

# Overexpression of Smad proteins, especially Smad7, in oral epithelial dysplasias

Yuk-Kwan Chen · Anderson Hsien-Cheng Huang ·  
Pei-Hsun Cheng · Shang-Hsun Yang · Li-Min Lin

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## Abstract

**Objective** Transforming growth factor  $\beta$ , via membrane-bound receptors and downstream Smad2–4, 7, can modulate tumorigenesis. Smad2 and Smad3 heterodimerize with Smad4, and the complex migrates to the nucleus to regulate the expression of target genes. Smad7 is a key negative regulator of this signaling pathway. This study aimed to examine Smad2–4, 7 expression and phosphorylated Smad2–3 (p-Smad2–3) in oral epithelial dysplasia and compared it with normal oral mucosa, hyperkeratosis/epithelial hyperplasia and squamous cell carcinoma (SCC). **Materials and methods** Immunohistochemical staining of Smad2–4, 7 and p-Smad2–3, was performed for 75 samples of human oral mucosa, including hyperkeratosis/epithelial hyperplasia ( $n=20$ ), mild epithelial dysplasia ( $n=11$ ), moderate to severe epithelial dysplasia ( $n=11$ ), and SCC ( $n=43$ ). Normal buccal mucosa samples ( $n=9$ ) were also included.

**Results** A significant increase in Smad7 expression was observed in the ascending order of samples of normal oral mucosa, hyperkeratosis/epithelial hyperplasia/mild oral epithelial dysplasia, moderate to severe oral epithelial dysplasia, and well-differentiated oral SCC/moderately to poorly differentiated oral SCC. Additionally, significant increases in Smad7 expression were noted as compared with expression of Smad2–4 and p-Smad2–3 in lesions of hyperkeratosis/epithelial hyperplasia, mild oral epithelial dysplasia, moderate to severe oral epithelial dysplasia, well-differentiated oral SCC, and moderately to poorly differentiated oral SCC.

**Conclusions** Our results indicate that Smad proteins, particularly Smad7, in oral epithelial dysplasia and SCC could contribute to the attenuation of Smads anti-proliferative signaling in cancer development.

**Clinical relevance** Smad7 could be a marker for risk of malignant transformation of oral epithelial dysplasia.

Y.-K. Chen · L.-M. Lin  
Division of Oral Pathology and Diagnosis,  
Kaohsiung Medical University Chung-Ho Memorial Hospital,  
Kaohsiung, Taiwan

A. H.-C. Huang  
Grace Dental Clinic,  
Kaohsiung, Taiwan

P.-H. Cheng · S.-H. Yang (✉)  
Department of Physiology and Institute of Basic Medical Sciences,  
College of Medicine, National Cheng Kung University,  
Tainan, Taiwan  
e-mail: syang@mail.ncku.edu.tw

L.-M. Lin (✉)  
Department of Dentistry, College of Dental Medicine,  
Kaohsiung Medical University,  
100 Shih-Chuan 1st Road,  
Kaohsiung, Taiwan  
e-mail: limin@cc.kmu.edu.tw

**Keywords** Smad7 · TGF- $\beta$  · Oral epithelial · Dysplasia · Oral squamous cell carcinoma

## Introduction

More than 90 % of all head and neck (HN) cancers are squamous cell carcinoma (SCC) [1]. HN cancers are consistently among the six most major global cancers and are one of the most important categories of cancer in most Asian countries, including Taiwan [1]. In Taiwan, oral SCC is the 4th most common type of diagnosed cancer in males and the 5th leading cause of cancer death in males [2]. Despite advances in the management of patients with this disease, the survival rate has not been considerably enhanced [3, 4]. A limited understanding of the mechanisms of tumor growth

as well as local and regional metastasis of HN cancers contributes to the poor prognosis for patients with this disease. Several mechanisms of carcinogenesis such as the transforming growth factor (TGF)- $\beta$  pathway have been elucidated as being involved in the development of oral SCC [5]; nonetheless, a complete understanding of the molecular pathogenesis of oral SCC development remains deficient.

The TGF- $\beta$  family modulates a wide variety of biological functions, including cell proliferation, differentiation, and apoptosis in many types of cells, including epithelial cells. These multifunctional functions of TGF- $\beta$  are elicited through oligomeric complex formation between two types (type I, T $\beta$ RI and type II, T $\beta$ RII) of membrane-bound serine–threonine kinase receptor. TGF- $\beta$  conveys signals by binding to T $\beta$ RII and stabilizes the heteromeric complex with T $\beta$ RI, and consequently, T $\beta$ RI is phosphorylated/activated by T $\beta$ RII. The phosphorylated/activated T $\beta$ RI then propagates the signals through interaction with three groups of Smads (receptor-regulated Smads (R-Smads), Smad2–3; common Smad (Co-Smad), Smad4; and inhibitor Smad (I-Smad), Smad7). Upon phosphorylation by T $\beta$ RI, R-Smads (Smad2–3) form heteromeric complexes with Co-Smad (Smad4) and move to the nucleus, where they modulate transcription of TGF- $\beta$  target genes [6]. I-Smad (Smad7) inhibits this signaling pathway by interfering with the activation of R-Smads. Smad7 forms a stable association with activated T $\beta$ RI, thereby preventing R-Smads from binding to and being activated by these receptors [7].

Reviewing the English-language literature regarding the TGF- $\beta$ -Smad signaling pathway in human oral SCCs, aberrant expressions of TGF- $\beta$ , T $\beta$ RI, and T $\beta$ RII [8], as well as Smad4, have been reported [9, 10]. However, to our knowledge, Smad proteins expression in human oral epithelial dysplasia has not yet been elucidated. Hence, the current study aimed to investigate the immunohistochemical (IHC) expression of Smad2–4, 7 as well as phosphorylated-Smad2–3 (p-Smad2–3) proteins, in human oral epithelial dysplasia, and also compared it with normal oral mucosa, hyperkeratosis/epithelial hyperplasia without epithelial dysplasia, and SCC in order to investigate the comprehensive role of Smad proteins in oral cancer formation.

## Materials and methods

### Tissue sample collection

Oral mucosa samples from a total of 75 patients (Table 1) were retrieved with the approval of the institutional review board of the Ethics Committee for Scientific Research on Human Beings at our institution. All the patients from whom the samples originated had the oral habits of betel-quid chewing, alcohol drinking, and cigarette smoking. Of

these 75 patients, 42 were cases of hyperkeratosis/epithelial hyperplasia ( $n=20$ ), mild epithelial dysplasia ( $n=11$ ), and moderate to severe epithelial dysplasia ( $n=11$ ). The histopathological characteristics of oral epithelial dysplasia include (1) basal layer hyperplasia, (2) nuclear enlargement and hyperchromatism, (3) loss of intercellular adhesion and normal polarization, (4) abnormal mitoses above the basal cell layer, (5) individual cell keratinization within the spinous layer, (6) cellular pleomorphism, (7) drop-shaped epithelial ridges, (8) irregular stratification, and (9) altered nuclear–cytoplasmic ratio [11]. Regarding these histological changes, the presence of basal cell hyperplasia, nuclear enlargement and hyperchromatism, and drop-shaped rete-ridges are regarded as the minimal criteria for the histological diagnosis of epithelial dysplasia [12]. The degree of dysplasia was graded in accordance with the following criteria [13]: (1) mild epithelial dysplasia, dysplastic alterations limited to the lower third of the buccal epithelium; (2) moderate epithelial dysplasia, dysplastic changes noted for up to two thirds of the thickness of the oral epithelium; and (3) severe epithelial dysplasia, dysplastic cells observed within more than two thirds but less than the whole thickness of the oral epithelium. Hyperplastic/hyperkeratotic oral epithelial lesions were included for clinical reasons, as leukoplakia, the well-known oral premalignant lesion, is most frequently associated with a histological diagnosis of epithelial hyperplasia/hyperkeratosis [14].

All cases of oral SCCs in this experiment were classified according to the primary site as described in the International Classification of Diseases (ICD 140–145) for Oncology (World Health Organization) [15]. The histological diagnoses of all samples were confirmed from hematoxylin and eosin-stained sections by two board-certified oral pathologists (Chen and Lin). Histological differentiation of oral SCC was also performed (well-differentiated,  $n=21$ ; moderately to poorly differentiated,  $n=12$ ). Samples of normal buccal mucosa ( $n=9$ ) from patients without the above-mentioned oral habits were also assessed in the current study.

### Semi-quantitative immunohistochemistry for Smad proteins

For the detection of Smad proteins, a standard avidin–biotin–peroxidase complex method [16] was used in the current study. A 4- $\mu$ m-thick section of each paraffin-embedded sample was mounted on a gelatin–chrome alum-coated slide. After deparaffinization in xylene (twice) and rehydration in a decreasing-concentration ethanol series (absolute, 95, 70, and 30 % ethanol and subsequently water), tissue sections were microwaved three times (5 min each time) in a citrate buffer (10 mM; pH 6.0) to retrieve antigenicity. Endogenous peroxidase activity was blocked with 3 % H<sub>2</sub>O<sub>2</sub> in methanol for 60 min. Prior to IHC staining, tissue

**Table 1** Clinical data of the patients in the present study

	Epithelial hyperplasia/ hyperkeratosis	Mild epithelial dysplasia	Moderate to severe epithelial dysplasia	Oral squamous cell carcinoma	
				Differentiation	
				Well	Moderately to poorly
Sex					
Male	18	10	9	20	12
Female	2	1	2	1	
Location					
Lip	4	7	3	3	1
Buccal mucosa	10	2	4	9	9
Tongue	6	2	4	9	2

sections were incubated for 60 min in a 10 % solution of normal goat serum to reduce non-specific staining for Smad2–4 as well as p-Smad2–3 proteins, while a blocking solution of 2 % dry milk in phosphate-buffered saline (PBS) was applied to those sections stained for Smad7 protein. The sections were then treated with the primary antibodies against Smad2 (1:100; cat. no.: 3122; Cell Signaling Technology®, Danvers, MA; rabbit/monoclonal), Smad3 (1:100; cat. no.: #9523; Cell Signaling Technology®; rabbit/monoclonal), p-Smad2 (1:100; cat. no.: sc-135644; Santa Cruz Biotechnology Inc, Santa Cruz, CA; rabbit/polyclonal), p-Smad3 (1:100; cat. no.: sc-130218; Santa Cruz Biotechnology Inc.; rabbit/polyclonal), Smad4 (1:100; cat. no.: #9515; Cell Signaling Technology®; rabbit/monoclonal), and Smad7 proteins (1:100; cat. no.: #H4092-M09; Abnova Corporation, Walnut, CA; mouse/monoclonal) overnight at 4°C. After subsequent rinsing with PBS (three times, 10 min each), tissue sections intended for Smad2–4 as well as p-Smad2–3 staining were incubated for 30 min at room temperature in the presence of biotin-conjugated goat anti-rabbit IgG (Vector, Burlingame, CA; 1:100). In contrast, the sections for Smad7 staining were treated with biotinylated anti-mouse IgG antibody (Vector; 1:100) for 30 min. After these procedures, all sections were again washed with PBS (three times, 10 min each) and then incubated with an avidin–biotin complex conjugated to horseradish peroxidase (Dako, Santa Barbara, CA) for a further 30 min. After washing with PBS (three times, 10 min each), peroxidase binding was visualized as brown reaction products as a result of the benzidine reaction. The sections were then counterstained with Mayer's hematoxylin. Each set of experiments included hamster buccal pouch carcinoma specimens known to express Smad proteins [17], which were employed as positive controls 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. The percentage of positive immunostaining (P) was scored as follows [17]: 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  $P \times I$  for each section. The two aforementioned oral pathologists independently evaluated the ISs of the IHC staining of each section. When a discrepancy existed between the two examiners, agreement was attained by mutual discussion. Inter-examiner agreement was tested using  $\kappa$  statistics [18]: a  $\kappa$  value of <0.40 was regarded as poor agreement; a value between 0.40 and 0.59, fair agreement; a value between 0.60 and 0.74, good agreement; and a value between 0.75 and 1.00, excellent agreement.

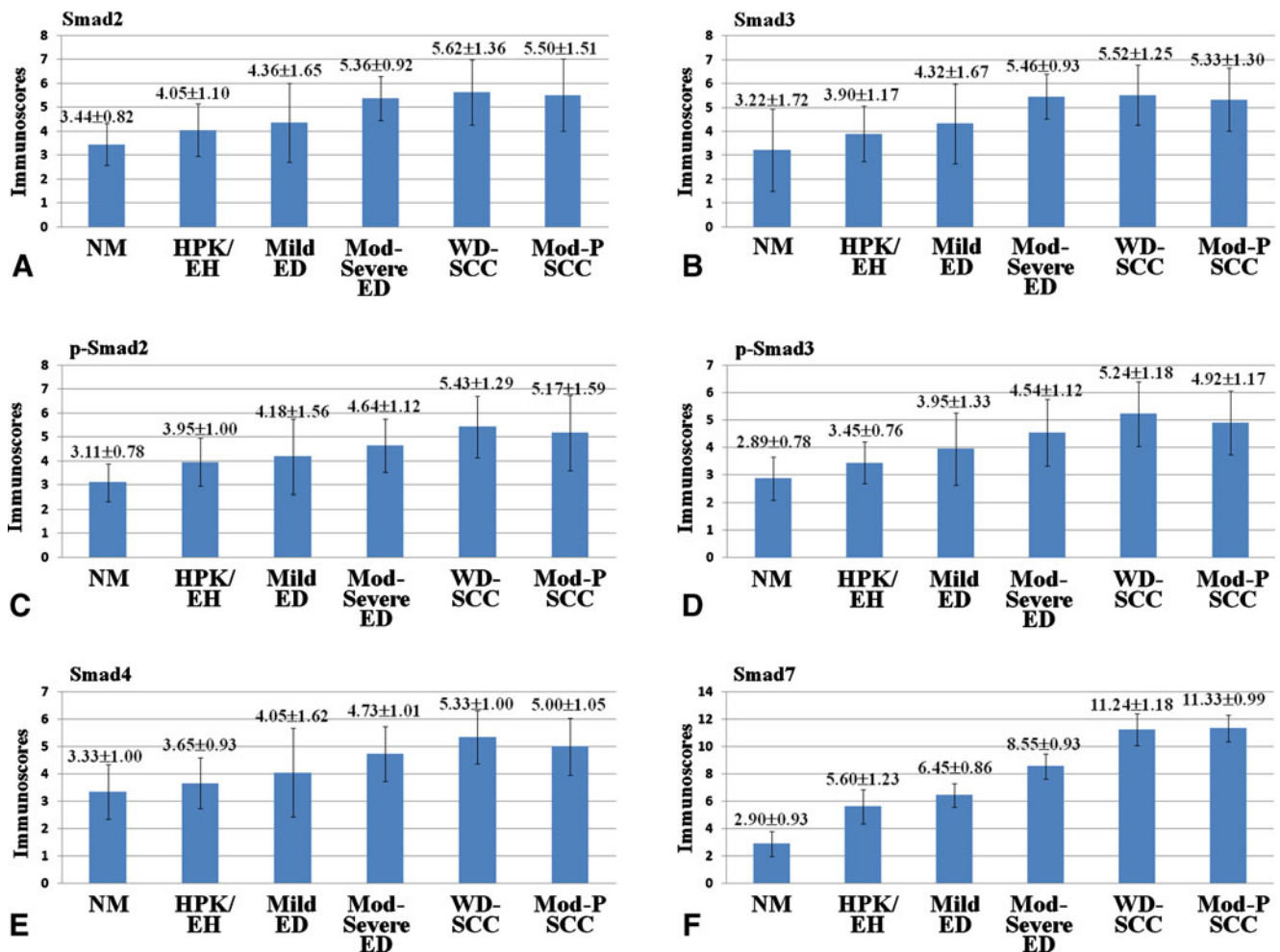
#### Statistical analyses

Statistical comparisons were performed using JUMP 8.0 software (SAS, Cary, NC). The means of the ISs in the different groups were analyzed using one-way ANOVA and Tukey–Kramer tests. The data were regarded as significant when the  $p$  value of <0.05.

#### Results

The ISs (mean±standard deviation) (Fig. 1a–f) and the IHC staining of Smad2–4, 7 and p-Smad2–3 for each type of lesion (Figs. 2, 3, 4, 5, 6, and 7)

The ISs of Smad2–3 (Fig. 1a–b), p-Smad2–3 (Fig. 1c–d), Smad4 (Fig. 1e), and Smad7 (Fig. 1f) were respectively elevated gradually from normal mucosa to hyperkeratosis/epithelial hyperplasia, mild oral epithelial dysplasia, moderate to severe oral epithelial dysplasia, and oral SCC. Inter-



**Fig. 1** Immunoscores (mean ± standard deviation) of Smad proteins (a Smad2, b Smad3, c phosphorated-Smad2 (p-Smad2), d p-Smad3, e Smad4, and f Smad7) for normal oral mucosa (NM), lesions of hyperkeratosis/epithelial hyperplasia (HPK/EH), mild oral epithelial

dysplasia (Mild ED), moderate to severe epithelial dysplasia (Mod-severe ED), well-differentiated squamous-cell carcinoma (WD-SCC) and moderately- to poorly-differentiated squamous-cell carcinoma (Mod-P SCC) in human oral mucosa

examiner agreement between the two examiners was excellent for the assessment of immunoscores, with a  $\kappa$  value of 0.88.

Cytoplasmic and/or nuclear staining for Smad2–4, 7 as well as p-Smad2–3, was observed. Representative IHC stainings for each type of lesion were shown in Figs. 2, 3, 4, 5, 6, and 7. No definitive staining was apparent for any of the negative control sections while definite positive immunostaining was evident for all of the positive control sections.

Statistical analyses of ISs of Smad2–4, 7 and p-Smad2–3 in individual lesions are shown in Table 2.

As observed from Table 2, statistical significances were noted for comparison of the following pairs:

*For Smad2* Moderate to severe epithelial dysplasia vs. normal mucosa; well-differentiated oral SCC vs. normal mucosa; moderately to poorly differentiated oral SCC vs. normal mucosa; epithelial hyperplasia/hyperkeratosis vs. well-differentiated oral SCC; epithelial hyperplasia/hyperkeratosis vs. moderately to

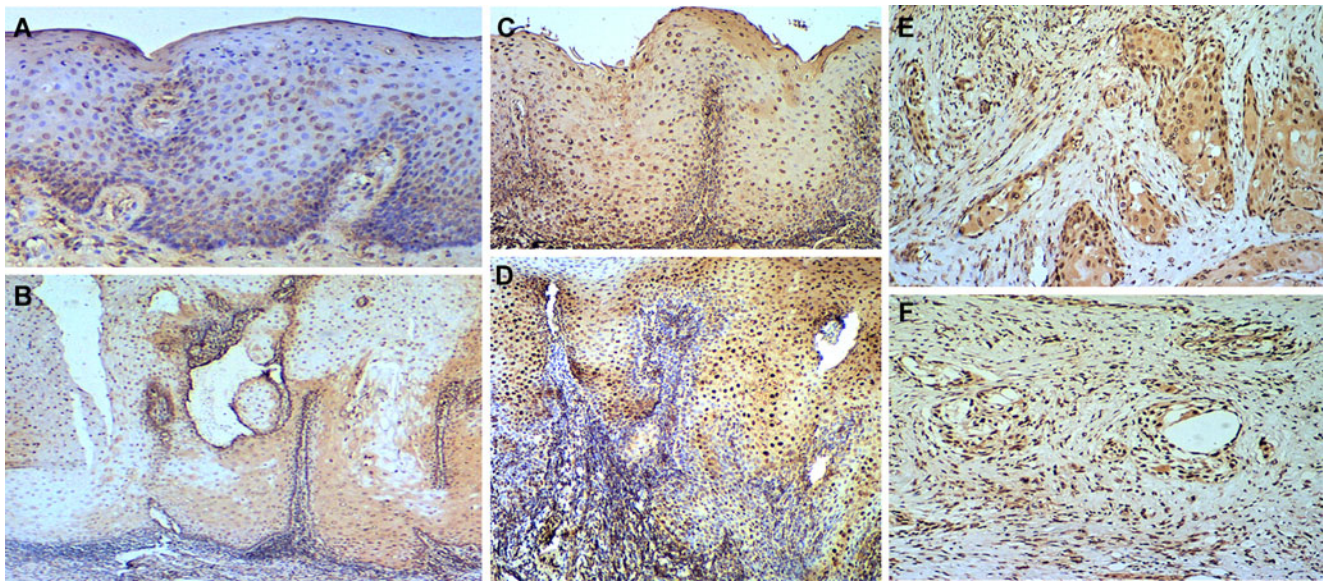
poorly differentiated oral SCC; mild epithelial dysplasia vs. well-differentiated oral SCC.

*For Smad3* Moderate to severe epithelial dysplasia vs. normal mucosa; well-differentiated oral SCC vs. normal mucosa; moderately to poorly differentiated oral SCC vs. normal mucosa; epithelial hyperplasia/hyperkeratosis vs. well-differentiated oral SCC; moderate to severe epithelial dysplasia vs. epithelial hyperplasia/hyperkeratosis.

*For p-Smad2* Well-differentiated oral SCC vs. normal mucosa; moderately to poorly differentiated oral SCC vs. normal mucosa; well-differentiated oral SCC vs. epithelial hyperplasia/hyperkeratosis; well-differentiated oral SCC vs. mild epithelial dysplasia.

*For p-Smad3* Moderate to severe epithelial dysplasia vs. normal mucosa, well-differentiated oral SCC vs. normal





**Fig. 2** Representative immunohistochemical expression of cytoplasmic and/or nuclear staining of Smad2 for normal mucosa (a), lesions of hyperkeratosis/epithelial hyperplasia (b), mild epithelial dysplasia (c), moderate to severe epithelial dysplasia (d), well-differentiated

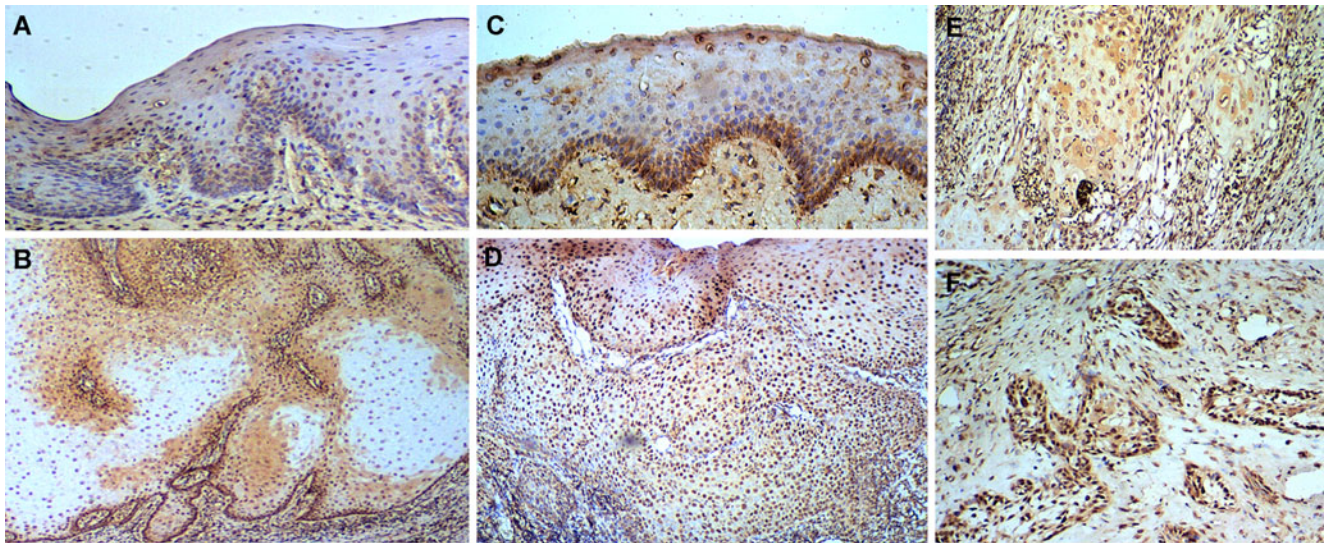
squamous cell carcinoma (e), and moderately to poorly differentiated squamous cell carcinoma (f) in human oral mucosa (avidin–biotin–peroxidase complex staining,  $\times 100$ )

mucosa, moderately to poorly differentiated oral SCC vs. normal mucosa, well-differentiated oral SCC vs. epithelial hyperplasia/hyperkeratosis, moderately to poorly differentiated oral SCC vs. epithelial hyperplasia/hyperkeratosis, and well-differentiated oral SCC vs. mild epithelial dysplasia.

*For Smad 4* Well-differentiated oral SCC vs. normal mucosa, moderately to poorly differentiated oral SCC vs. normal mucosa, epithelial hyperplasia/hyperkeratosis vs. well-differentiated oral SCC, epithelial hyperplasia/hyperkeratosis

vs. moderately to poorly differentiated oral SCC, and mild epithelial dysplasia vs. well-differentiated oral SCC.

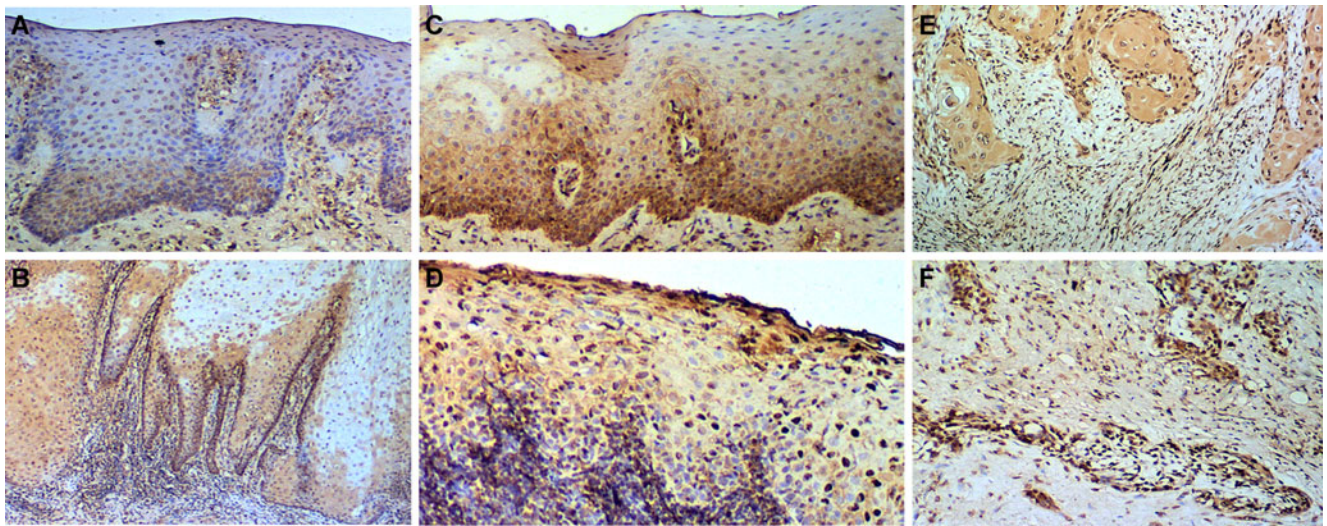
*For Smad7* Epithelial hyperplasia/hyperkeratosis vs. normal mucosa, mild epithelial dysplasia vs. normal mucosa, moderate to severe epithelial dysplasia vs. normal mucosa, well-differentiated oral SCC vs. normal mucosa, moderately to poorly differentiated oral SCC vs. normal mucosa; epithelial hyperplasia/hyperkeratosis vs. moderate to severe epithelial dysplasia, epithelial hyperplasia/hyperkeratosis



**Fig. 3** Representative immunohistochemical expression of cytoplasmic and/or nuclear staining of Smad3 for normal mucosa (a), lesions of hyperkeratosis/epithelial hyperplasia (b), mild epithelial dysplasia (c), moderate to severe epithelial dysplasia (d), well-differentiated

squamous cell carcinoma (e), and moderately to poorly differentiated squamous cell carcinoma (f) in human oral mucosa (avidin–biotin–peroxidase complex staining,  $\times 100$ )





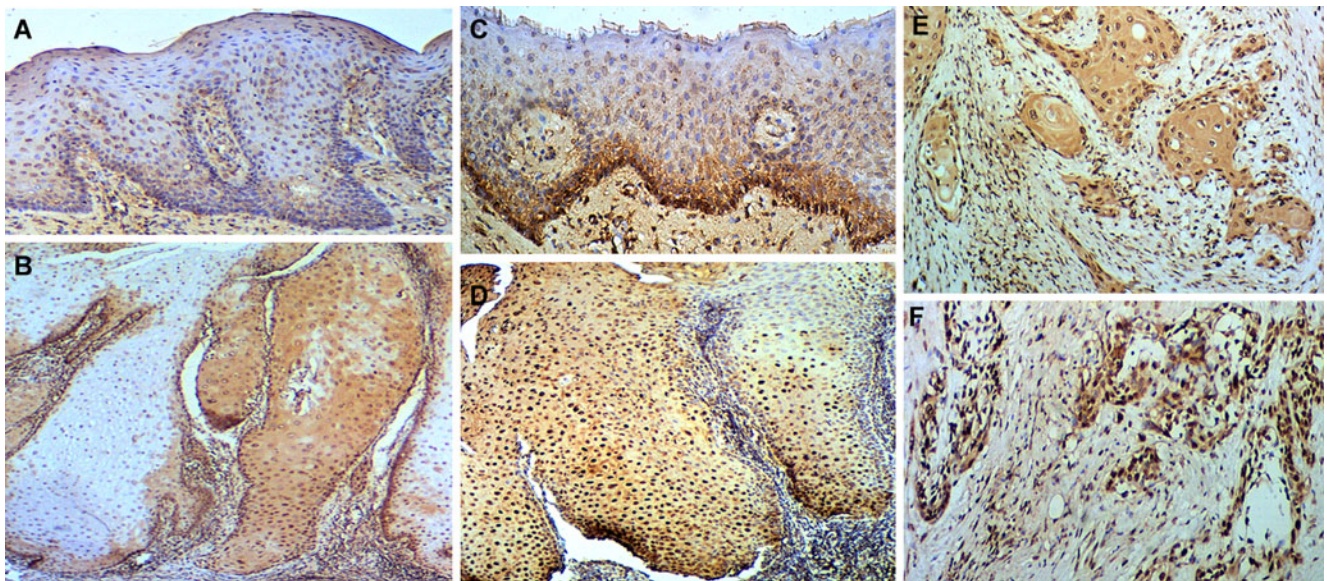
**Fig. 4** Representative immunohistochemical expression of cytoplasmic and/or nuclear staining of phosphorylated-Smad2 for normal mucosa (**a**), lesions of hyperkeratosis/epithelial hyperplasia (**b**), mild epithelial dysplasia (**c**), moderate to severe epithelial dysplasia (**d**),

well-differentiated squamous cell carcinoma (**e**), and moderately to poorly differentiated squamous cell carcinoma (**f**) in human oral mucosa (avidin–biotin–peroxidase complex staining,  $\times 100$ )

vs. well-differentiated oral SCC; epithelial hyperplasia/hyperkeratosis vs. moderately to poorly differentiated oral SCC, mild epithelial dysplasia vs. moderate to severe epithelial dysplasia, mild epithelial dysplasia vs. well-differentiated oral SCC, mild epithelial dysplasia vs. moderately to poorly differentiated oral SCC, moderate to severe epithelial dysplasia vs. well-differentiated oral SCC, and moderate to severe epithelial dysplasia vs. moderately to poorly differentiated oral SCC.

Moreover, Smad7 expression was observed in the ascending order of samples of normal oral mucosa, hyperkeratosis/epithelial hyperplasia/mild oral epithelial dysplasia, moderate to severe oral epithelial dysplasia, and well-differentiated oral SCC/moderately to poorly differentiated oral SCC.

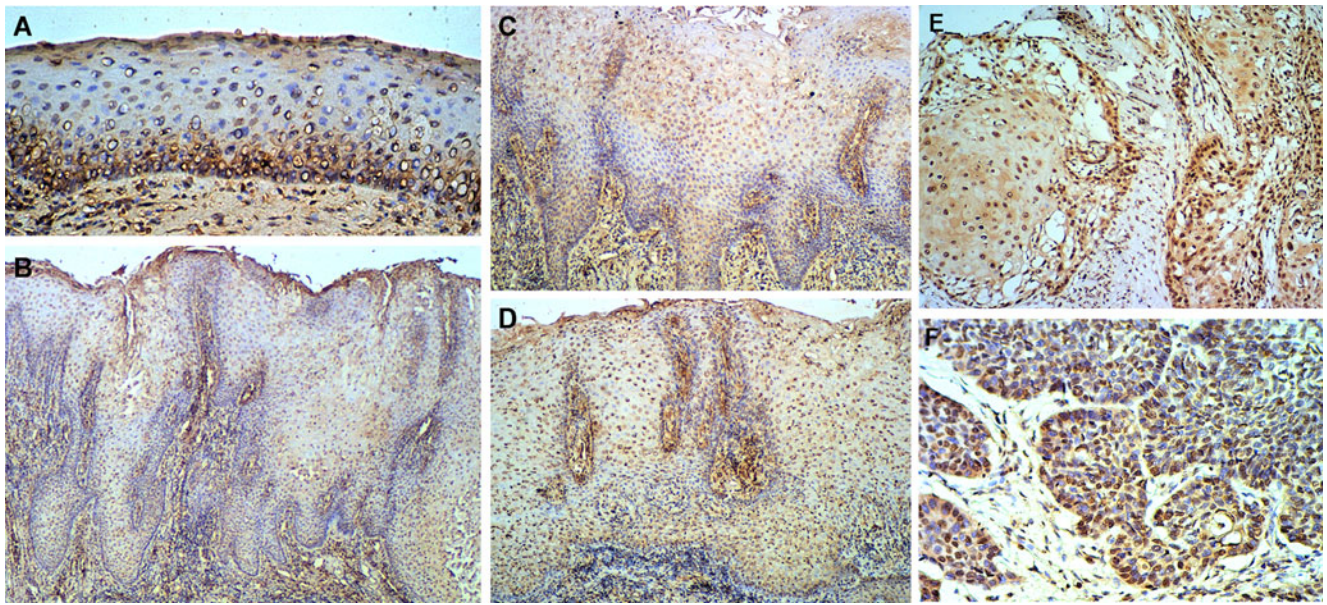
Statistical analyses of the ISs of Smad2–4, 7 and p-Smad2–3 in comparison with each of other Smad for each type of lesion are shown in Table 3.



**Fig. 5** Representative immunohistochemical expression of cytoplasmic and/or nuclear staining of phosphorylated Smad3 for normal mucosa (**a**), lesions of hyperkeratosis/epithelial hyperplasia (**b**), mild epithelial dysplasia (**c**), moderate to severe epithelial dysplasia (**d**),

well-differentiated squamous cell carcinoma (**e**), and moderately to poorly differentiated squamous cell carcinoma (**f**) in human oral mucosa (avidin–biotin–peroxidase complex staining,  $\times 100$ )





**Fig. 6** Representative immunohistochemical expression of nuclear staining of Smad4 for normal mucosa (**a**), lesions of hyperkeratosis/epithelial hyperplasia (**b**), mild epithelial dysplasia (**c**), moderate to severe epithelial dysplasia (**d**), well-differentiated squamous cell

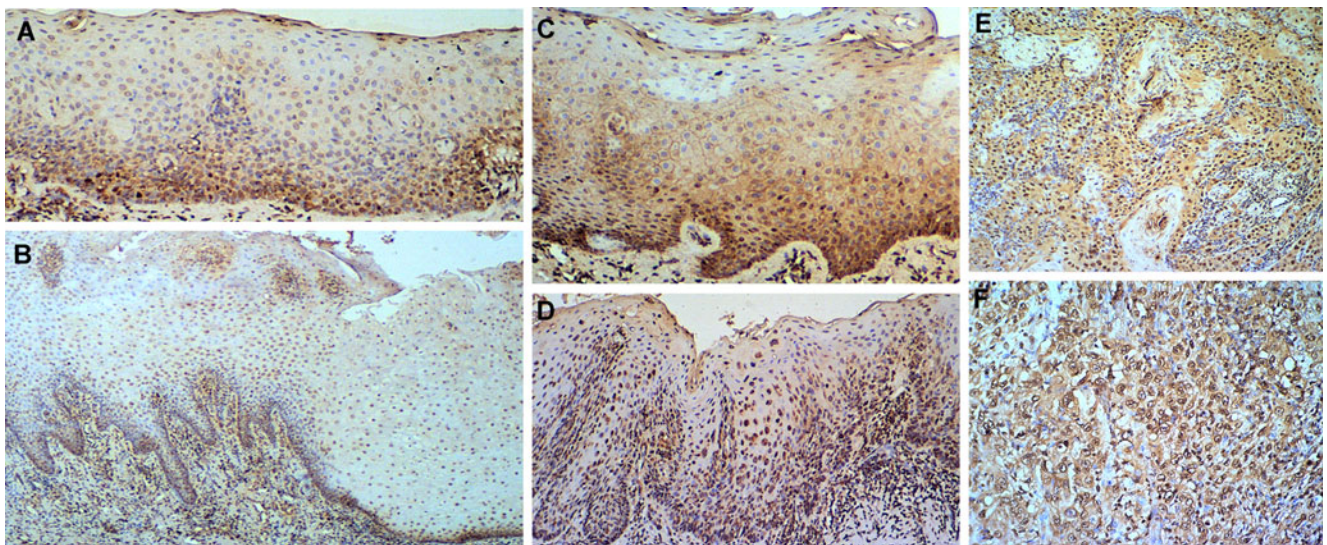
carcinoma (**e**), and moderately to poorly differentiated squamous cell carcinoma (**f**) in human oral mucosa (avidin–biotin–peroxidase complex staining,  $\times 100$ )

As noted from Table 3, statistical significances were confirmed for comparison of the following pairs:

For lesions of epithelial hyperplasia/hyperkeratosis, mild epithelial dysplasia, moderate to severe epithelial dysplasia, well-differentiated oral SCC and moderately to poorly differentiated oral SCC: Smad7 vs. Smad2, Smad7 vs. Smad3, Smad7 vs. p-Smad2, Smad7 vs. p-Smad3, and Smad7 vs. Smad4.

## Discussion

Smad proteins play a pivotal role in the intracellular signaling of the TGF- $\beta$  superfamily of extracellular polypeptides, which initiate signaling to regulate a wide variety of biological processes such as embryogenesis, organogenesis, and tumor formation [7]. However, little is known about the expression of Smad proteins in lesions of human oral



**Fig. 7** Representative immunohistochemical expression of nuclear staining of Smad7 for normal mucosa (**a**), lesions of hyperkeratosis/epithelial hyperplasia (**b**), mild epithelial dysplasia (**c**), moderate to severe epithelial dysplasia (**d**), well-differentiated squamous cell

carcinoma (**e**), and moderately to poorly differentiated squamous cell carcinoma (**f**) in human oral mucosa (avidin–biotin–peroxidase complex staining,  $\times 100$ )

**Table 2** Comparison of immunoscores (mean±standard deviation) of the Smad proteins in individual lesions

Smad2		Smad3		p-Smad2		p-Smad3		Smad4		Smad7	
Lesion <sup>a</sup>	<i>p</i> value	Lesion <sup>a</sup>	<i>p</i> value	Lesion <sup>a</sup>	<i>p</i> value	Lesion <sup>a</sup>	<i>p</i> value	Lesion <sup>a</sup>	<i>p</i> value	Lesion <sup>a</sup>	<i>p</i> value
5 vs. 1	0.0012*	5 vs. 1	0.0008*	5 vs. 1	0.0003*	5 vs. 1	<0.0001*	5 vs. 1	0.0006*	6 vs. 1	<0.0001*
6 vs. 1	0.0089*	4 vs. 1	0.0061*	6 vs. 1	0.0065*	6 vs. 1	0.0011*	5 vs. 2	0.0002*	5 vs. 1	<0.0001*
4 vs. 1	0.0216*	6 vs. 1	0.0093*	5 vs. 2	0.0055*	5 vs. 2	<0.0001*	6 vs. 1	0.0196*	6 vs. 2	<0.0001*
5 vs. 2	0.0037*	5 vs. 2	0.0035*	5 vs. 3	0.0252*	4 vs. 1	0.0164*	6 vs. 2	0.0241*	4 vs. 1	<0.0001*
6 vs. 2	0.0405*	4 vs. 2	0.0370*	4 vs. 1	0.1013	6 vs. 2	0.0065*	5 vs. 3	0.0061*	5 vs. 2	<0.0001*
5 vs. 3	0.0301*	6 vs. 2	0.0562	6 vs. 2	0.1131	5 vs. 3	0.0037*	4 vs. 1	0.0923	6 vs. 3	<0.0001*
4 vs. 2	0.0991	5 vs. 3	0.0535	3 vs. 1	0.2993	4 vs. 2	0.1026	4 vs. 2	0.1450	5 vs. 3	<0.0001*
6 vs. 3	0.1724	4 vs. 3	0.2267	6 vs. 3	0.2843	3 vs. 1	0.1606	6 vs. 3	0.2102	3 vs. 1	<0.0001*
4 vs. 3	0.3282	3 vs. 1	0.3375	2 vs. 1	0.5889	6 vs. 3	0.1644	3 vs. 1	0.6347	4 vs. 2	<0.0001*
3 vs. 1	0.5028	6 vs. 3	0.3143	5 vs. 4	0.5694	5 vs. 4	0.5537	4 vs. 3	0.6087	6 vs. 4	<0.0001*
2 vs. 1	0.8647	2 vs. 1	0.8192	4 vs. 2	0.7175	4 vs. 3	0.7040	5 vs. 4	0.7268	2 vs. 1	<0.0001*
3 vs. 2	0.9727	3 vs. 2	0.9204	6 vs. 4	0.9220	2 vs. 1	0.8077	3 vs. 2	0.8799	5 vs. 4	<0.0001*
5 vs. 4	0.9954	5 vs. 6	0.9989	4 vs. 3	0.9312	3 vs. 2	0.6857	5 vs. 6	0.9682	4 vs. 3	<0.0001*
6 vs. 4	0.9999	4 vs. 6	0.9999	5 vs. 6	0.9933	6 vs. 4	0.9670	2 vs. 1	0.9839	3 vs. 2	0.1004
5 vs. 6	0.9999	5 vs. 4	1.0000	3 vs. 2	0.9921	5 vs. 6	0.9671	6 vs. 4	0.9932	6 vs. 5	0.9999

*p* phosphorylated\**p*<0.05, statistical significance<sup>a</sup> (1) Normal mucosa, (2) epithelial hyperplasia/hyperkeratosis, (3) mild epithelial dysplasia, (4) moderate to severe epithelial dysplasia, (5) well-differentiated oral squamous cell carcinoma, (6) moderately to poorly differentiated oral squamous cell carcinoma



**Table 3** Comparisons of immunoscores (mean±standard deviation) of individual Smads with each other Smad for each type of lesion

Normal mucosa	Epithelial hyperplasia/ hyperkeratosis		Mild epithelial dysplasia		Moderate to severe epithelial dysplasia		Well-differentiated OSCC		Moderately to poorly differentiated OSCC	
	Smad	p value	Smad	p value	Smad	p value	Smad	p value	Smad	p value
Smad2 vs. p-Smad3	0.8763	Smad7 vs. p-Smad3	<0.0001*	Smad7 vs. p-Smad3	<0.0001*	Smad7 vs. p-Smad3	<0.0001*	Smad7 vs. p-Smad3	0.0000*	Smad7 vs. p-Smad3
Smad2 vs. Smad7	0.8763	Smad7 vs. Smad4	<0.0001*	Smad7 vs. Smad4	<0.0001*	Smad7 vs. p-Smad2	<0.0001*	Smad7 vs. Smad4	0.0000*	Smad7 vs. Smad4
Smad4 vs. p-Smad3	0.9482	Smad7 vs. Smad3	<0.0001*	Smad7 vs. p-Smad2	<0.0001*	Smad7 vs. Smad4	<0.0001*	Smad7 vs. p-Smad2	0.0000*	Smad7 vs. p-Smad2
Smad4 vs. Smad7	0.9482	Smad7 vs. p-Smad2	<0.0001*	Smad7 vs. Smad3	<0.0001*	Smad7 vs. Smad2	<0.0001*	Smad7 vs. Smad3	0.0000*	Smad7 vs. Smad3
Smad2 vs. p-Smad2	0.9850	Smad7 vs. Smad2	0.0001*	Smad7 vs. Smad2	<0.0001*	Smad7 vs. Smad3	<0.0001*	Smad7 vs. Smad2	0.0000*	Smad7 vs. Smad2
Smad3 vs. p-Smad3	0.9850	Smad2 vs. p-Smad3	0.4576	Smad2 vs. p-Smad3	0.9409	Smad3 vs. p-Smad3	0.3151	Smad2 vs. p-Smad3	0.9103	Smad2 vs. p-Smad3
Smad3 vs. Smad7	0.9850	p-Smad2 vs. p-Smad3	0.6547	Smad3 vs. p-Smad3	0.9639	Smad3 vs. p-Smad2	0.4328	Smad2 vs. Smad4	0.9728	Smad2 vs. Smad4
Smad2 vs. Smad3	0.9977	Smad3 vs. p-Smad3	0.7480	Smad2 vs. Smad4	0.9798	Smad2 vs. p-Smad3	0.4328	Smad3 vs. p-Smad3	0.9728	Smad3 vs. p-Smad3
Smad4 vs. p-Smad2	0.9977	Smad2 vs. Smad4	0.8298	Smad3 vs. Smad4	0.9899	Smad3 vs. Smad4	0.5640	Smad2 vs. p-Smad2	0.9957	Smad2 vs. p-Smad2
p-Smad2 vs. p-Smad3	0.9977	p-Smad2 vs. Smad4	0.9433	p-smad2 vs. p-Smad3	0.9957	Smad2 vs. p-Smad2	0.5640	p-Smad2 vs. p-Smad3	0.9957	Smad3 vs. Smad4
p-Smad2 vs. Smad7	0.9977	Smad3 vs. Smad4	0.9739	Smad2 vs. p-Smad2	0.9985	Smad2 vs. Smad4	0.6959	Smad2 vs. p-Smad2	0.9957	p-Smad2 vs. p-Smad3
Smad2 vs. Smad4	0.9999	Smad4 vs. p-Smad3	0.9904	Smad3 vs. p-Smad2	0.9996	Smad4 vs. p-Smad3	0.9984	p-Smad2 vs. Smad4	0.9998	Smad2 vs. Smad3
Smad4 vs. Smad3	0.9999	Smad2 vs. Smad3	0.9975	p-smad2 vs. Smad4	0.9996	Smad3 vs. Smad2	0.9999	Smad2 vs. Smad3	0.9998	p-Smad2 vs. Smad4
Smad3 vs. p-Smad2	0.9999	Smad2 vs. p-Smad2	0.9996	Smad4 vs. p-Smad3	0.9999	Smad4 vs. p-Smad2	0.9999	Smad3 vs. p-Smad2	0.9998	Smad3 vs. p-Smad2
Smad7 vs. p-Smad3	1.0000	p-smad2 vs. Smad3	1.0000	Smad2 vs. Smad3	1.0000	p-Smad2 vs. p-Smad3	0.9999	Smad4 vs. p-Smad3	0.9998	Smad4 vs. p-Smad3

OSCC oral squamous cell carcinoma, p phosphorylated

\* $p < 0.05$ , statistical significance

epithelial dysplasia. Thus, the present study, to our knowledge, may be the first to demonstrate aberrant expression of the Smad proteins in human oral epithelial dysplastic lesions, particularly Smad7 protein. Smad7 is an inhibitor of the TGF- $\beta$ -activated signaling pathway. This inhibitory Smad (Smad7) has been shown to function as an intracellular antagonist of TGF- $\beta$  family signaling and is associated with several cancers such as pancreatic, colonic and gastric cancers. Smad7 has been found to be overexpressed in human pancreatic cancer, and upregulation of Smad7 leads to loss of TGF- $\beta$ -induced growth inhibition of pancreatic cancer cells *in vitro* [19]. Smad7 overexpression has been identified in colorectal tumors and Smad7 upregulation has been found to be associated with a poor prognosis [20]. Aberrant Smad7 expression has been found to be significantly related to pulmonary carcinogenesis and progression, particularly in highly metastatic lung cell lines [21]. Furthermore, overexpression of Smad7 has been demonstrated in HNSCC cell line [22]. Upregulation of Smad7 mRNA has also been reported in HNSCCs [23–27]. However, Smad7 protein expression in human oral epithelial dysplasia has not yet been comprehensively reported. Although oral lichen planus (classified as a potentially malignant disorder [28]) was not the objective of this study, there has been a study of Smad7 protein in oral lichen planus in which a subset of patients developed HNSCC, with biopsies showing diagnoses varying from hyperkeratosis to epithelial dysplasia or HNSCC [29]; however, the degree of dysplasia was not explicitly indicated. Hence, the current study may be, to our knowledge, the first to demonstrate significant increases in Smad7 protein in the order of normal mucosa, hyperkeratosis/epithelial hyperplasia/mild epithelial dysplasia, moderate to severe epithelial dysplasia, and SCC, indicating that Smad7 upregulation is an early event in the development of human oral SCC. Additionally, the previously reported animal findings of increased Smad7 expression in both precancerous and cancerous stages in chemically induced hamster buccal pouch [17] and mouse cutaneous carcinogenesis [30] have been verified in the current investigation of human oral epithelial dysplasia and SCC.

Reviewing the English-language literature, aberrant Smad4 expression has been documented in HNSCC specimens including oral SCCs [10, 31, 32]. Moreover, Smad4 mutation [9] and Smad4 defects [22] have been demonstrated in HNSCC cell lines. Bornstein et al. [33] have also indicated that Smad4 loss in gene-knockout mice HNSCC model generated spontaneous cancers with increased genomic instability and hence, suggesting that Smad4 would be an important gatekeeper gene in HNSCC [34]. On the other hand, most recently, it has been reported that HNSCC patients expressed inactivated Smad2–3 had a significantly better overall survival while loss of Smad4 expression did not implicate prognostic significance [35]. Furthermore,

frequent abnormal Smad2–3 as well as p-Smad2–3 expression has been documented in HNSCC specimens including oral SCCs [31, 32]. Dysregulated Smad2–3 expression and Smad2 mutation has been demonstrated in HNSCC cell lines [9]. Significantly, five methylated tumor suppressor genes: septin 9 (SEPT9), sodium-coupled monocarboxylate transporter 1 (SLC5A8), functional smad-suppressing element on chromosome 18 (FUSSEL18), early B cell factor 3 (EBF3), and iroquois homeobox 1 (IRX1) have been confirmed to be associated in the TGF- $\beta$ 1-Smad signaling pathway [36], and in which, Smad2 was able to enhance IRX1 promoter activity significantly [37]. In the current study, we identified that Smad2–4 as well as p-Smad2–3 expression was respectively elevated gradually from normal oral mucosa to hyperkeratosis/epithelial hyperplasia, mild oral epithelial dysplasia, moderate to severe oral epithelial dysplasia, and oral SCC. Thus, taken altogether, it indicates that dysregulated TGF- $\beta$ 1-Smad signaling can occur as a result of various kinds of defects in multiple components of the TGF- $\beta$ 1 signaling pathway in human oral SCC formation.

Perhaps one of the most essential biological effects of TGF- $\beta$  is its capability to restrain proliferation of many cell types, including oral keratinocytes. TGF- $\beta$  inhibits progression of oral keratinocytes from the G1 to the S phase of the cell cycle. Thus, in oral epithelial cells, TGF- $\beta$  is a homeostasis-guarding factor that restricts growth of the normal oral epithelium. Multiple lines of evidence imply that neoplastic transformation of oral cancers can result in loss of a growth inhibitory response to TGF- $\beta$ . Under the accumulated harmful effects of oral carcinogens including betel-quid and/or cigarettes, aberrant expressions of Smad2–4, p-Smad2–3, and particularly Smad7 proteins in human oral epithelial dysplasia, as demonstrated in the current study, have been implied to induce uncontrolled TGF- $\beta$  activity in human normal oral keratinocytes, which is consequently associated with the initiation and progression of oral SCCs. Therefore, disruption of the normal TGF- $\beta$  balance in human oral keratinocytes by overexpression of Smad proteins, as verified in this study, can lead to the development of TGF- $\beta$ -associated oral SCCs, and this could be one of the potential explanations for the association between overexpression of Smad proteins and human oral SCC development. Furthermore, Smad7, being an inhibitory Smad, has been reported to be rapidly induced by TGF- $\beta$  family members in several cell types, indicating a key role of Smad7 in feedback regulation of TGF- $\beta$  signaling [38]. Dysregulation of this feedback regulation of TGF- $\beta$  activity results in cancerous states.

In conclusion, the current study, to our knowledge, was the first to detect a significant overexpression of Smad7 protein in the order of normal mucosa to epithelial dysplasia to SCC, not only enhancing our understanding of Smad7 protein in oral malignancy, but also suggesting the relevance



of this protein as a putative marker for risk of malignant transformation of lesions of oral epithelial dysplasia to SCC, if, in the future, this can be confirmed by further studies in which control samples from individuals with exposure to risk factors (carcinogens) similar to the affected individuals but without disease are preferably to be employed, because this decreases the possibility of identifying changes indicative of only exposure but not transformation.

**Conflict of interest** The authors declare that they have no conflicts of interest.

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