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ORIGINAL ARTICLE

Immunohistochemical expression of SHH, PTC, SMO and GLI1 in glandular odontogenic cysts and dentigerous cysts

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OBJECTIVE: To investigate expression of the sonic hedgehog (SHH) signaling pathway components in glandular odontogenic cysts (GOCs), and dentigerous cysts (DCs).
MATIERIALS AND METHODS: Immunohistochemical staining for SHH, patched (PTC), smoothened (SMO), and the transcriptional factor GLI1 were investigated in the 12 GOCs specimens and 20 DCs.
RESULTS: In GOCs and DCs, immunoreactivity for SHH, PTC, SMO, and GLI1 were detected in the epithelial cytoplasm. Each of the genes of the SHH signaling pathway was expressed in similar patterns in the epithelial lining of the cysts. The expression of SHH, PTC, SMO, and GLI1 was significantly higher in epithelia than that of subepithelial fibroblasts ($P < 0.01$). No statistical difference among the labeling index of the epithelial lining among the different cyst types could be revealed.
CONCLUSIONS: The findings suggest that the proteins of the SHH signaling pathway are predominantly located within the epithelial components of GOCs and DCs. SHH signaling pathway may play a role in epithelial lining formation.
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Keywords: glandular odontogenic cysts; immunohistochemistry; SHH; PTC; SMO; GLI1

Introduction

Glandular odontogenic cyst (GOC) of the jaws is a rare histopathologic entity presenting potentially aggressive (Kramer *et al*, 1991; Kaplan *et al*, 2008). Padayachee and Van Wyk (1987) initially considered it to be a sialodontogenic cyst. In 1988, Gardner *et al* proposed the term GOC. In the classification of the World Health Organization (WHO, Kramer *et al,* 1991) this lesion is listed as a developmental odontogenic epithelial cyst with lacking evidence of salivary gland origin. Histopathologically, GOC is characterized by a cyst wall lining of cuboidal to thin squamous through pseudostratified focally ciliated columnar epithelium with duct or gland-like spaces, containing extracellular mucous with a positive reaction to alcian blue and periodic Schiff staining. However, because of microscopical similarities, GOC is apt to misdiagnosis with dentigerous cysts (DCs) especially when mucous metaplasia is present. The etiology and pathogenesis of GOC still remains uncertain (Kaplan *et al*, 2008).

The Sonic hedgehog (SHH) signaling pathway plays a critical role in tooth development (Dassule *et al*, 2000; Cobourne *et al*, 2004; Zhang *et al*, 2005, 2008). The two transmembrane proteins PTC and SMO are interacting, and GLI1 is a known transcriptional factor of SHH signal pathway. Without ligand SHH, PTC inhibits SMO activating transcriptional factor GLI1. If SHH binds to PTC, then its inhibition of SMO is altered. This allows SMO activating the GLI1 transcription factors to regulate the downstream target gene transcription (Zhang *et al*, 2005). Previous studies have demonstrated that SHH signaling pathway genes play a important role during tooth development, tumorigenesis and pathogenesis of keratocystic odontogenic tumors (KCOTs) (Ohki *et al*, 2004; Zhang *et al*, 2006a, 2008; Sun *et al*, 2008). However, studies investigating the association of SHH signaling pathways within GOCs and DCs are somewhat scant. It is essential to discovery novel molecular events of this rare and aggressive disease, which may allow for a better understanding of the pathogenesis and more importantly to develop new strategies of therapeutics. The objective of this study was to investigate the protein distribution of the SHH signaling pathway in GOCs and DCs.

Materials and methods

Sample collection

The samples in this study comprised the specimens of 12 patients suffering from GOCs and 20 from DCs out of

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the database of the Department of Oral Pathology, School and Hospital of Stomatology, Wuhan University, China. Hematoxylin and eosin-stained sections of these specimens were reviewed by an oral pathologist to confirm the diagnoses according to the criteria stated in the guidelines of the WHO histological typing of odontogenic tumors (Kramer *et al*, 1991). The clinical and radiographic features of 12 GOCs have been previously reported (Shen *et al*, 2006). Sections of 4-μm thickness were series cut from the 4% formalin-fixed paraffin embedded blocks and mounted on PLL-coated glass slides.

Immunohistochemistry

Immunohistochemistry was performed according to our previous reported (Zhang *et al*, 2006a). Briefly, sections were deparaffinized in xylene, hydrated through graded alcohol and washed out with deionized water. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 15 min. Antigen retrieval was performed by pressure cooker in 10 mM citrate buffer for 15 min. After bovine serum albumin treated for 15 min to block non-specific binding, the sections were incubated with primary antibodies (Table 1) at 4°C overnight. The labeled streptavidin–biotin method was performed to bind the primary antibodies with use of a LSAB kit (Dako, Denmark, Carpinteria, CA, USA). The slides were visualized by diaminobenzidine solution and weakly counterstained with hematoxylin. Ten KCOTs were selected as positive control. Negative controls were prepared by replacing primary antibodies with phosphate buffered saline and were confirmed to be unstained.

Evaluation

The immunoreactivity was evaluated in terms of location, pattern, and intensity. Immunoreactions were independently analyzed by two investigators unaware of the clinical data. Four high power fields within the epithelial lining, mucous cell, and subepithelial fibroblasts were randomly counted, according to our previous reported methods (Zhang *et al*, 2006a) with some modification. The tissues were examined and classified based on the staining intensity (0, no staining; 1, mild staining; 2, moderate staining; and 3, intense staining) and the percentage of positive cells (0, $<$ 10%; 1, 10–25%; 2, 25–50%; 3, 50–75%; and 4, 75–100% of stained cells). The percentage of positive cells multiply the staining intensity was considered as the final staining scores. Mean staining scores of four high power field was calculated in each specimen and value was expressed by mean $\pm$ s.d.

Table 1 Primary antibodies applied in this study

	Clonality	*Antibody source*	*Dilution*
SHH	Monoclonal (rat)	R&D	2 μg ml^{-1}
PTC	Polyclonal (goat)	Santa Cruz	1:50
SMO	Polyclonal (goat)	Santa Cruz	1:50
GLI1	Polyclonal (goat)	Santa Cruz	1:80

Statistical analyses

Data were analyzed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA) statistical package. The statistical significance of differences in the percentages of cases with different reactivity levels for SHH, PTC, SMO, and GLI1 was analyzed by Pearson's Chi-squared test. A P-value < 0.05 was considered to be statistically significant.

Results

Histological features of GOCs

All specimens consisted of luminal epithelia and surrounding connective tissue. The cystic spaces were lined by non-keratinized stratified squamous epithelium that varied in thickness. It exhibited a flat interface with the subjacent stroma. The surface layer of the epithelium was composed of eosinophilic cuboidal or low-columnar cells being sometimes ciliated. Spherical epithelial thickenings were noted, with some irregular papillary projections into the lumen. Often intraepithelial micro-cysts- or glandular-like structures were lined by eosinophilic cuboidal cells similar to those seen in the epithelial surface, being filled with mucous-like material. The basal cells presented no polarity of the nuclei and were occasionally vacuolated. No mitotic figures were detected in all 12 cases GOCs. Scant irregularly shaped calcifications were revealed in the fibrous stroma in other two specimens. In all but two cases, the underlying connective tissue consisted of dense fibrous tissue, free of chronic inflammatory cells. In some specimens, erosion of the surrounding bone could be detected.

Immunoreactivity for SHH, PTC, SMO, and GLI1 in GOCs

In all 12 GOC specimens immunoreactivity was detected for SHH, PTC, SMO, and GLI1 (Figure 1a–d) mainly located within the epithelial cytoplasm, but also could be detected in nuclear. Immunoreactivity of SHH, PTC, SMO, and GLI-1 was detected homogeneously and intensely in the basal cell layers and plaque-like areas (Figure 1a,c), and heterogeneously and less intensely in the superficial cuboid (Figure 1d) and suprabasal layers of epithelium. The mucous-producing cells (Figure 1d) of the cystic lining revealed lacking or weak immunoreactivity. SHH, PTC, SMO, and GLI-1 was expressed in similar patterns in the epithelial lining of the cysts and significantly higher than in subepithelial fibroblasts (Table 2, $P < 0.01$).

Immunoreactivity for SHH, PTC, SMO, and GLI1 in DCs

The epithelial lining of DCs disclosed homogenously immunostain for SHH, PTC, SMO, and GLI-1(Figure 2a–d). In five DCs with mucous metaplasia, the mucous-producing cells (Figure 2a–d) did not express any SHH, PTC, SMO, and GLI1. Subepithelial fibroblasts in cystic linings of DCs showed faint positive expressions of SHH, PTC, SMO, and GLI-1. There was a highly significant difference between the reactivity for

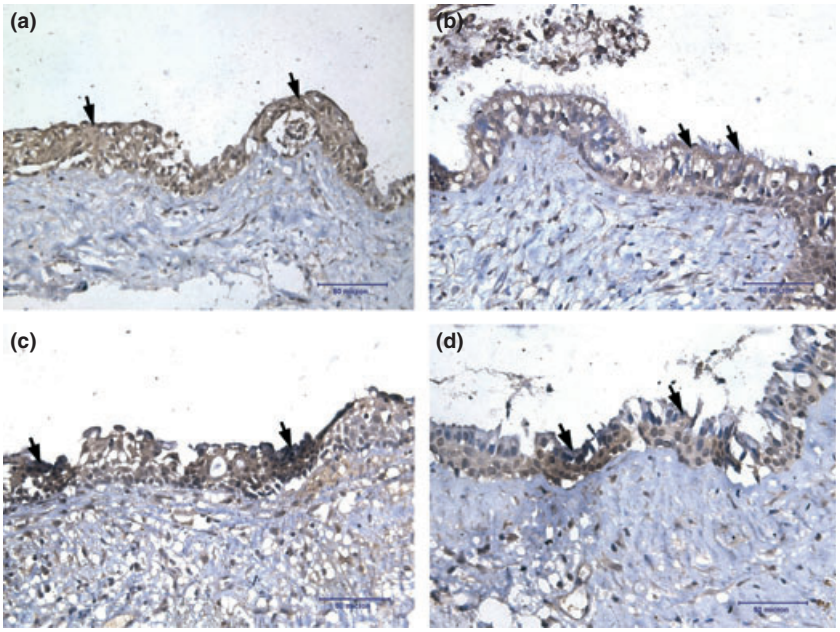

Figure 1 Immunohistochemical reactivity of SHH signaling pathway in glandular odontogenic cysts. Epithelial lining cells (arrow) are positive for SHH **(a)**, PTC **(b)**, SMO **(c)** and GLI **(d)**

Table 2 Labeling index for SHH, PTC, SMO, and GLI among the different cysts

	SHH	*PTC*	*SMO*	*GLI*
GOCs (n = 12)				
Epithelial lining	4.3 ± 1.1	3.6 ± 1.9	3.9 ± 2.8	3.1 ± 2.3**·*
Mucous cell	0.4 ± 1.3	0.8 ± 1.7	0.6 ± 0.3	0.9 ± 0.7
Fibroblasts	0.7 ± 0.5	1.2 ± 0.8	1.7 ± 1.3	1.0 ± 0.6
DCs (n = 20)				
Epithelial lining	2.1 ± 1.4	1.6 ± 1.0	2.9 ± 1.8	2.3 ± 1.9 **·**
Mucous cell[a]	0.2 ± 0.2	0.3 ± 0.3	0.3 ± 0.1	0.4 ± 0.2
Fibroblasts	0.5 ± 0.2	0.4 ± 0.3	0.8 ± 0.6	0.7 ± 0.4

Values are mean ± s.d. (score of staining); *$P < 0.05$,**$P < 0.01$.
[a]In five cases.

SHH, PTC, SMO, and GLI1 between the cystic lining and the subepithelial fibroblasts (Table 2, $P < 0.01$). There were no statistic difference among the immunoreactivity of SHH, PTC, SMO, and GLI-1 in GOCs and DCs.

Discussion

As a mediator of epithelial–mesenchymal interactions, SHH signaling pathway is not only participating in the odontogenesis (Dassule *et al*, 2000; Cobourne *et al*, 2004; Zhang *et al*, 2005) but also in the odontogenic tumorigenesis. Expression of SHH, PTC, SMO, and GLI1 was detected in the odontogenic tumors (Zhang

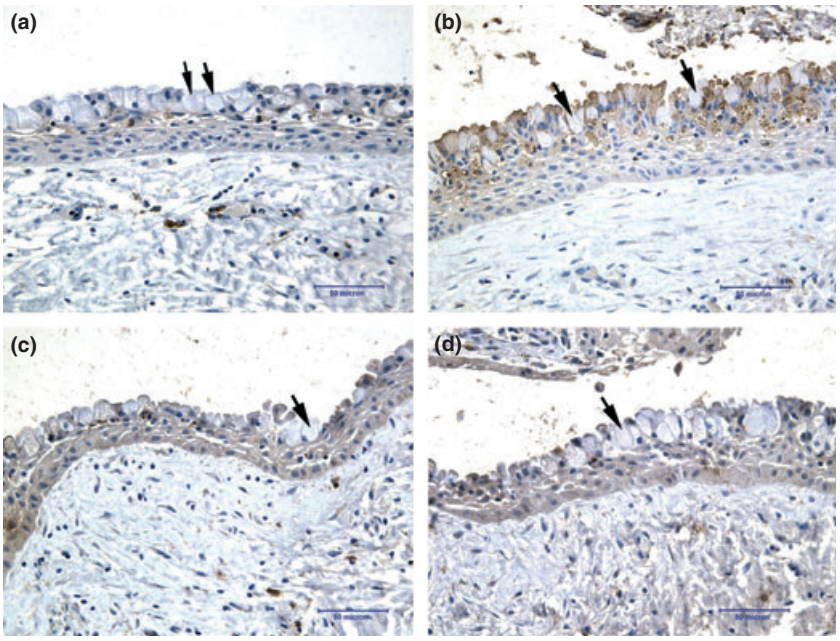

Figure 2 Immunohistochemical reactivity of SHH signaling pathway in dentigerous cysts. Epithelial lining cells are positive for SHH **(a)**, PTC **(b)**, SMO **(c)**, and GLI **(d)**, whereas mucous-producing cells show scarce expression of SHH signaling proteins (arrow)

et al, 2006a) as well as in KCOT (Ohki *et al*, 2004). In this study, GOCs showed homogenously SHH, PTC, SMO, and GLI1 expression predominantly in odontogenic cystic lining, suggesting that the SHH signaling pathway may play a role in epithelial lining formation during the pathogenesis. The results of SHH signaling pathway staining GOC might suggest a probably common mechanism within the pathogeneses of these three types of cysts. This would be consistent with previous reports of epithelial expression of SHH signaling pathway in odontogenic tumors and KCOTs (Kumamoto *et al*, 2004; Ohki *et al*, 2004; Zhang *et al*, 2006a). The results here presented showed no statistically significant differences between the GOCs and KCOTs. Barreto *et al* (2002) reported the immunoreactivity of PTC localized in GOC superficial layer cells in a single case and found no mutation of the PTC gene when using direct sequencing. The role of SHH, PTC, SMO, and GLI1 in GOC pathogenesis is far more clear, further genetic studies of the SHH signaling pathway in larger series of GOC samples will be needed to establish associations between SHH signaling pathway and pathologic changes in odontogenic epithelium.

Recent research revealed that heterozygosity loss of PTC was observed in approximately 50% of dentigerous cysts, probably indicating that PTC mutation might be involved in the pathogenesis of DCs (Levanat *et al*, 2000). The DCs and GOCs may present similar histological features such as mucous-producing cell (Kaplan *et al*, 2008). In this study, it was detected that mucous-producing cells in both types of cysts were lacking expression of SHH signaling pathway, being consistent with expression patterns of cytokeratin in these two types of cysts (Shen *et al*, 2006).

The involvement of SHH signaling pathway in the pathogenesis of GOC and DC may provide a potential molecular therapy target for those two entities (Taipale *et al*, 2000; Williams *et al*, 2003; Romer *et al*, 2004; Zhang *et al*, 2006b). Until now several synthetic molecules acting antagonistically to SMO have been reported probably presenting importance as potential drugs. For example, a chemical compound extracted from lilies, called cyclopamine, inactivates the transmembrane SMO protein and is able to reverse the oncogenic mutations of in vitro cultured cell lines (Taipale *et al*, 2000). A synthetic CUR611212 was described which inhibits the SHH signaling pathway by binding to SMO (Williams *et al*, 2003). CUR611212 acts as an anti-proliferative agent in cells whose proliferation is promoted due to activation of the SHH signaling pathway. Its physiochemical properties such as small size, ease of synthesis and solubility make it a desirable candidate for drug development (Williams *et al*, 2003). A prolonged inactivation of the SHH signaling pathway after a single application make it possible to treatment GOC and DC.

In summary, we investigate the immunolocation of SHH protein in the GOCs and DCs. The result suggested that the epithelial lining of GOCs and DCs were positive to SHH, PTC, SMO and GLI with similar expression pattern. There is no statistical significant of SHH protein expression between GOCs and DCs. The GOCs and DCs may share similar pathogenesis process and treatment strategies. There is also some limitation in this study because of the small number of GOC specimen.

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