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TGF-β1, Smad-2/-3, Smad-1/-5/-8, and Smad-4 signaling factors are expressed in ameloblastomas, adenomatoid odontogenic tumors, and calcifying cystic odontogenic tumors: an immunohistochemical study

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OBJECTIVES: The TGF-β/Smad signaling pathway regulates diverse cellular functions, including tooth development, and is involved in numerous pathological processes such as tumorigenesis. The aim of this study was to investigate the immunoexpression of the TGF-β/Smad signaling pathway members in ameloblastoma (AM), calcifying cystic odontogenic tumor (CCOT), and adenomatoid odontogenic tumor (AOT).

MATERIALS AND METHODS: This retrospective cross-sectional study included 65 tissue specimens: 34 AMs, 13 CCOTs, and 18 AOTs. Serial sections were immunohistochemically stained with TGF-β1, Smad-4, Smad-1/-5/-8, and Smad-2/-3 antibodies, and a semiquantitative measurement of the positive cells was carried out by two oral pathologists using a 0–3 scale (0: no immunoreactivity, 1: <20% positive cells, 2: 20–50% positive cells, 3: >50% positive cells).

RESULTS: All biomarkers studied were found significantly decreased in AM compared to CCOT and AOT. AOT and CCOT expressed Smad-1/-5/-8 more strongly compared to AM (OR = 11.66, $P < 0.001$ and OR = 5.34, $P = 0.013$, respectively), and Smad-2/-3 immunostaining was found significantly increased in CCOT (OR = 10.42, $P = 0.001$) and AOT (OR = 5.16, $P < 0.004$) compared to AM. Similarly, Smad-4 was expressed more strongly in AOT and CCOT compared to AM ($P = 0.001$), while AOT demonstrated a fivefold higher chance to express TGF-β1 compared to AM ($P = 0.011$).

CONCLUSION: TGF-β/Smad signaling pathway is activated in AM, AOT, and CCOT. The statistically significant reduced TGF-β1/Smad immunoexpression in AM compared to AOT/CCOT could be associated with the more aggressive biological behavior of AM including increased cell proliferation and reduced apoptosis and differentiation. Thus, the biomarkers TGF-β, Smad-4, Smad-1/-5/-8, and Smad-2/-3 could serve as supplementary diagnostic indices between odontogenic tumors of high and low neoplastic dynamics.

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Keywords: adenomatoid odontogenic tumor; ameloblastoma; calcifying cystic odontogenic tumor; Smad; TGF-β

Introduction

The transforming growth factor beta (TGF-β) superfamily comprises a large group of structurally related molecules that are involved in critical cellular functions, such as proliferation, differentiation, motility, extracellular matrix production, angiogenesis, and apoptosis (1–5). The main members of this superfamily are TGF-β, bone morphogenetic proteins (BMP), activin, nodals, and anti-Mullerian hormone (6–8).

Human TGF-β has three isoforms (TGF-β1, -β2, and -β3) encoded by distinct genes (9–12), with TGF-β1 being the most common (6). After binding to type I (TβRI) and type II (TβRII) serine/threonine kinase receptors, TGF-β signals are transferred to cell nucleus through Smad transcriptional factors, causing stimulation or inhibition of target genes (13). Smads, named after the founder members Mad (the Drosophila gene Mothers Against Decapentaplegic) (14) and Sma (the Mad homologs observed in the nematode Caenorhabditis elegans) (15), may, also, be activated by numerous upstream molecules of pleiotropic function and are thought to constitute the core-point for transcriptional regulation of multiple genes with diverse activities (6,16).

Smads are categorized into R-Smads, the receptor-activated Smads that include Smad-1, Smad-2, Smad-3, Smad-5 and Smad-8; Co-Smad or Smad-4 that forms

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transfer complexes with activated phosphorylated R-Smads to bring them into the nucleus and initiate the transcriptional activity; and I-Smads, the inhibitory Smad-6 and Smad-7 that inhibit the signaling activity of the R-Smads (16). In general, Smad-2 and Smad-3 act as substrates for TGF-β, activin and nodal receptors, and Smad-1, Smad-5, and Smad-8 are activated by BMP-2, BMP-4, and BMP-7 (16). R-Smads are activated via receptor-mediated phosphorylation and are subject to a constant nucleocytoplasmic shuttling, as phosphorylation decreases their affinity for cytoplasmic anchors and increases their affinity for nuclear factors (16,17). When R-Smads are dephosphorylated, they translocate into the cytoplasm for another round of receptor-mediated phosphorylation, according to the receptor activation state (16,18).

TGF-β is involved in all stages of tooth development, mostly through paracrine and autocrine regulation of epithelial–mesenchymal interactions that result in terminal differentiation of ameloblasts and odontoblasts, as well as the regulation of proliferation and homeostasis of odontogenic epithelium (19–23). *In vitro* studies show that TGF-β1, TGF-β2, and TGF-β3 isoforms are co-expressed in developing teeth (22–24). TGF-β1 is localized in the stellate reticulum and dental lamina; TGF-β2 in pre-ameloblasts, ameloblasts, and the epithelial cells of ameloblastomas; and TGF-β3 in ameloblasts and dental papilla (22). TGF-β1 is also expressed in the developing alveolar bone, cementum, and periodontontal ligament (25). Smads are essential for the control of tooth formation and growth (26,27). Smad-4 is expressed in all stages of odontogenesis in the dental epithelium and mesenchyme (27), as well as in Hertwig's epithelial root sheath (HERS) and the epithelial rests of Malassez (ERM) (27,28). Smad-2, Smad-3, and Smad-4 normally show low expression in odontoblasts and pulp cells (29), but Smad-2 and -3 expression increases in reparative dentin, probably due to the stimulation of TGF-β1 signaling (30). Smad-1, Smad-5, and Smad-8 are strongly expressed by the dental epithelium and mesenchyme (26,27).

The TGF-β/Smad signaling pathway is involved in oncogenesis with a dual action: It terminates cell cycle and induces apoptosis during initiation and early tumor formation (31,32), but it later promotes tumorigenesis and enhances invasiveness and tumor progression (31–33). The TGF-β-induced oncogenesis is usually caused by mutations of TGF-β, its receptors, or Smads, which disable components of the TGF-β signaling pathway (31,32,34). Alterations in various points of the TGF-β/Smad signaling pathway have been described in many cancer types (33,35–41). As Smad-4 is a key point of the pathway that acts as a tumor suppressor gene (42), its alteration or dysfunction negatively affects the TGF-β-induced tumor suppression and may lead to carcinogenesis (43). In fact, *Smad-4* mutations have been found in various cancers, such as pancreatic, breast, lung, or colorectal (44–47), and decreased Smad-4 expression is associated with a poor prognosis in patients with squamous cell carcinoma of the esophagus and the oral mucosa (48,49). A role for Smad-2 and Smad-3 in carcinogenesis is, also, strongly suggested. For example, a missense mutation in Smad-2 and Smad-3 genes was found in colorectal cancer. This genetic alteration leads to inhibition of the Smad-2/-3 protein nuclear transfer and subsequent decrease of its function during TGF-β-induced transcriptional activation (50,51).

As the TGF-β/Smad signaling pathway is involved in both tooth development and oncogenesis, we investigated the immunohistochemical expression of TGF-β1, phosphorylated Smad-2/-3 (pSmad-2/-3), phosphorylated Smad-1/-5/-8 (pSmad-1/-5/-8), and Smad-4 in ameloblastomas (AM), adenomatoid odontogenic tumors (AOT), and calcifying cystic odontogenic tumors (CCOT).

Materials and methods

Sixty-five cases of epithelial odontogenic tumors were retrospectively studied: 34 AM (7 follicular, 6 plexiform, 5 follicular/plexiform, 8 follicular/acanthomatous, 5 unicystic luminal, and 3 unicystic intraluminal), 18 AOT, and 13 CCOT. Diagnosis was based on standard microscopic criteria (52,53). The main clinical features of all cases are summarized in Table 1.

Immunohistochemistry

Five-micron-thick serial sections of formalin-fixed and paraffin-embedded tissues were immunostained in the Leica BOND-MAX™ fully automated immunohistochemistry system (Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK), by applying the NovoLink™ Polymer Detection System (Leica Biosystems Newcastle Ltd). For epitope retrieval, a high temperature technique with citrate buffer was utilized. The sections were incubated in 3% hydrogen peroxide (Novocastra™ Peroxidase Block; Novocastra™ Leica Biosystems) to neutralize endogenous peroxidase activity; treated with Novocastra™ Protein Block to reduce non-specific binding of primary and polymer; incubated with primary antibodies; and treated with Novocastra™ Post Primary Block, containing 10% (v/v) animal serum in tris-buffered saline, to enhance penetration of the subsequent polymer reagent. Consequently, poly-HRP anti-mouse/rabbit IgG reagent (NovoLink™ Polymer)

Table 1 Main clinical features of 34 cases of AM, 18 cases of AOT, and 13 cases of CCOT

	AM	*AOT*	*CCOT*	*Total*
N	34	18	13	65
Gender				
Male	13	7	8	28
Female	17	10	4	31
N/A	4	1	1	6
Age				
7–14	2	7	2	11
15–29	3	7	4	14
30–44	9	3	2	14
45–82	16	0	3	19
N/A	4	1	2	7
Location				
Mandible	20	7	5	32
Maxilla	8	10	7	25
N/A	6	1	1	8

AM, ameloblastoma; AOT, adenomatoid odontogenic tumor; CCOT, calcifying cystic odontogenic tumor; N/A, not available.

containing 10% (v/v) animal serum in tris-buffered saline was applied to localize the primary antibody, and the reaction product was visualized by incubation with the substrate/chromogen 3,3′–diaminobenzidine (DAB) prepared from Novocastra™ DAB Chromogen and Novolink™ DAB Substrate Buffer (Polymer), as a brown precipitate. Finally, the sections were counterstained with Novocastra™ Hematoxylin (0.02%).

Primary antibodies used were mouse monoclonocal antibody against human TGF-β1 (1:20, TGFB17; Novocastra™ Leica Microsystems); rabbit polyclonocal antibody against phosphorylated Smad-1/-5/-8 (1:100, anti-phosphoSmad-1/Smad5/Smad8, phospho-specific Ser463/465; Millipore™, Billerica, MA, USA); goat polyclonocal antibody against phosphorylated Smad-2/-3 (1:50, sc-11769, p-Smad-2/-3 Ser423/425; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA); and rabbit monoclonocal antibody against Smad-4 (1:100, EP618Y; Millipore™). Positive internal controls for TGF-β1, Smad-4, and Smad-2/-3 primary antibodies were normal oral mucosa sections (54). Smad-1/-5/-8 expression in squamous epithelium, including oral epithelium, has not been mapped. However, in our samples, pSmad-1/-5/-8 expression was nuclear as expected. For negative control, the primary antibodies were substituted with non-immune serum of the same specificity.

Immunohistochemical scoring

Each section was scored blindly by two investigators, and the extent of immunoreactivity in the epithelium was recorded using a 0–3 scale, according to the method of Scheper et al. (55): 0, negative; 1, low (<20% positive cells); 2, moderate (20–50% positive cells); and 3, strong (>50% positive cells). Discordant results were reviewed by both investigators, and a consensus was reached.

Statistical analysis

For statistical analysis, Stata10.1 software package (Stata Corp., TX, USA) was used, with statistical significance at $P < 0.05$. The immunoreactivity scores for TGF-β1, Smad-1/-5/-8, Smad-2/-3, and Smad-4 among the three tumor types were compared with Chi-square test. The potential correlation of each score with the type of odontogenic tumor, age, and gender of the patients, and location of the lesion was studied by univariable and multivariable ordinal logistic regression (proportional odds model), and the correlation between the scores was further evaluated with the Kendall's tau-b coefficient and its corresponding *P*-value.

Results

Results regarding the odontogenic epithelium immunoreactivity are summarized in Table 2. Reaction of stromal cells was not evaluated.

TGF-β1

Normal oral epithelium (Fig. 1a) exhibited a predominantly granular cytoplasmic TGF-β1 immunoexpression. A similar TGF-β1 expression pattern, low (35.3%), moderate (20.6%), or strong (32.4%), was seen in 30/34 AM in the ameloblast-like and stellate-reticulum-like cells (Fig. 1b).

Table 2 Immunoexpression score for TGF-β1, pSmad-2/-3, pSmad-1/-5/-8, and Smad-4

	AM	*AOT*	*CCOT*	*Total*
N (%)	34 (100.0%)	18 (100.0%)	13 (100.0%)	65 (100.0%)
TGF-β1				
0	4 (11.8)	1 (5.6)	2 (15.4)	7 (10.8)
1	12 (35.3)	1 (5.6)	1 (7.7)	14 (21.5)
2	7 (20.6)	3 (16.7)	2 (15.4)	12 (18.5)
3	11 (32.4)	12 (66.7)	7 (53.8)	30 (46.2)
N/A	0 (0.0)	1 (5.6)	1 (7.7)	2 (3.1)
Smad-2/-3				
0	19 (55.9)	2 (11.1)	2 (15.4)	23 (35.4)
1	11 (32.4)	11 (61.1)	4 (30.8)	26 (40.0)
2	3 (8.8)	5 (27.8)	6 (46.2)	14 (21.5)
3	1 (2.9)	0 (0.0)	1 (7.7)	2 (3.1)
Smad-1/-5/-8				
0	3 (8.8)	0 (0.0)	0 (0.0)	3 (4.6)
1	12 (35.3)	1 (5.6)	1 (7.7)	14 (21.5)
2	13 (38.2)	6 (33.3)	7 (53.8)	26 (40.0)
3	4 (11.8)	9 (50.0)	4 (30.8)	17 (26.2)
N/A	2 (5.9)	2 (11.1)	1 (7.7)	5 (7.7)
Smad-4				
0	1 (2.9)	0 (0.0)	0 (0.0)	1 (1.5)
1	7 (20.6)	1 (5.6)	0 (0.0)	8 (12.3)
2	15 (44.1)	1 (5.6)	0 (0.0)	16 (24.6)
3	11 (32.4)	15 (83.3)	12 (92.3)	38 (58.5)
N/A	0 (0.0)	1 (5.6)	1 (7.7)	2 (3.1)

AM, ameloblastoma; AOT, adenomatoid odontogenic tumor; CCOT, calcifying cystic odontogenic tumor. N/A: not available, due to insufficient material.
0: no positive cells; 1: <20% positive cells; 2: 20–50% positive cells; 3: >50% positive cells.

Almost all AOT (17/18) demonstrated varying TGF-β1 expression in the cells of the duct-like and rosette-like structures that were chiefly strong (53.8%) (Fig. 1c). The majority of CCOT (11/13) expressed TGF-β1 in the ameloblast-like basal cells and overlying epithelial cells that was chiefly strong (66.7%) (Fig. 1d). The possibility for strong TGF-β1 expression was increased almost fivefold in AOT (OR = 4.85, *P*-value = 0.011) and twofold in CCOT (OR = 2.44, *P*-value = 0.178), compared to AM.

pSmad-2/-3

pSmad-2/-3 immunostaining in normal oral epithelium (Fig. 2a) was predominantly nuclear. Both the ameloblast-like and stellate-reticulum-like cells were stained in 15/34 AM (Fig. 2b), but reaction was mostly low (32.4%). Most AOT (61.1%) demonstrated low pSmad-2/-3 expression in the duct-like and rosette-like structures (Fig. 2c), while 11/13 CCOT showed expression throughout their lining that was chiefly moderate (46.2%) (Fig. 2d). The possibility for moderate/strong pSmad-2/-3 expression was increased almost fivefold in AOT (OR = 5.16, $P = 0.004$) and 10-fold in CCOT (OR = 10.42, $P = 0.001$), compared to AM.

pSmad-1/-5/-8

pSmad-1/-5/-8 immunostaining was predominantly nuclear and localized in all epithelial components of AM (Fig. 3a), AOT (Fig. 3b), and CCOT (Fig. 3c), when positive. Considering AM, 29/32 were positive and chiefly low or moderately reactive (35.3% and 38.2%, respectively), while

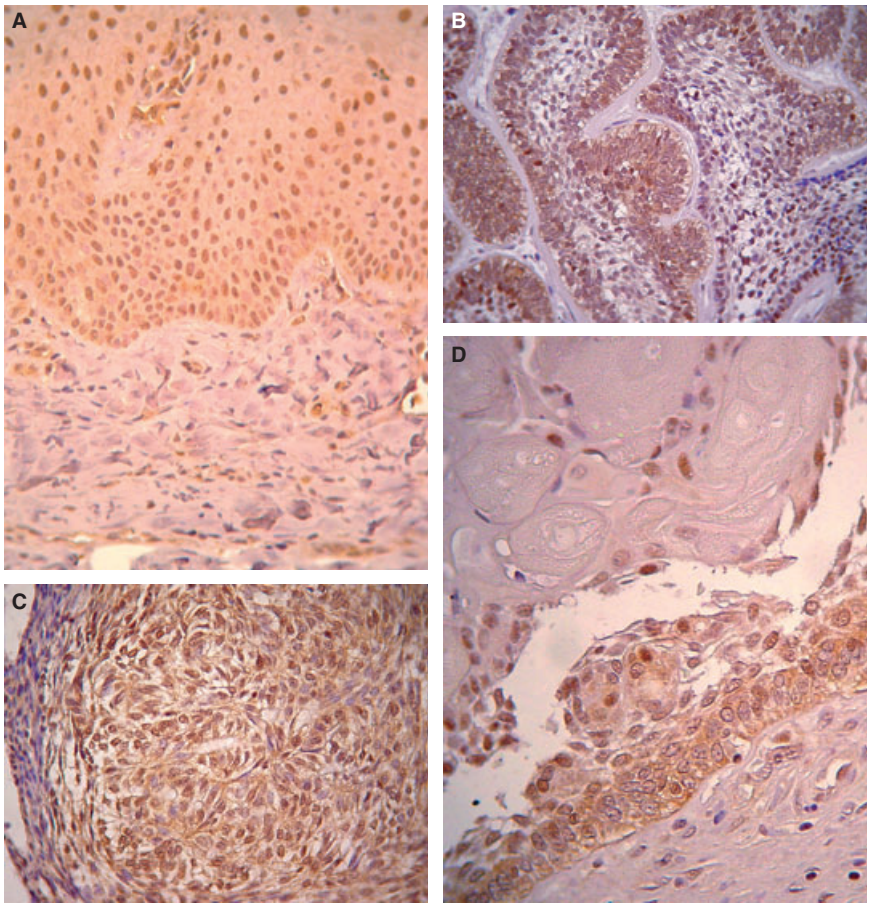

Figure 1 (a) TGF-β1 predominantly cytoplasmic immunoexpression in normal oral mucosa. (b) TGF-β1 predominantly cytoplasmic immunoexpression in follicular ameloblastoma. (c) TGF-β1 predominantly cytoplasmic immunoexpression in adenomatoid odontogenic tumor. (d) TGF-β1 predominantly cytoplasmic immunoexpression in calcifying cystic odontogenic tumor.

all AOT and CCOT were positive, mostly strongly or moderately (50% and 53.8%, respectively). The possibility for strong pSmad-1/-5/-8 expression was increased almost 12-fold in AOT (OR = 11.66, *P*-value = 0.001) and five-fold in CCOT (OR = 5.34, *P*-value = 0.013), compared to AM.

Smad-4

Smad-4 immunostaining was nuclear/cytoplasmic in normal oral epithelium (Fig. 4a). In 33/34 AM, Smad-4 overall expression was mostly moderate (44.1%) and localized at the ameloblast-like and stellate-reticulum-like cells (Fig. 4b). In AOT (Fig. 4c) and CCOT (Fig. 4d), Smad-4 immunostaining was mostly strong (82.3% and 92.3%, respectively), while ghost cells were non-reactive. Non-parametric test for trend showed that CCOT and AOT were more likely to demonstrate strong reactivity for Smad-4 compared to AM ($P < 0.001$).

Correlations

Overall, the percentage of the TGF-β1-positive cells was significantly higher compared to the pSmad-2/-3-positive cells ($P < 0.001$), and their correlation was not statistical significant (Kendall's tau-b = 0.199, $P = 0.073$). In particular, TGF-β1 immunostaining was significantly increased in AM and AOT ($P < 0.001$), compared to pSmad-2/-3. The overall correlation between TGF-β1 and Smad-4 expression was statistically significant (Kendall's tau-b = 0.430, $P < 0.001$), with Smad-4 being significantly stronger than TGF-β1 ($P < 0.001$).

The percentages of the pSmad-2/-3- and Smad-4-positive cells showed a statistically significant positive correlation of moderate power ($P < 0.001$, Kendall's tau-b = 0.425), while in most cases Smad-4 immunoexpression was significantly higher than pSmad-2/-3 ($P < 0.001$).

Similarly, the percentages of the pSmad-1/-5/-8- and Smad-4-positive cells showed a statistically significant positive correlation of moderate power ($P < 0.001$, Kendall's tau-b = 0.582), while in most cases Smad-4 immunoexpression was significantly higher or equal to pSmad-1/-5/-8 ($P < 0.001$).

No correlation was found between the histological type of ameloblastoma and TGF-β1, pSmad-2/-3, pSmad-1/-5/-8, or Smad-4 expression.

Discussion

In the present study, TGF-β1, functional pSmads-2/-3 and pSmads-1/-5/-8, and Smad-4 proteins were immunohistochemically detected in the epithelial cells of AMs, AOTs,

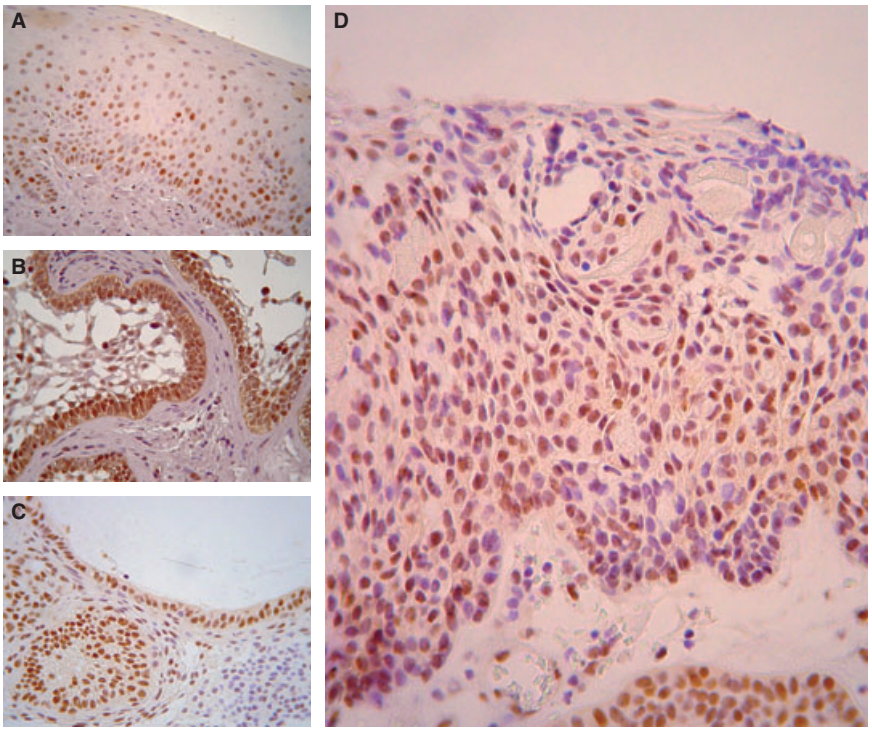

Figure 2 (a) pSmad-2/-3 predominantly nuclear and sporadically cytoplasmic immunostaining in normal oral mucosa. (b) pSmad-2/-3 predominantly nuclear and sporadically cytoplasmic immunostaining in follicular ameloblastoma. (c) pSmad-2/-3 predominantly nuclear and sporadically cytoplasmic immunostaining in adenomatoid odontogenic tumour. (d) pSmad-2/-3 predominantly nuclear and sporadically cytoplasmic immunostaining in calcifying cystic odontogenic tumor.

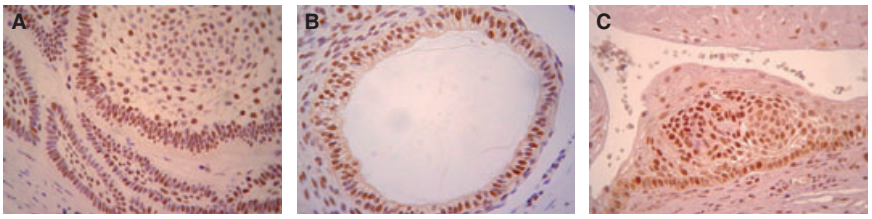

Figure 3 (a) pSmad1/-5/-8 predominantly nuclear and sporadically cytoplasmic immunostaining in follicular ameloblastoma. (b) pSmad1/-5/-8 predominantly nuclear and sporadically cytoplasmic immunostaining in adenomatoid odontogenic tumor. (c) pSmad1/-5/-8 predominantly nuclear and sporadically cytoplasmic immunostaining in calcifying cystic odontogenic tumor.

and CCOTs. Immunoreactivity in the normal oral epithelium for the antibodies tested was similar to that previously reported (55,56) except for pSmad-1/-5/-8 that has not been studied immunohistochemically in normal oral mucosa before, and it demonstrated a nuclear immunostaining pattern along the epithelial layers of the oral mucosa. pSmads immunolocalization was primarily nuclear, although some cytoplasmic reaction was also observed, possibly due to the nucleocytoplasmic shuttling of pSmads (16,17).

TGF-β and TGF-β1 have been previously studied in odontogenic tumors (20,21,34,57). Heikinheimo et al. (57) found decreased expression of *TGF-β1* in AM, compared to human fetal tooth germs. In the study of Takata et al. (21), marked nuclear TGF-β expression was seen in the epithelial cells of six of seven desmoplastic AMs, but not in 10 follicular and plexiform variants, suggesting involvement of TGF-β in the desmoplastic matrix formation. Kumamoto et al. (20) reported marked cytoplasmic TGF-β immunoreactivity in the ameloblast-like cells of AMs, the pseudoglandular cells of duct-like structures in AOTs, and in the epithelial cells of CCOTs. Iezzi et al. (34) reported cytoplasmic TGF-β1 immunoreactivity in both ameloblast-like and stellate-reticulum-like cells in 4 of 10 unicystic and peripheral AMs, and 11 of 19 solid AMs. TGF-β1 expression was significantly elevated in stromal cells of solid AMs, compared to unicystic/peripheral ones, and this was associated with the 'acquisition of an aggressive behavior'. The results of our study are comparable to those of previous reports (22,36). In addition, the percentage of cells expressing TGF-β1 in AM was significantly lower than that in AOT and CCOT, five- and twofold, respectively. In oral epithelium, loss of TGF-β1 expression is an early event during malignant transformation, as *TGF-β1* normally acts as a tumor suppressor gene, and has stronger inhibitory effect on epithelial growth compared to the other TGF-β

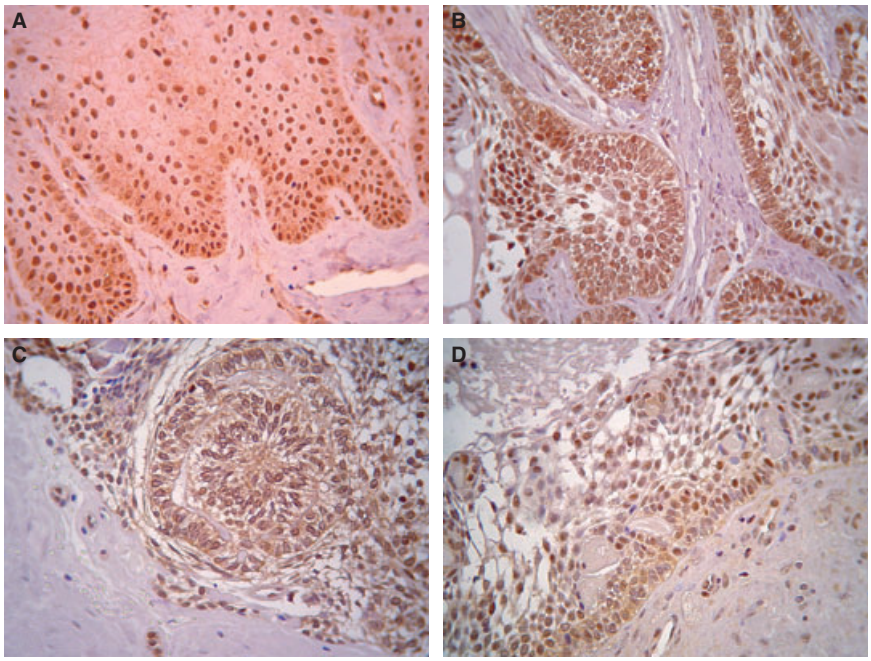

Figure 4 (a) Smad-4 nuclear and cytoplasmic immunostaining in (A) normal oral mucosa. (b) Smad-4 nuclear and cytoplasmic immunostaining in follicular ameloblastoma. (c) Smad-4 nuclear and cytoplasmic immunostaining in adenomatoid odontogenic tumor. (d) Smad-4 nuclear and cytoplasmic immunostaining in calcifying cystic odontogenic tumor.

isoforms (58). In contrast, TGF-β1 is increased in terminal differentiation of mucosal keratinocytes (59), while decreased expression of TGF-β1 and its receptors has been inversely correlated to differentiation in oral squamous cell carcinomas (60) and other cancers (35,37). Thus, lower expression of TGF-β1 in AM may be associated to its more primitive phenotype, characterized by increased proliferation and decreased differentiation.

Smad expression in odontogenic tumors has been previously studied in spontaneous developing keratocystic odontogenic tumors (KCOT) in mice that have been associated with ablation of Smad-4 (28). In the present study, pSmads-2/-3, pSmads-1/-5/-8, and Smad-4 were expressed in AM, AOT, and CCOT. In parallel with TGF-β1, pSmads-2/3, and Smad-4 expression was generally higher in AOT and CCOT compared to AM, and this could be indirectly associated with reduced apoptosis and enhanced cell proliferation (61–63). In fact, previous studies have shown reduced expression of apoptotic factors in AM, compared to AOT and CCOT (61–63). It has been found that AM has two relatively distinct patterns, an outer anti-apoptotic layer that could be considered the oncogenic and proliferative frontal of the tumor as reduced apoptosis is one of the major events during tumorigenesis and an inner pro-apoptotic differentiating site (63). In total, AM demonstrates an increased oncogenic activity compared to AOT and CCOT (53) that could be attributed to disturbed TGF-β1/Smad-2/-3 signaling. Therefore, it has been shown that TGF-β acts as a potent tumor suppressor in the early stages of tumor progression, while later functioning more to enhance the malignant phenotype (10).

Statistical analysis showed a lack of positive correlation between TGF-β1 and pSmads-2/-3 expression although Smads-2/-3 is a substrate of TGF-β1 (16). In contrast, there was a statistically significant correlation between TGF-β1 expression and Smad-4, as well as pSmads-2/-3 and Smad-4. Smad-4 is a key point of the TGF-β/Smad pathway that could have been activated by other molecules as well; thus, correlation between TGF-β1 and Smad-4 could have been secondary to other molecular events. A disturbance of the TGF-β/Smad pathway at the pSmad-2/-3 level could be attributed to disturbed TGF-β1 signaling (e.g., functional inactivation of TβRs) (10,31,41), enhanced inhibitory effect of Smad-7 (64) or Smads-2/-3 disturbance such as mutation (31, 47, 50, 65). Smad-7 is an inhibitor of Smad-2 and Smad-3 that interferes with TβRI receptor, causing inhibition of phosphorylation of Smad-2 and Smad-3 and thus preventing TGF-β-mediated responses (64). Mutant Smad-3 may block the activation of wild-type Smad-2 and Smad-3 (66). Mutations or deletions of *Smad-2, Smad-3,* and *Smad-4* have been described in aggressive metastatic colorectal cancer (50, 51, 65), while in head and neck cancer loss of *Smad-2* is considered an important event (67). In oral squamous cell carcinomas, low expression of pSmad-2 and TβRII has been associated with increased tumor aggressiveness, possibly due to the loss of the protective anti-oncogenic signaling transduced by TGF-β through Smad-2 (68).

Moreover, various kinases such as the mitogen-activated protein kinase Erk (69) can also phosphorylate the linker region of Smad-2/-3 thus preventing the R-Smads' accumulation in the nucleus and suppressing their activity (69,70). In particular, kinase-induced phosphorylation of the Smad-2/-3 linker region by Erk1/2 MAP kinase, cyclin-dependent kinase, and p38 MAP kinase has been found in ameloblastomas (54,61,71–73). Although that could explain the weak positive correlation between TGF-β1 and Smad-2/-3, not in our study the antibody used detects exclusively

the TGF-β-induced dually phosphorylated Smad-2 and Smad-3 at C-terminal Ser423 and Ser425 residues.

Overall, the decreased pSmads-2/-3 expression in AM compared to AOT and CCOT found in the present study may be associated with its aggressive behavior.

In the present study, Smad-4 immunoreactivity was mostly cytoplasmic and nuclear, as has been previously described in head and neck carcinomas (48,74). CCOT and AOT were more likely to demonstrate a strong reactivity for Smad-4 compared to AM. Low expression levels of Smad-4 are associated with Smad-4 mutations in carcinomas of the pancreas (75,76), gastrointestinal tract (43,49,77,78), lung and breast (6), and head and neck (48,79). Gao et al. (28) suggested that in experimentally induced KCOT, *Smad-4* acted as a tumor suppressor gene for the BMP/Smad pathway and its reduction allowed the activation of Sonic hedgehog (Shh) signaling in odontogenic epithelium (HERS, ERM). The Shh pathway has been shown to be activated in most AMs (80) and some AOTs and CCOTs (81). Smad-4-mediated TGF-β/BMP signaling is additionally required for Nfic (nuclear factor Ic) expression in the cranial neural crest-derived dental mesenchyme, a vital factor for normal odontogenesis (27). It is apparent that disturbance in Smad-4 expression, regardless of its causes, may negatively affect other critical pathways of odontogenic tissue homeostasis.

Smads-1, Smads-5, and Smads-8 are mainly activated by the BMP-2, BMP-4, and BMP-7 (16). BMPs are considered to be involved in the pathogenesis of odontogenic tumors by affecting differentiation of neoplastic odontogenic epithelium via epithelial–mesenchymal interactions (82), and BMP-2, BMP-4, and BMP-7 have been detected in the epithelial and stromal cells of AM and AOT (82). Expression of pSmads-1/-5/-8 was seen in the odontogenic tumors studied, but was more common and strong in AOT and CCOT. This may be associated with the ability of both tumors to form calcified dental tissues, a process mediated by BMPs (83).

The mechanism underlying the differences in TGF-β1 and Smad expression levels among the three groups of odontogenic tumor studied remains unresolved. To clarify the instrumental or epiphenomenal role of these molecules, the corresponding chromosomal loci status should be examined. Studies have shown genetic alterations of several chromosomes mainly in AMs, such as loss of chromosome 21 (84) and aberration in chromosome 22 (85). However, the chromosomes hosting TGF-β1 and Smads, namely 19q for TGF-β1 5q for Smad-8, 18q for Smad-2 and Smad-4, 15q for Smad-3 and Smad-5, and 4q for Smad-1 (86), have not been examined in odontogenic tumors, and there are no data regarding their stability and overall status. As 18q21 is frequently deleted or rearranged in a variety of human cancers (87), it could possibly become a reliable starting point for the further study of the actual role of the TGF-β1/Smad factors in odontogenic tumors.

In conclusion, the present study showed that TGF-β/Smad signaling pathway is activated in AM, AOT, and CCOT. There was a statistically significant difference in the expression of TGF-β1 and Smads between AM and AOT/CCOT that could be associated with the proliferation and differentiation of the latter, but the effect of this difference on the pathogenesis and/or biological behavior of the tumors needs further investigation.

References

1. Attisano L, Wrana JL. Signal transduction by the TGF-beta superfamily. *Science* 2002; **296**: 1646–7.
2. Chang H, Brown CW, Matzuk MM. Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocr Rev* 2002; **23**: 787–823.
3. Massague J. How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* 2000; **1**: 169–78.
4. Hogan BL. Bone morphogenetic proteins in development. *Curr Opin Genet Dev* 1996; **6**: 432–8.
5. Kingsley DM. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev* 1994; **8**: 133–46.
6. Massague J. TGFb signal transduction. *Annu Rev Biochem* 1998; **67**: 753–91.
7. Vale W, Hsueh A, Rivier C et al. The inhibin/activin family of hormones and growth factors. In: Sporn MB, Roberts AB, eds. *Peptide growth factors and their receptors II*. Berlin: Springer-Verlag, 1991; 211–48.
8. Cate RL, Mattaliano RJ, Hession C, et al. Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. *Cell* 1986; **45**: 685–98.
9. Prime SS, Pring M, Davies M, Paterson IC. TGF-beta signal transduction in oro-facial health and non-malignant disease (part I). *Crit Rev Oral Biol Med* 2004; **15**: 324–36.
10. Prime SS, Davies M, Pring M, Paterson IC. The role of TGF-β in epithelial malignancy and its relevance to the pathogenesis of oral cancer (Part II). *Crit Rev Oral Biol Med* 2004; **15**: 337–47.
11. Barton DE, Foellmer BE, Du J, Tamm J, Derynck R, Francke U. Chromosomal mapping of genes for transforming growth factors-β2 and -β3 in man and mouse: dispersion of the TGF-β gene family. *Oncogene Res* 1988; **3**: 323–31.
12. Fujii D, Brissenden JE, Derynck R, Francke U. Transforming growth factor-β gene maps to human chromosome 19 long arm and to mouse chromosome 7. *Somat Cell Mol Genet* 1986; **12**: 281–8.
13. Heldin CH, Miyazono K, ten Dijke P. TGF-β signaling from cell membrane to nucleus through SMAD proteins. *Nature* 1997; **390**: 465–71.
14. Raftery LA, Twombly V, Wharton K, Gelbart WM. Genetic screens to identify elements of the decapentaplegic signalling pathway in Drosophila. *Genetics* 1995; **139**: 241–54.
15. Savage C, Das P, Finelli AL, et al. Caenorhabditis elegans genes sma-2, sma-3, and sma-4 define a conserved family of transforming growth factor beta pathway components. *Proc Natl Acad Sci USA* 1996; **93**: 790–4.
16. Massagué J, Seoane J, Wotton D. Smad transcription factors. *Genes Dev* 2005; **19**: 2783–810.
17. Shi Y, Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 2003; **113**: 685–700.
18. Inman GJ, Hill CS. Stoichiometry of active smad-transcription factor complexes on DNA. *J Biol Chem* 2002; **277**: 51008–16.
19. Yoshida M, Kumamoto H, Ooya K, Mayanagi H. Immunohistochemical analysis of mixed and mesenchymal odontogenic tumors. *Oral Med Pathol* 2003; **8**: 125–32.
20. Kumamoto H, Yoshida M, Ooya K. Immunohistichemical detection of hepatocyte growth factor, transforming growth factor-β and their receptors in epithelial odontogenic tumors. *J Oral Pathol Med* 2002; **31**: 539–48.

21. Takata T, Miyauchi M, Ogawa I, et al. Immunoexpression of transforming growth factor β in desmoplastic ameloblastoma. *Virchows Arch* 2000; **436**: 319–23.
22. Heikinheimo K, Happonen R-P, Miettinen PJ, Ritvos O. Transforming growth factor beta 2 in epithelial differentiation of developing teeth and odontogenic tumors. *J Clin Invest* 1993; **91**: 1019–27.
23. Vaahtokari A, Vainio S, Thesleff I. Associations between transforming growth factor β1 RNA expression and epithelial-mesenchymal interactions during tooth morphogenesis. *Development* 1991; **113**: 985–94.
24. D'Souza RN, Litz M. Analysis of tooth development in mice bearing a TGF-beta 1 null mutation. *Connect Tissue Res* 1995; **32**: 41–6.
25. Gao J, Symons AL, Bartold PM. Expression of transforming growth factor-beta 1 (TGF-beta 1) in the developing periodontium of rats. *J Dent Res* 1998; **77**: 1708–16.
26. Huang XF, Chai Y. TGF-ß signaling and tooth development. *Chin J Dent Res* 2010; **13**: 7–15.
27. Huang X, Xu X, Bringas P Jr, Hung YP, Chai Y. Smad-4-Shh-Nfic signaling cascade-mediated epithelial-mesenchymal interaction is crucial in regulating tooth root development. *J Bone Miner Res* 2010; **25**: 1167–78.
28. Gao Y, Yang G, Weng T, et al. Disruption of *Smad-4* in odontoblasts causes multiple keratocystic odontogenic tumors and tooth malformation in mice. *Mol Cell Biol* 2009; **29**: 5941–51.
29. Lucchini M, Romeas A, Couble ML, Bleicher F, Magloire H, Farges JC. TGF beta 1 signaling and stimulation of osteoadherin in human odontoblasts in vitro. *Connect Tissue Res* 2002; **43**: 345–53.
30. Hwang YC, Hwang IN, Oh WM, Park JC, Lee DS, Son HH. Influence of TGF-beta1 on the expression of BSP, DSP, TGF-beta1 receptor I and Smad proteins during reparative dentinogenesis. *J Mol Histol* 2008; **39**: 153–60.
31. Massague J, Blain SW, Lo RS. TGF-β signaling in growth control, cancer and heritable disorders. *Cell* 2000; **103**: 295–309.
32. Reiss M. Transforming growth factor-beta and cancer: a love-hate relationship? *Oncol Res* 1997; **9**: 447–57.
33. Dong L, Wang YX, Li SL, et al. TGF-beta1 promotes migration and invasion of salivary adenoid cystic carcinoma. *J Dent Res* 2011; **90**: 804–9.
34. Iezzi G, Piattelli A, Rubini C, et al. Expression of transforming growth factor β1 in ameloblastomas. *J Craniofac Surg* 2008; **19**: 1618–21.
35. Mhawech-Fauceglia P, Kesterson J, Wang D, et al. Expression and clinical significance of the transforming growth factor-β signalling pathway in endometrial cancer. *Histopathology* 2011; **59**: 63–72.
36. Teixeira AL, Araújo A, Coelho A, et al. Influence of TGFB1 + 869T>C functional polymorphism in non-small cell lung cancer (NSCLC) risk. *J Cancer Res Clin Oncol* 2011; **137**: 435–9.
37. Iancu IV, Botezatu A, Goia-Ruşanu CD, et al. TGF-beta signalling pathway factors in HPV-induced cervical lesions. *Roum Arch Microbiol Immunol* 2010; **69**: 113–8.
38. Do TV, Kubba LA, Du H, Sturgis CD, Woodruff TK. Transforming growth factor-beta1, transforming growth factor-beta2, and transforming growth factor-beta3 enhance ovarian cancer metastatic potential by inducing a Smad3-dependent epithelial-to-mesenchymal transition. *Mol Cancer Res* 2008; **6**: 695–705.
39. Sun L, Diamond ME, Ottaviano AJ, Joseph MJ, Ananthanarayan V, Munshi HG. Transforming growth factor-beta 1 promotes matrix metalloproteinase-9-mediated oral cancer invasion through snail expression. *Mol Cancer Res* 2008; **6**: 10–20.
40. Logullo AF, Nonogaki S, Miguel RE. Transforming growth factor β1 (TGF-β) expression in head and neck squamous cell carcinoma patients as related to prognosis. *J Oral Pathol Med* 2003; **32**: 139–45.
41. DeCoteau JF, Knaus PI, Yankelev H, et al. Loss of functional cell surface transforming growth factor beta (TGF-beta) type 1 receptor correlates with insensitivity to TGF-beta in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 1997; **94**: 5877–81.
42. Levy L, Hill CS. Smad-4 dependency defines two classes of transforming growth factor {beta} (TGF-{beta}) target genes and distinguishes TGF-{beta}-induced epithelial-mesenchymal transition from its antiproliferative and migratory responses. *Mol Cell Biol* 2005; **25**: 8108–25.
43. Xu X, Brodie SG, Yang X, et al. Haploid loss of the tumor suppressor Smad-4/Dpc4 initiates gastric polyposis and cancer in mice. *Oncogene* 2000; **19**: 1868–74.
44. Zhao S, Ammanamanchi S, Brattain M, et al. Smad-4-dependent TGF-beta signaling suppresses RON receptor tyrosine kinase-dependent motility and invasion of pancreatic cancer cells. *J Biol Chem* 2008; **283**: 11293–301.
45. Halder SK, Rachakonda G, Deane NG, Datta PK. Smad 7 induces hepatic metastasis in colorectal cancer. *Br J Cancer* 2008; **99**: 957–65.
46. Katsuno Y, Hanyu A, Kanda H, et al. BMP signaling enhances invasion and bone metastasis of breast cancer cells through Smad pathway. *Oncogene* 2008; **27**: 6322–33.
47. Yanagisawa K, Uchida K, Nagatake M, et al. Heterogenecities in the biological and biochemical function of Smad 2 and Smad 4 mutants naturally occurring in human lung cancers. *Oncogene* 2000; **19**: 2305–11.
48. Wang X, Sun W, Bai J, et al. Growth inhibition induced by transforming growth factor-beta1 in human oral squamous cell carcinoma. *Mol Biol Rep* 2009; **36**: 861–9.
49. Natsugoe S, Xiangming C, Matsumoto M, et al. Smad-4 and transforming growth factor beta1 expression in patients with squamous cell carcinoma of the esophagus. *Clin Cancer Res* 2002; **8**: 1838–42.
50. Ku JL, Park SH, Yoon KA, et al. Genetic alterations of the TGF-beta signaling pathway in colorectal cancer cell lines: a novel mutation in Smad3 associated with the inactivation of TGF-beta-induced transcriptional activation. *Cancer Lett* 2007; **247**: 283–92.
51. Eppert K, Scherer SW, Ozcelik H, et al. MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell* 1996; **86**: 543–52.
52. Neville BW, Damm DD, Allen CM, Bouquot JE. *Oral and maxillofacial pathology*, 3rd edn. Philadelphia: WB Saunders Company, 2009.
53. Barnes L, Eveson JW, Reichart P, et al., eds. *World Health Organization classification of tumours: pathology and genetics of head and neck tumours*. Lyon, France: IARC Press, 2005.
54. Danielsson K, Wahlin YB, Coates PJ, Nylander K. Increased expression of Smad proteins, and in particular Smad3, in oral lichen planus compared to normal oral mucosa. *J Oral Pathol Med* 2010; **39**: 639–44.
55. Scheper MA, Chaisuparat R, Nikitakis NG, Sauk SS. Expression and alterations of the PTEN/AKT/mTOR pathway in ameloblastomas. *Oral Dis* 2008; **14**: 561–8.
56. Chen Y, Zhang W, Geng N, et al. MMPs, TIMP-2, and TGF-beta1 in the cancerization of oral lichen planus. *Head Neck* 2008; **30**: 1237–45.
57. Heikinheimo K, Jee KJ, Niini T, et al. Gene expression profiling of ameloblastoma and human tooth germ by means of a cDNA microarray. *J Dent Res* 2002; **81**: 525–30.

58. Mincione G, Marcadntonio MC, Artese L, et al. Loss of expression of TGF-β1, TβRI, and TβRII correlates with differentiation in human oral squamous cell carcinomas. *Int J Oncol* 2008; **32**: 323–31.
59. Min BM, Woo KM, Lee G, Park NH. Terminal differentiation of normal human oral keratinocytes is associated with enhanced cellular TGF-s and phospholipase C-γ1 levels and apoptotic cell death. *Exp Cell Res* 1999; **49**: 377–85.
60. Meng W, Xia Q, Wu L, et al. Downregulation of TGF-beta receptor types II and III in oral squamous cell carcinoma and oral carcinoma-associated fibroblasts. *BMC Cancer* 2011; **11**: 88–101.
61. Kumamoto H. Molecular pathology of odontogenic tumors. *J Oral Pathol Med* 2006; **35**: 65–74.
62. Fregnani ER, Pires FR, Quezada RD, Shih IeM, Vargas PA, de Almeida OP. Calcifying odontogenic cyst: clinicopathological features and immunohistochemical profile of 10 cases. *J Oral Pathol Med* 2003; **32**: 163–70.
63. Sandra F, Nakamura N, Mitsuyasu T, Shiratsuchi Y, Phishi M. Two relatively distinct patterns of ameloblastoma: an anti-apoptotic proliferating site in the outer layer (periphery) and a pro-apoptotic differentiating site in the inner layer (centre). *Histopathology* 2001; **39**: 93–8.
64. Nakao A, Afrakhte M, Moren A, et al. Identification of Smad7 a TGFb-inducible antagonist of TGF-b signalling. *Nature* 1997; **389**: 631–5.
65. Zhu Y, Richardson JA, Parada LF, Graff JM. Smad3 mutant mice develop metastatic colorectal cancer. *Cell* 1998; **94**: 703–14.
66. Kawabata M, Imamura T, Inoue H, et al. Intracellular signaling of the TGF-beta superfamily by Smad proteins. *Ann NY Acad Sci* 1999; **886**: 73–82.
67. Qiu W, Schonleben F, Li X, Su GH. Disruption of transforming growth factor beta-Smad signaling pathway in head and neck squamous cell carcinoma as evidenced by mutations of SMAD2 and SMAD4. *Cancer Lett* 2007; **245**: 163–70.
68. Peng H, Shintani S, Kim Y, Wong DT. Loss of p12CDK2-AP1 expression in human oral squamous cell carcinoma with disrupted transforming growth factor-β-Smad signaling pathway. *Neoplasia* 2006; **8**: 1028–36.
69. Kretzschmar M, Doody J, Timokhina I, Massagué J. A mechanism of repression of TGFbeta/Smad signaling by oncogenic Ras. *Genes Dev* 1999; **13**: 804–16.
70. Heldin CH, Moustakas A. Role of Smads in TGFβ signaling. *Cell Tissue Res* 2012; **347**: 21–36.
71. Kumamoto H, Ooya K. Immunohistochemical detection of phosphorylated JNK, p38 MAPK, and ERK5 in ameloblastic tumors. *J Oral Pathol Med* 2007; **36**: 543–9.
72. Kumamoto H, Takahashi N, Ooya K. K-Ras gene status and expression of Ras/mitogen-activated protein kinase (MAPK) signaling molecules in ameloblastomas. *J Oral Pathol Med* 2004; **33**: 360–7.
73. Kumamoto H, Kimi K, Ooya K. Detection of cell cycle-related factors in ameloblastomas. *J Oral Pathol Med* 2001; **30**: 309–15.
74. Xie W, Aisner S, Baredes S, Sreepada G, Shah R, Reiss M. Alterations of Smad expression and activation in defining 2 subtypes of human head and neck squamous cell carcinoma. *Head Neck* 2012. doi: 10.1002/hed.22924. [Epub ahead of print]
75. Wilentz RE, Iacobuzio-Donahue CA, Argani P, et al. Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. *Cancer Res* 2000; **60**: 2002–6.
76. Hahn SA, Schutte M, Hoque A, et al. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 1996; **271**: 350–3.
77. Xiangming C, Natsugoe S, Takao S, et al. Preserved Smad-4 expression in the transforming growth factor beta signaling pathway is a favorable prognostic factor in patients with advanced gastric cancer. *Clin Cancer Res* 2001; **7**: 277–82.
78. Miyaki M, Iijima T, Konishi M, et al. Higher frequency of Smad-4 gene mutation in human colorectal cancer with distant metastasis. *Oncogene* 1999; **18**: 3098–103.
79. Pring M, Prime S, Parkinson EK, Paterson I. Dysregulated TGF-beta 1-induced Smad signaling occurs as a result of defects in multiple components of the TGF-beta signaling pathway in human head and neck carcinoma cell lines. *Int J Oncol* 2006; **28**: 1279–85.
80. Kumamoto H, Ohki K, Ooya K. Expression of Sonic hedgehog (SHH) signaling molecules in ameloblastomas. *J Oral Pathol Med* 2004; **33**: 185–90.
81. Zhang L, Chen XM, Sun ZJ, Bian Z, Fan MW, Chen Z. Epithelial expression of SHH signalling pathway in odontogenic tumors. *Oral Oncol* 2006; **42**: 398–408.
82. Kumamoto H, Ooya K. Expression of bone morphogenetic proteins and their associated molecules in ameloblastomas and adenomatoid odontogenic tumors. *Oral Dis* 2006; **12**: 163–70.
83. Gao YH, Yang LJ, Yamaguchi A. Immunohistochemical demonstration of bone morphogenetic protein in odontogenic tumors. *J Oral Pathol Med* 1997; **26**: 273–7.
84. Jääskeläinen K, Jee KJ, Leivo I, Saloniemi I, Knuutila S, Heikinheimo K. Cell proliferation and chromosomal changes in human ameloblastoma. *Cancer Genet Cytogenet* 2002; **136**: 31–7.
85. Stenman G, Sandros J, Mark J, Happonen RP. Observations by G-banding in benign odontogenic tumors. *Cancer Genet Cytogenet* 1986; **19**: 253–9.
86. Available at: http/www.ncbi.nlm.nih.gov (accessed on 29 October, 2012).
87. Nakao A, Röijer E, Imamura T, et al. Identification of Smad2, a human Mad-related protein in the transforming growth factor beta signaling pathway. *J Biol Chem* 1997; **272**: 2896–900.

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