Aberration of a TGF-β Signaling Molecule, Smad4, in Oral Squamous Cell Carcinoma

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Abstract:Although carcinogenesis of oral squamous cell carcinoma (OSCC) have been studied by many investigators in the past decade, the data about its molecular mechanism remain fragmented. The objective of the present study was to investigate the expression of a signaling molecule of TGF-β pathway, Smad4 in OSCC in comparison with normal oral mucosa. The expression of Smad4 in tissue samples of OSCC and oral normal mucosa was studied by means of immunohistochemical technique. We also compared the expression of Smad4 protein in SCC cell lines and normal oral keratinocytes by Western blot analysis. The rate of Smad4 expression in OSCC tissue samples was only 60% while 82% in tissue samples of normal oral mucosa. A reduction of Smad4 expression was clearly shown in all SCC cell lines as compared with normal oral keratinocytes. These findings indicate that the aberration of TGF-β pathway as evidenced by a reduction or deletion of Smad4 expression may promote carcinogenesis of OSCC.

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

Introduction

Oral squamous cell carcinoma (OSCC) is a serious problem of oral health worldwide including the northern Thailand population[(1)]. It has long been known that carcinogenesis of OSCC is multi-step and involved with many genetic alterations. In the past decades, several attempts have been performed in order to elucidate the molecular mechanism of OSCC development. As a result, a number of molecules, for example, p53, Ki67 [(2)], $p27^{KIP1}$, $p12^{DOC-1}$, $p16^{INK4a}$, $p21^{WAF1/CIP1}$, and cyclin D1 [(3,4)] have been found to be aberrant in OSCC, suggesting their involvement in carcinogenesis of OSCC. Nevertheless, the full picture of OSCC progression and metastasis remains fragmented in many ways.

TGF-β, a multifunctional growth factor, is known to regulate 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 the process, paradoxically, TGF-β functions predominantly as an oncogene to promote the progression to aggressive metastatic disease[(6,7)]. Reduction or loss 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. To address this question, we here investigated the expression of a downstream signaling molecule of TGF-β, Smad4, in OSCC tissue samples and cell lines in comparison with that of the normal counterparts.

Materials and methods

Sample collection and immunohistochemistry

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

The study of Smad4 expression was conducted by a technique of immunohistochemistry with the use of the mouse monoclonal anti-Smad4 (dilution, 1:50; sc-7966; Santa Cruz Biotechnology, CA) antibody. The specificity of the Smad4 antibody has previously been examined[(8)]. Briefly, deparaffinized sections were immersed in 3% hydrogen peroxide solution for 15 min to block endogenous peroxidase activity. Subsequently, sections were submitted to a water bath treatment in Antigen Retrieval Solution[â] (Dako, Denmark) for 40 min, allowed to cool down for 20 min, washed in 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, incubated with biotinylated secondary antibody and streptavidin by using Vectastain[â] Universal Quick Kit (Vector, Burlingame, CA). Chromogen was developed by using Immunon[â] AEC substrate system (Immunon, Pittsburg, PA) for 15 min. Sections were 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 experimental sections except that the primary antibody was omitted and replaced with normal serum or buffer.

Cell culture

Five oral squamous cell carcinoma cell lines were used in the present study. The SCC4 cell line was obtained from ATCC, SCC25, SCC66, SCC68 and SCC111 were kind gifts from Dr. J. Rheinwald (Brigham and Women Hospital, Boston, MA). The tumors from which these lines were derived did not undergo any treatment prior to establishment. All SCC lines were grown in SCC/J2 media (Ham F12: DMEM; 1:1) [(15)]. Monolayer cultures of human normal oral keratinocytes (NOK) were prepared from gingival tissues obtained from oral surgeries and grown in keratinocyte serum free medium (Keratinocyte-SFM, GIBGO BRL, Gaithersburg, MD) supplemented with epidermal growth

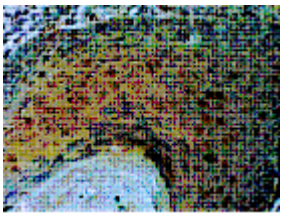

Fig.1

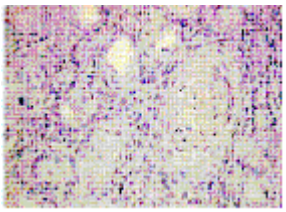

Fig.2

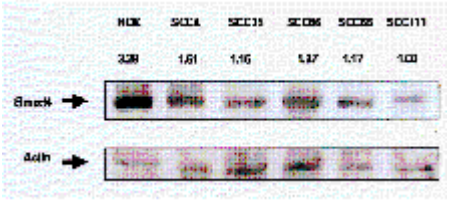

Fig.4

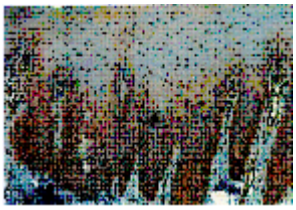

Fig.3

factor and bovine pituitary extract [16].

Antibodies and immunological methods

The antibodies were purchased from and used according to the protocols provided by the following suppliers: actin (Sigma-Aldrich, A2066, St. Louis, MO), Smad4 (Santa Cruz Biotechnology, sc-7966, CA). Protein lysates were prepared in EBC buffer (50 mM Tris-HCl, 120 mM NaCl, 1% (v/v) Nonidet P40, pH 8.0), 0.5 mM PMSF, 1 mg/ml aprotinin and leupeptin, 2 mM NaF, 0.5 mM Na-orthovanadate. After gel electrophoresis, immunoblot analysis was performed 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 quantification of signals from each band was analyzed by ImageJ program and and normalized by corresponding actin band.

Results and Discussion

Immunohistochemistry

The results revealed that the expression of Smad4 was preserved in 60% of OSCC cases and 82% of normal oral mucosa, respectively. The immunostaining of Smad4 was strongly localized in the nuclei and weakly stained in the cytoplasm (Fig. 1). One of the Smad4-negative OSCC cases was shown in Figure 2. The immunostaining of Smad4 in normal oral epithelium was illustrated in Figure 3 where the staining was localized particularly in the basal and spinous cell layers. Negative control sections were stained appropriately.

Western blot analysis of the Smad4 protein in SCC lines

Since immunohistochemistry result revealed that the Smad4 protein was undetected in some OSCC cases, we investigated the steady state protein levels of the Smad4 protein in some SCC lines. The tumor origin of each SCC line was not undergone any treatment prior to surgery. Therefore, the result detected directly revealed the nature of SCC and was not modified by any treatment. NOK was used as a normal control for the normal level of Smad4. As seen in Figure 4, Smad4 steady state protein levels were lower in all SCC lines compared to the NOK.

Collectively, these findings indicate that an aberration of Smad4 is evident in OSCC and may involve in progression and metastasis of the disease.

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