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Original

Aberrant expression of Smad4, a TGF-β signaling molecule, in oral squamous cell carcinoma

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Abstract: Although carcinogenesis of oral squamous cell carcinoma (OSCC) has been studied by many investigators in the past decade, the available evidence about its molecular mechanism is inconclusive. The objective of the present study was to compare expression of Smad4, a signaling molecule of the transforming growth factor β (TGF-β) pathway, between OSCC and normal oral mucosa. We assayed expression of Smad4 in OSCC and normal oral mucosa by performing immunohistochemistry using paraffin-embedded tissue samples. We also compared expression of Smad4 protein between OSCC lines and normal oral keratinocytes, using Western blot analysis. Smad4 expression was observed in only 60% of OSCC tissue samples, whereas it was observed in 82% of normal oral mucosa samples. Reduced Smad4 expression was clearly observed in all OSCC lines, compared with normal oral keratinocytes. These findings suggest that aberration of the TGF-β pathway, as indicated by a reduction or absence of Smad4 expression, promotes carcinogenesis of OSCC. (J. Oral Sci. 48, 105-109, 2006)

Keywords: oral squamous cell carcinoma; transforming growth factor β; Smad4.

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Introduction

Oral squamous cell carcinoma (OSCC) is a serious oral health problem worldwide (1). It has long been known that carcinogenesis of OSCC is multi-step and involves many genetic alterations. In the past few decades, there have been several studies of the molecular mechanisms of OSCC development. These studies indicate that the molecules p53, Ki67 (2), $p27^{KIP1}$, $p12^{DOC-1}$, $p16^{INK4a}$, $p21^{WAF1/CIP1}$ and cyclin D1 (3,4) are expressed in an aberrant manner in OSCC, suggesting that these molecules are involved in carcinogenesis of OSCC. Nevertheless, the complete process of OSCC progression and metastasis remains unclear.

Transforming growth factor β (TGF-β), a multifunctional growth factor, regulates growth and differentiation of many cell types (5). The role of TGF-β in tumorigenesis is rather complicated. Currently, it is believed that TGF-β functions as a tumor suppressor early in tumorigenesis, when epithelial cell responsiveness to TGF-β is still relatively normal. Later in tumorigenesis, TGF-β paradoxically functions predominantly as an oncogene promoting progression to aggressive metastatic disease (6,7). Reduction or absence of TGF-β receptors and/or downstream signaling molecules, also known as Smads, has been observed in several human cancers including esophageal cancer (8,9), follicular thyroid tumor (10), pancreatic cancer (11,12), colorectal cancer (13), and head and neck cancer (14). However, little is known about the expression of TGF-β signaling molecules, particularly in OSCC. In the present study, we examined expression of Smad4, which is a downstream signaling molecule of

TGF-β, in OSCC tissue samples and cell lines and their normal counterparts.

Materials and Methods

Immunohistochemistry of Smad4 protein in OSCC tissue samples

Fifteen paraffin-embedded tissue samples of OSCC and 11 paraffin-embedded tissue samples of normal oral mucosa were obtained from the archives of the Oral Pathology Laboratory, Faculty of Dentistry, Chiang Mai University, Thailand.

We assayed Smad4 by performing immunohistochemistry using a mouse monoclonal anti-Smad4 antibody (sc-7966; dilution, 1:50; Santa Cruz Biotechnology, CA). The specificity of this anti-Smad4 antibody is described elsewhere (8). Briefly, deparaffinized sections were immersed in 3% hydrogen peroxide solution for 15 min to block endogenous peroxidase activity. Then, sections were given water bath treatment in Antigen Retrieval Solution® (Dako, Denmark) for 40 min, allowed to cool for 20 min, washed in Tris-buffered saline (TBS), and incubated with 5% normal serum for 10 min to block nonspecific binding. Sections were then incubated with the mouse monoclonal anti-Smad4 antibody overnight at 4˚C. On the following day, sections were washed in TBS, and were then incubated with biotinylated secondary antibody and streptavidin using a Vectastain® Universal Quick Kit (Vector, Burlingame, CA). The chromogen was developed using an Immunon® 3-amino-9-ethylcarbazole (AEC) substrate system (Immunon, Pittsburg, PA) for 15 min. Sections were then counterstained with hematoxylin, and mounted. The slides were viewed and photographed under an epifluorescence microscope (Olympus, Tokyo, Japan). Negative control sections were processed identical to the experimental sections, except that the primary antibody was omitted and replaced with normal serum or buffer.

The expression of Smad4 was semiquantitatively analyzed. Cells were considered to be positive if there was any staining of the cytoplasm and/or nuclei, regardless of the staining intensity. The percentage of immunoreactive nuclei was evaluated, and the samples were scored according to the following criteria: - = < 10%; + = 10-50%; 2+ = 50-90%; 3+ = > 90%.

Western blot analysis of Smad4 protein in OSCC cell lines

Five OSCC cell lines were used in the present study. The SCC4 cell line was obtained from ATCC (American Type Culture Collection). Cell lines SCC25, SCC66, SCC68 and SCC111 were donated by Dr. J. Rheinwald (Brigham and Women's Hospital, Boston, MA). The tumors from which these lines were derived did not undergo any treatment prior to establishment. All OSCC lines were grown in SCC/J2 media (Ham's F12/Dulbecco's Modified Eagle Medium (DMEM) ratio, 1:1) (15). Monolayer cultures of human normal oral keratinocytes (NOK) were prepared from gingival tissue obtained from oral surgeries, and were grown in keratinocyte serum-free medium (Keratinocyte-SFM, GIBGO BRL, Gaithersburg, MD) supplemented with epidermal growth factor and bovine pituitary extract (16). The antibodies were purchased from the following suppliers, and were used according to the suppliers' protocols: actin (A2066), Sigma-Aldrich (St. Louis, MO); Smad4 (sc-7966), Santa Cruz Biotechnology (Santa Cruz, CA).

Protein lysates of the OSCC cell lines were prepared in EBC buffer (50 mM Tris-HCl, 120 mM sodium chloride (NaCl), 1% [v/v] Nonidet P40, pH 8.0) containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml aprotinin, 1 mg/ml leupeptin, 2 mM sodium fluoride (NaF), and 0.5 mM sodium-orthovanadate. The lysates were gel-electrophoresed, followed by immunoblot analysis using polyvinylidene fluoride membranes (Immobilon P, Millipore, Billerica, MA). Antigen-antibody complexes were detected using horseradish peroxidase-linked sheep anti-mouse and donkey anti-rabbit secondary antibodies (Amersham Corp., Pitcataway, NJ) and enhanced chemiluminescence (Renaissance, NEN Dupont, Boston, MA). In some cases, the membranes were stripped and reprobed according to the manufacturer's protocol. The signals from each band were quantified using ImageJ software, and were normalized to the corresponding actin band.

Results

Immunohistochemistry of Smad4 protein in OSCC tissue samples

The expression of Smad4 was preserved in 60% of OSCC tissue samples and 82% of normal oral mucosa samples (Table 1). The immunostaining of Smad4 was strongly localized in the nucleus, with weak staining in the cytoplasm (Fig. 1A). One of the Smad4-negative OSCC tissue samples is shown in Fig. 1B. The immunostaining of Smad4 in normal oral epithelium is illustrated in Fig. 1C, in which the staining is localized mainly in the basal

Table 1 Smad4 expression in normal oral mucosa and OSCC

	Smad4 expression				
	–	+	++	+++	N
Normal oral mucosa	2	1	2	6	11
OSCC	6	0	3	6	15

and spinous cell layers. Negative control sections showed no staining.

Western blot analysis of Smad4 protein in OSCC cell lines

Because immunohistochemistry indicated an absence of Smad4 protein in some of the OSCC tissue samples, we measured steady-state protein levels of Smad4 protein in the OSCC lines. All tumors from which the OSCC lines were derived had not been treated by radiation or chemotherapy before surgery, and the excised tumors did not undergo any treatment prior to the establishment of the cell lines. Therefore, the present results can be considered to accurately reflect the characteristics of OSCC. NOK was used as a normal control for comparison of levels of Smad4. After immunoblot analysis, the intensity of each band reflecting the Smad4 steady-state protein level from each cell line was quantified using ImageJ software and illustrated using a bar graph. Smad4 steady-state protein levels were lower in all OSCC lines than in the NOK (Fig. 2).

Discussion

The signaling pathway of TGF-β in epithelial cells is very complex. It is believed that TGF-β regulates a plethora of developmental processes. Under normal circumstances, TGF-β transmits its signal into the cell via the tetrameric cell surface receptors: TGF-β receptors I and II (17). Activated TGF-β receptors I and II phosphorylate the cytoplasmic signaling proteins Smad2 and Smad3, which then form complexes with Smad4 and translocate into the nucleus, where they function as transcription factors of target genes. Negative feedback in transduction of the TGF-β signal is believed to be autoregulated by the inhibitory protein Smad7 (18).

Disruption of the components of TGF-β signal transduction (the ligand, the receptors, the signal transducers and their transcriptional targets) causes a wide variety of human diseases including cancers, chondrodysplasia and pulmonary hypertension (19). In a study of a mouse multistage model of skin carcinogenesis, TGF-β1 was not detectable in chemically induced papillomas with a high frequency of malignant progression (20). Targeted expression of a dominant-negative type-II TGF-β receptor (DN-TβR-II) in mouse skin leads to an increase in carcinoma incidence and a decrease in tumor latency (21). Expression of the activated, phosphorylated form of Smad2 (Smad2-P) has been found to be absent in approximately 70% of head and neck tumors (14).

Aberrant expression of Smad4, which has been shown to be a tumor suppressor, has been observed in several types

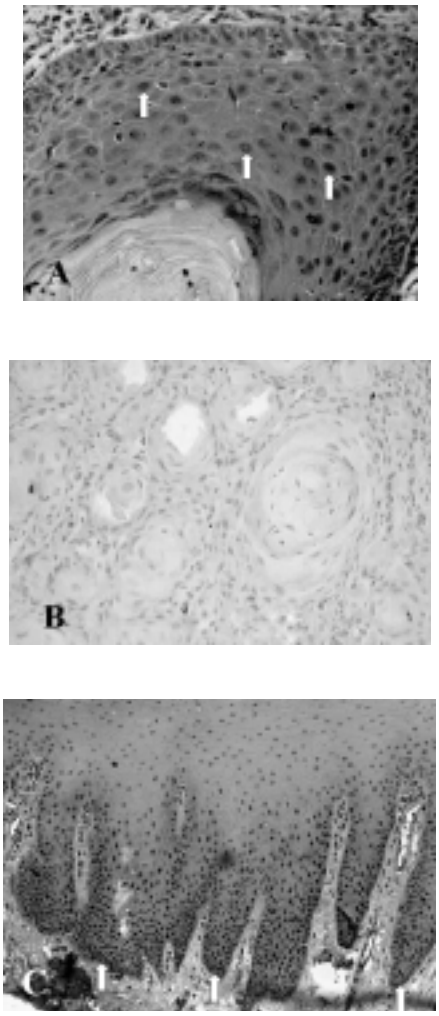

Fig. 1 A) OSCC cells strongly express Smad4 in the nuclei (arrows) (original magnification, ×200). B) A Smad4-negative OSCC tissue sample (original magnification, ×100). C) Smad4 is strongly expressed in the nuclei and cytoplasm of the basal and lower spinous cell layers of normal oral epithelium (arrows) (original magnification, ×100).

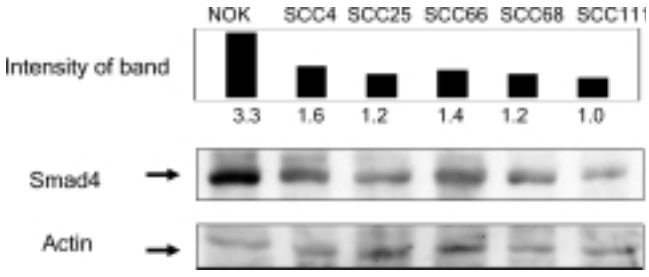

Fig. 2 Immunoblot analysis of Smad4 protein expression in OSCC lines. Protein lysate samples (20 μg) were subjected to immunoblot analysis. Normal oral keratinocytes (NOK) were used as a normal control. The intensity of each band was quantified, and the intensities are shown in the bar graphs above the bands.

of malignant tumor, including tumors of the pancreas (22), colorectum (23), breast (24), lung (25), ovary (26), head and neck (14,27), and esophagus (8). The present immunohistochemical results indicate that expression of Smad4 was absent in 40% of OSCC tissue samples, compared with only 18% of normal oral mucosa tissue samples. These findings indicate that loss of Smad4 expression occurs more frequently in OSCC than in normal oral mucosa, and suggest that loss of tumor suppression is involved in carcinogenesis of some OSCC cases. Previous studies of Smad4 expression in various cancers have produced varied results. For example, absence of Smad4 expression has been observed in 23.5 to 55% of pancreatic adenocarcinoma (28,29), 67.8% of esophageal squamous cell carcinoma (8), and 22 to 38.5% of head and neck squamous cell carcinoma (14,27). In a study of correlation between expression of Smad4 and prognosis of patients with esophageal squamous cell carcinoma, those patients with preserved expression of Smad4 had a higher rate of early-stage carcinoma and fewer lymph node metastases than those with reduced Smad4 expression (8). In another study of esophageal squamous cell carcinoma, there was a significant inverse correlation between Smad4 expression and both depth of invasion and pathological stage (9). In a study of pancreatic adenocarcinoma, absence of Smad4 expression was observed more frequently in patients at TNM stage IV than in patients at lower stages, and absence of Smad4 expression was observed more frequently in patients with poorly differentiated adenocarcinoma than in patients with well or moderately differentiated adenocarcinoma (28). Moreover, studies indicate that pancreatic adenocarcinoma patients with Smad4 expression survive longer than those without Smad4 expression (28,29).

The present Western blot analysis indicated that expression of Smad4 protein was reduced in all OSCC cell lines investigated. It was recently reported that 2 carcinoma-derived human oral keratinocyte cell lines have undetectable levels of Smad4 protein (30). These cell lines are resistant to TGF-β1-induced growth arrest. In addition, transient transfection of Smad4 into one of these cell lines restored TGF-β1-induced growth inhibition and Smad-dependent transcriptional activation. Consistent with these previous findings, the downregulation of Smad4 protein in the present OSCC lines may play a role in regulation of the TGF-β pathway. Collectively, these findings indicate that aberrant expression of Smad4 is common in human cancers, including OSCC, and may be involved in progression and metastasis of the disease.

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