-induced HBPCM.

TGF-β1 cytokine signaling is mediated through two serine threonine kinase receptors, TβRI and TβRII.[4,5] Reduced TβRII expression in particular has been found to occur more often throughout oral epithelial tumor progression.[22] Signaling via TβRII has been suggested to have a tumor-inhibitory function in tumorigenesis.[16] Notably, the ratio of TβRII:TβRI has been claimed to determine the balance between growth inhibition and other effects of TGF-β1, with a low TβRII level resulting in escape from growth inhibition, whilst the other effects of TGF-β1 remain unchanged. In the current study, a loss of TβRII expression of pouch keratinocytes commencing after 9-week of DMBA treatment was observed; on the other hand, there was no significant alteration in TβRI expression in the 9-week/14-week DMBA-treated buccal-pouch mucosa as compared with untreated/mineral oil-treated/3-week DMBA-treated pouches in the present study. Hence, these findings may indicate attenuation of TβRII signaling resulting in increased resistance to TGF-β1-mediated growth suppression, enhanced cell proliferation, and hence an increased transformation capacity of the DMBA-altered pouch keratinocytes. In addition, these findings obtained in the HBCPM are compatible with data obtained from studies of human oral SCCs.[22–25] Although mutations in individual members of the TGF-β1/Smad pathway are rare, TβRII expression has been identified to be affected by mutation in the promoter region of the gene.[26] Activated oncogene (such as *ras*) activity[27] and epigenetic modulation[28] have also been found to affect TβRII expression. Further studies are needed to explore which mechanism(s) may be associated with loss of TβRII expression in DMBA-induced HBPCM.

Smad proteins are key molecules in TGF-β1 signaling, eventually regulating both TGF-β1 tumor suppressive and oncogenic effects.[29] The roles of Smad2–3 in the development of chemically-induced cutaneous tumors have been investigated in mice.[30] Regarding DMBA-induced HBPCM, however, data on Smads are still scarce. The data (downregulation of Smad2–4 and upregulation of Smad7) derived from the current study provide evidence that Smad signaling is associated with the tumorigenesis of HBPCM. In human studies, loss of Smad2 expression and Smad3 mutation has been found to be possibly attributed to downregulation of the TGF-β1/Smad pathway.[19,31] Reduced Smad4 expression has been observed in oral SCC.[32] Immunohistochemical studies of head and neck SCC samples have demonstrated alterations of individual Smads,[19,33] and *in vitro* study has provided evidence that Smad signaling may enhance invasiveness in head and neck SCC.[34] Moreover, the mRNA expression of Smad1–8 was examined in patients with oral SCC, and Smad2 and Smad6 were found to be potential prognostic factors for survival prediction.[35] Hence, taken together, the results for Smad proteins obtained from HBPCM are partly compatible with the findings of human studies.[19,31–35]

It should be noted that there was a slight but not significant decrease in TGF-β1 expression as compared with the untreated/mineral oil-treated/3-week DMBA-treated pouch mucosa in the 9-week/14-week DMBA-treated pouch mucosa (Table 1), which implies that TGFβ-1 could still, at least theoretically, exert a growth-inhibitory effect during the late stage of DMBA-induced HBPCM. We speculate that such an inhibitory effect of TGF-β1 could be compensated by impairment of the TGF-β1/Smad signaling pathway, as demonstrated in the present study. Further studies of the detailed mechanism of TGF-β1/Smad signaling on endogenous target genes such as cell cycle regulatory proteins (p15, p21, and p27, etc.) may help to elucidate this interesting issue.[36]

In conclusion, our data provide evidence that a disruption in TGF-β1/Smad signaling occurs as a result of reduced expression of TßRII and Smad2–4 and elevated expression of Smad7 in the TGF-β1 signaling pathway in DMBA-induced hamster buccal-pouch carcinogenesis, consequently leading to loss of response to TGF-β1 growth-suppressive effects on dysplastic pouch keratinocytes. In future, quantitative measurements of the aberrant expression of TGF-β1/Smad signaling pathway using more specific real-time quantitative polymerase chain reaction are recommended in order to acquire a more confirmative conclusion.

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