High-Level Expression of the Smad Ubiquitin Ligase Smurf2 Correlates with Poor Prognosis in Patients with Esophageal Squamous Cell Carcinoma

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Abstract

Transforming growth factor β (TGF-β) regulates growth of various cells, and inactivation of the TGF-β signaling pathway contributes to tumor progression. Smad2 is phosphorylated and activated by TGF-β, resulting in the antiproliferative effects of TGF-β signaling. Smurf2 (Smad ubiquitination regulatory factor 2) was identified as the Smad ubiquitin ligase that induces the ubiquitination and degradation of Smad2. This study was undertaken to elucidate the relationships between Smurf2 expression and the clinicopathological characteristics of patients with esophageal squamous cell carcinoma (SCC) and the correlation between Smurf2 and Smad2 expression. Surgical specimens obtained from 80 patients with esophageal SCC were subjected to immunohistochemical staining. Our data indicated that high-level expression of Smurf2 correlated with depth of invasion, lymph node metastasis, and a poor survival rate. We also found an inverse correlation between the expression of Smurf2 and Smad2. Western blotting analysis of esophageal SCC-derived cell lines revealed similar inverse correlations. We demonstrated that high-level expression of Smurf2 appears to correlate with tumor development and poor prognosis in patients with esophageal SCC and that alteration of Smad2 expression in the TGF-β signaling pathway may be induced by enhancement of Smad2 degradation mediated by high-level expression of Smurf2.

Introduction

Members of the TGF-β superfamily are multifunctional proteins that regulate a wide range of cellular processes, such as growth, differentiation, apoptosis, and morphogenesis. TGF-β and related proteins bind to two different types of serine/threonine kinase receptors, termed type I and type II receptors. Type I receptors are activated by type II receptors upon ligand binding and mediate specific intracellular signals that are transmitted by Smad proteins (1). Eight different Smad proteins have been identified in mammals, and they are classified into three subgroups: (a) receptor-regulated Smads (R-Smads); (b) common partner Smads (Co-Smads); and (c) inhibitory Smads (I-Smads). R-Smads are phosphorylated and activated by various type I receptors of the TGF-β superfamily (2), and then they form heteromeric complexes with Co-Smads and translocate into the nucleus. Nuclear Smad complexes regulate transcription of target genes. Among the R-Smads, Smad2 and Smad3 act in the TGF-β and activin pathways, whereas Smad1, Smad5, and Smad8 act in the bone morphogenetic protein pathways. I-Smads, including Smad6 and Smad7, bind to type I receptors and compete with R-Smads for activation, resulting in inhibition of TGF-β superfamily signaling (3).

TGF-β strongly inhibits cell proliferation, and carcinomas show loss of the growth-inhibitory responses to TGF-β (4). Resistance to TGF-β is associated with functional inactivation of TGF-β-specific receptors or Smads (5). Inactivating mutations of Smads have been detected in human cancers (6, 7). Smad2 is a specific intracellular mediator of TGF-β signaling, and activated Smad2 (P-Smad2) resulting from the action of TGF-β may be involved in growth inhibition (8). In a previous study, we observed that altered expression of P-Smad2 correlated with tumor progression in patients with esophageal SCC,4 but mutation of the mad2 genes was rare (9). Lack of P-Smad2, rather than gene mutation, may be a more likely mechanism for inactivation of the TGF-β-specific receptors and related proteins that regulate the phosphorylation of Smad2, but the mechanism responsible for such inactivation in esophageal SCC is still unclear.

Ubiquitin-dependent protein degradation plays key roles in various biological processes, including signal transduction, cell cycle progression, and transcriptional regulation (10). Ubiquitination of proteins is induced by an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. In the ubiquitin-proteasome pathway, ubiquitin ligases play crucial roles in the recognition of target proteins and subsequent protein degradation (11). Smurf1 was originally identified as the ubiquitin ligase that induces the ubiquitination and degradation of bone morphogenic protein-specific Smad1 and Smad5 (12). In contrast, Smurf2, a Smurf1-related ubiquitin ligase, targets TGF-β pathway-restricted Smad2 (13, 14). Furthermore, Smurf2 interacts with Smad7, and the resulting Smurf-Smad7 complexes then associate with TβRI and enhance their turnover (15).

In this study, we explored the hypothesis that the mechanism responsible for altered expression of P-Smad2 might be attributable to Smad2 degradation and high-level expression of its ubiquitin ligase, Smurf2. However, it remains to be elucidated whether expression of Smurf2 is abnormal and whether there is a correlation between Smurf2 and Smad2 expression in carcinomas. Therefore, we examined Smurf2 expression and its correlations with Smad2 expression and degradation in esophageal SCC.

Materials and Methods

Patients. Surgical specimens were obtained from patients (69 males and 11 females) who had esophageal SCC and underwent potentially curative surgery without preoperative therapy at the Department of Surgery I, Gunma University Faculty of Medicine between 1983 and 2000. The patients’ ages ranged from 40 to 78 years, and their mean age was 61.6 years. Tumor stages were classified according to the fifth edition of the TNM (tumor-node-metastasis) classification of the International Union Against Cancer.

Immunohistochemical Staining of Smurf2. Resected specimens were fixed with 10% formaldehyde and embedded in paraffin blocks. Immunohistochemical staining of the sections was performed by the standard avidin-biotin peroxidase complex method described previously (16). Briefly, the sections were incubated with an anti-Smurf2 polyclonal antibody (Upstate Biotechnology, Lake Placid, NY) at a dilution of 1:100 and counterstained lightly with hematoxylin. A negative control to each section was prepared by substituting normal rabbit serum for the primary antibody. No staining was detected in any control section.

Evaluation of Smurf2 Expression. The expression of Smurf2 in esophageal tumor cells was compared with that in normal esophageal epithelium. When tumor cells, especially those at the tumor front, were stained more strongly than or as strongly as normal epithelial cells, the sample was classified as Smurf2 positive, and when tumor cells were stained more weakly than normal epithelial cells or not stained at all, the sample was classified as Smurf2 negative.

Cell Culture. Five established cell lines derived from esophageal SCC were used: TE-series 1, 2, 8, 13, and 15 (gifts from Dr. T. Nishihira; Tohoku University, Sendai, Japan; Ref. 17). These TE-series cells were cultured in RPMI 1640 (Sigma, St. Louis, MO) containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin).

Cell Extraction and Western Blotting. Lysates from exponentially growing cell lines were prepared in a buffer comprising 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride and subjected to Western blotting, as described previously (16). The protein concentrations were determined with a BCA Protein Assay Kit (Pierce, Rockford, IL). A 30-µg aliquot of protein from each cell line was subjected to electrophoresis on a 10% Ready-Gel (Bio-Rad, Tokyo, Japan), followed by electroblotting onto a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The proteins were immunoblotted using anti-Smurf2 (Upstate Biotechnology), anti-Smad2-P (Upstate Biotechnology), anti-Smad7 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-TβR-I (Santa Cruz Biotechnology) antibodies. An anti-β-actin (Sigma) antibody served as the control.

Statistical Analysis. Statistical analysis was performed using the χ² test, Fisher’s exact test, and the Mann-Whitney t test. Patient survival curves were calculated using the Kaplan-Meier method, and analysis of survival was performed using the log-rank test.

Results

Correlations between Clinicopathological Findings and Smurf2 Expression. In normal esophageal squamous epithelium, immunostaining of Smurf2 was detected in the cytoplasm and nuclei of basal and suprabasal terminally differentiated keratinocytes (Fig. 1A). In primary esophageal SCCs, Smurf2-positive staining was also detected in the cytoplasm and nuclei (Fig. 1B).

The correlations among the clinicopathological characteristics of patients with esophageal SCC and Smurf2 expression are summarized in Table 1. The SCCs of 45 of 80 patients (56.3%) expressed Smurf2, whereas those of 35 patients (43.7%) did not. There were significant correlations between Smurf2 expression and depth of invasion (P = 0.0353) and lymph node metastasis (P = 0.0522). However, there was no significant association with patient age, sex, tumor location, tumor differentiation, distant metastasis, or pathological stage. The survival rates of patients with Smurf2-positive tumors were significantly lower than those of patients with Smurf2-negative tumors (P = 0.0123; Fig. 1C). The 5-year survival rates of patients with and without Smurf2 immunostaining were 29% and 69%, respectively. However, according to multivariate analysis using a Cox proportional hazards model, Smurf2-positive immunostaining was not identified as an independent prognostic factor (data not shown).

Regression analysis showed a significant inverse correlation between Smurf2 and P-Smad2 expression for tumor cells that were positive or negative immunostaining (P = 0.0280; Table 2).

Expression of Smurf2, P-Smad2, Smad7, and TβR-I in SCC-derived Cell Lines. We characterized the expression of Smurf2, P-Smad2, Smad7, and TβR-I at the protein level in five esophageal SCC-derived cell lines. Although all these cell lines of the TE-series...
normal esophageal epithelium and was related to the depth of invasion, lymph node metastasis, and poor prognosis. Western blotting showed a differential expression level of Smurf2 in the esophageal SCC-derived cell line, and its level tended to be related to the pathological stage of the original tumor (17). Furthermore, immunohistochemical staining and Western blotting revealed an inverse correlation between the expression of Smurf2 and Smad2-P in both resected esophageal specimens and the cell lines. These findings suggest that Smurf2 might promote tumor development in esophageal SCC, regulating P-Smad2 expression through its degradation and resisting TGF-β-induced growth-inhibitory effects.

It has been reported that the degradation of P-Smad2 may involve Ubch5 ubiquitin-conjugating enzymes, whereas the ubiquitin ligase(s) involved in this process is/are not known (20). Because Ubch5-related enzymes can function in concert with ubiquitin ligases, it is likely that some member of the ubiquitin-conjugating enzymes family may be involved in the Smurf2-mediated degradation of P-Smad2.

P-Smad2 was seen in the nuclei of both normal esophageal epithelium and SCC (3) because Smad2 translocates to the nucleus after it has been phosphorylated and activated by TGF-β. We detected Smurf2 in both the cytoplasm and nuclei of normal esophageal epithelium and SCCs. In a previous study on subcellular localization, Smurf2 was detected predominantly in the cytoplasm (14), whereas in another study, it was detected predominantly in the nuclei (15). Moreover, the targets of Smurf2 are referred to activated or not activated Smad2, Smad7, and TβRI (13–15). However, in our study, the TβRI expression levels of the cell lines were similar, and the Smad7 expression levels were similar except in the one cell line. Both TβRI and Smad7 expression showed no inverse correlations with Smurf2 expression. We found that the total Smad2 expression levels were also similar, as described previously (16), and showed no such correlations with Smurf2 expression. Therefore, in esophageal SCC, Smurf2 may mainly target P-Smad2 and regulate its steady-state level.

In conclusion, high-level expression of Smurf2 may affect the development of esophageal SCC, especially in relation to depth of invasion and lymph node metastasis, and may be associated with poor prognosis. The chief target substrate of Smurf2 was thought to be P-Smad2. However, we will investigate the mechanism responsible for high-level Smurf2 expression in aggressive esophageal SCC.

**Discussion**

Smads are intracellular mediators of TGF-β superfamily signaling, and Smad2 may play a crucial role in the antiproliferative effects of TGF-β (8). Previous studies on Smad2 expression in human cancers demonstrated that lack of P-Smad2 was related to histological differentiation of head and neck carcinomas (18) and to tumor progression in patients with esophageal SCC (4). However, the mechanisms responsible for altered expression of P-Smad2 in carcinomas remain unclear. Recently, Smurf2 was identified as the Smad ubiquitin ligase that induces the ubiquitination and degradation of Smad2 (13, 14). Therefore, we used immunohistochemical and Western blotting techniques to examine the relationships between Smurf2 expression and the clinicopathological characteristics of patients with esophageal SCC and the correlation between Smurf2 and activated Smad2 expression.

Immunohistochemical staining showed that the Smurf2 expression level was higher in tumor tissue [especially at the tumor front, where the proliferative activity of esophageal SCC is high (19)] than in normal esophageal epithelium and was related to the depth of invasion, lymph node metastasis, and poor prognosis. Western blotting showed a differential expression level of Smurf2 in the esophageal SCC-derived cell line, and its level tended to be related to the pathological stage of the original tumor (17). Furthermore, immunohistochemical staining and Western blotting revealed an inverse correlation between the expression of Smurf2 and Smad2-P in both resected esophageal specimens and the cell lines. These findings suggest that Smurf2 might promote tumor development in esophageal SCC, regulating P-Smad2 expression through its degradation and resisting TGF-β-induced growth-inhibitory effects.

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**Table 1 The correlation between clinicopathological characteristics and Smurf2 expression**

<table>
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<tr>
<th>Parameters</th>
<th>Smurf2 (+) (n = 45)</th>
<th>Smurf2 (-) (n = 35)</th>
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<td>Age (mean ± SD; yrs)</td>
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<td>62.3 ± 1.5</td>
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<td></td>
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<td></td>
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<td></td>
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<td></td>
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* Numbers in parentheses are percentages.

**Table 2 The correlation between expression of Smurf2 and P-Smad2**

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Acknowledgments

We thank Dr. T. Nishihira for the TE-series cell lines.

References

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