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Notch Signaling and Ghost Cell Fate in the Calcifying Cystic Odontogenic Tumor

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Abstract
Notch signaling is an evolutionarily conserved mechanism that enables adjacent cells to adopt different fates. Ghost cells (GCs) are anucleate cells with homogeneous pale eosinophilic cytoplasm and very pale to clear central areas (previous nucleus sites). Although GCs are present in a variety of odontogenic lesions notably the calcifying cystic odontogenic tumor (CCOT), their nature and process of formation remains elusive. The aim of this study was to investigate the role of Notch signaling in the cell fate specification of GCs in CCOT. Immunohistochemical staining for four Notch receptors (Notch1, Notch2, Notch3 and Notch4) and three ligands (Jagged1, Jagged2 and Delta1) was performed on archival tissues of five CCOT cases. Level of positivity was quantified as negative (0), mild (+), moderate (2+) and strong (3+). Results revealed that GCs demonstrated overexpression for Notch1 and Jagged1 suggesting that Notch1-Jagged1 signaling might serve as the main transduction mechanism in cell fate decision for GCs in CCOT. Protein localizations were largely membranous and/or cytoplasmic. Mineralized GCs also stained positive implicating that the calcification process might be associated with upregulation of these molecules. The other Notch receptors and ligands were weak to absent in GCs and tumoral epithelium. Stromal endothelium and fibroblasts were stained variably positive.

Key words: Notch, Jagged, Delta, Calcifying cystic odontogenic tumor (CCOT), Ghost cells

Introduction

Notch signaling is an evolutionarily conserved mechanism that enables adjacent cells to adopt different fates [1]. The *Drosophila* Notch gene encodes a transmembrane receptor with a large extracellular domain carrying multiple epidermal growth factor-like repeats and a cytoplasmic domain required for signal transduction [1]. In vertebrates, there are four Notch receptor proteins (Notch1, Notch2, Notch3, and Notch4) and five membrane-bound ligand proteins (Delta1, Delta2, Delta4, Jagged1, and Jagged2) [1]. Signals exchanged between neighboring cells through binding of ligand with its cognate receptor initiates short range events including differentiation, proliferation, and apoptotic events at all stages of development, thus controlling organ formation and morphogenesis [1]. Deregulation of Notch signaling has been implicated in developmental abnormalities and neoplasias [2].

Ghost cells are large pale anucleate cells with homogeneous pale eosinophilic cytoplasm and very pale to clear central areas instead of a basophilic nucleus [3]. They tend to form small clusters or large masses. Although characteristic of calcifying cystic odontogenic tumors (CCOT) [4], ghost cells are also found in other odontogenic lesions namely ameloblastoma [5] odontoma [6] and ameloblastic fibro-odontoma [7], and in nonodontogenic tumors such as pilomatrixoma [8], a tumor with hair matrix cell differentiation, and craniopharyngioma, a tumor of the pituitary gland [9]. Several theories of ghost cell formation have been put forth including that these cells are most likely abnormal keratinized bodies, or they might represent simple cell degeneration or a form of enamel matrix; or might be apoptotic odontogenic cells or represent different stages of normal and abnormal keratin formation resulting from metaplastic transformation of odontogenic tumors [4]. The World Health Organization Classification of Head and Neck Tumors considered ghost cells as transitory squamous cells at various stages of development [10]. However to date the true nature of these ghost cells remains unresolved. The calcifying process of ghost cells also remains ill-understood.

Odontogenic tumors form a special research interest in this region because these neoplasms represent a clinically significant group of jaw tumors that are both challenging to diagnose and treat. Our group has worked on the demographic and immunoprofile of some of these tumors in the hope of gaining a better

understanding on the development and progression of these neoplasms [11-23]. In recent years, we have focused our attention on the oncogenic role of Notch signaling in the tumorigenesis of ameloblastomas [19-20], squamous odontogenic tumor [21], calcifying epithelial odontogenic tumor [22] and CCOT [23]. In the latter study which was based on a single case report, we observed that Notch1 positivity was detected in both the epithelial and ectomensenchymal tumoral components suggesting that this molecule might play some roles in the cytological differentiation and acquisition of tissue-specific characteristics. These preliminary findings prompted us to examine a further five new cases of CCOT for both Notch receptor (Notch1-4) and ligand (Jagged1-2 and Delta1) activity specifically to clarify the role of Notch signaling in the fate of ghost cells.

Materials and Methods

Tissue Samples

The test sample consisted of five CCOT cases that fulfilled the World Health Organization diagnostic criteria [10]. These were obtained from patients (four female and one male) whose ages ranged from 13-55 years (mean age: 23.5 years). All cases were intrabony in location (3 mandibular and two maxillary lesions) and presented as cystic CCOT characterized by ameloblastomatous lining epithelium, ghost cells and calcifications [6].

From the archival formalin-fixed, paraffin-embedded tissue blocks of these cases, new five micron thick sections were cut for staining with hematoxylin-eosin and Congo red, and for immunohistochemistry with primary antibodies directed against Notch (1-4), Jagged(1-2) and Delta 1 (Table 1).

Immunohistochemistry

Immunostaining was performed using the Envision technique as previously described [19, 20]. Briefly, pretreatment of deparaffinized sections for antigen retrieval was done by microwaving (99°C) in 10 nM of citrate buffer (pH 6, 20 min). For blocking endogenous peroxidase, the sections were immersed in 0.3% methanol containing 3% hydrogen peroxide for 20 min and rinsing in 0.05 M Tris-buffered saline (TBS) (5 min, two times) before immersing in blocking solution (Dako Corporation, Carpintera, CA, USA) for 20 min at room temperature. Subsequently the sections were incubated with the primary antibody (Table 1) for 1 h at room temperature. Immunoreactions were performed using the Envision Kit (Dako Corporation, Carpintera, CA, USA). The antigenic sites were visualized using diaminobenzidine (DAB) substrate chromogen (Dako Corporation, Carpintera, CA, USA) and counterstained with Mayer's hematoxylin. Appropriate positive controls were applied. For negative control, sections were treated as above but without the primary antibody. All the control sections were negative.

Table 1. Antibodies, sources and dilutions.

Antibody	Clonality	Catalogue ID	Source	Dilution
Notch1	Mouse monoclonal	LS-C16925	LifeSpan BioSciences Inc., Seattle, WA, USA	1:500
Notch2	Rabbit polyclonal	LS-B3399	LifeSpan BioSciences Inc., Seattle, WA, USA	1:500
Notch3	Rabbit polyclonal	LS-B1621	LifeSpan BioSciences Inc., Seattle, WA, USA	1:500
Notch4	Rabbit polyclonal	LS-C40785	LifeSpan BioSciences Inc., Seattle, WA, USA	1:500
Jagged1	Rabbit polyclonal	LS-B2442	LifeSpan BioSciences Inc., Seattle, WA, USA	1:500
Jagged2	Rabbit polyclonal	Ab60041	Abcam Inc., Cambridge, MA, USA	1:500
Delta1	Rabbit polyclonal	LS-B72	LifeSpan BioSciences Inc., Seattle, WA, USA	1:500

Table 2. Immunoexpression of Notch receptors and ligand proteins in calcifying cystic odontogenic tumors (n = 5).

CCOT components	*Notch1*	*Notch2*	*Notch3*	*Notch4*	*Jagged1*	*Jagged2*	*Delta1*
Basal pre-ameloblast-like cells	+	-	-	+	-	-	-
Suprabasal stellate reticulum-like cells	+	-	-	+	+	-	-
Transitory squamous cells	+	-	-	+	+	-	
Ghost cells	2+	-	-	+	3+	-	-
Calcifications	+	-	+	+	+	-	-
Connective tissue stroma	-	-	+	+	-	-	-
Dentinoid	NA	NA	NA	NA	NA	NA	NA
Odontome	NA	NA	NA	NA	NA	NA	NA

CCOT, Calcifying cystic odontogenic tumor; -, negative; +, mild (<25% positivity); 2+, moderate (25-50% positivity); 3+, strong (>50% positivity); NA, Not available

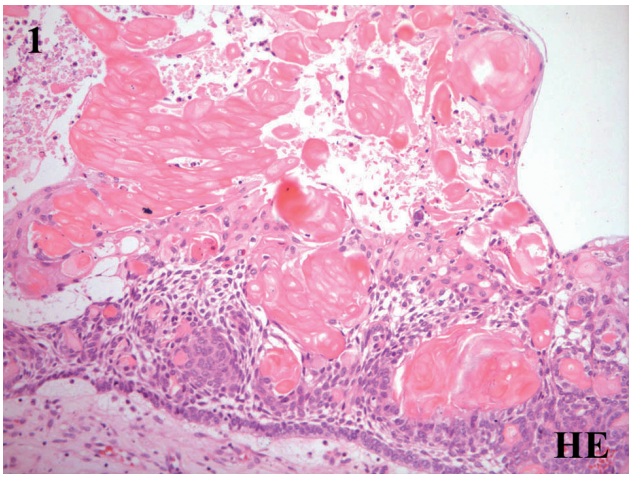
Immunohistochemical Analysis

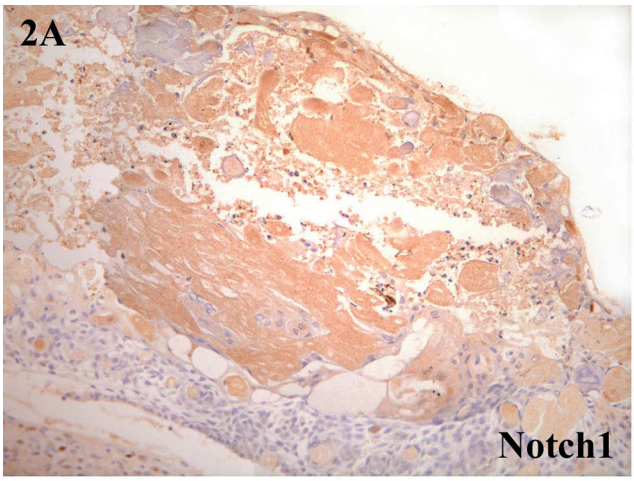
Notch receptor and ligand protein distribution pattern and levels of staining intensity in all five CCOT cases were evaluated using descriptive and semiquantitative methods (19, 20). In the latter, the immunostained sections were systematically sampled and the level of expression for Notch (1-4), Jagged (1-2) and Delta1 was quantified according to the staining intensity and percentage of immunopositive tumoral components present (CCOT neoplastic epithelial cells, ghost cells and calcifications): (-) negative when none of these tumoral components are positively stained; (+) mild when staining was present in focal areas (<25%); (++) moderate when staining was evident in significant areas (25-50%); and (+++) strong when staining is present in extensive areas (>50%). Stromal immunoreactivity was also assessed in a similar manner.

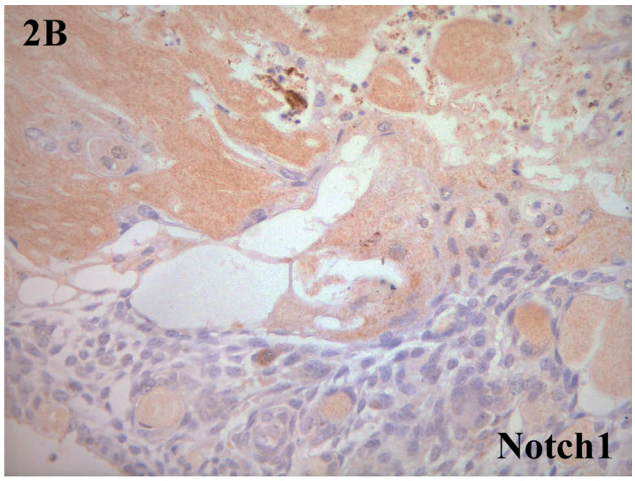
Results

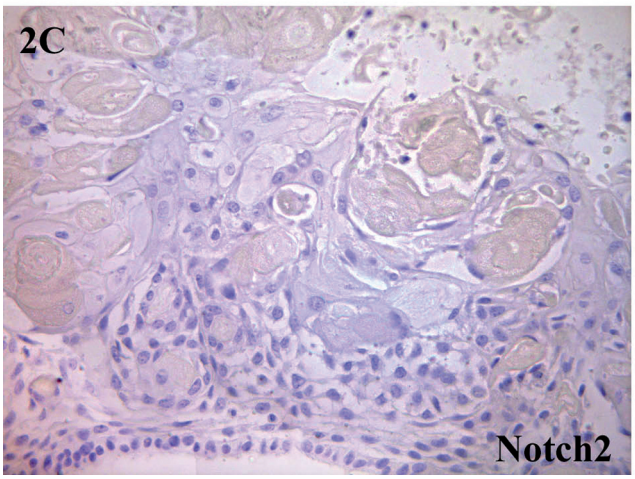
Microscopic Findings

All five cases of CCOT studied here were of the cystic type. Histological examination revealed lesional tissues composed of fibrous wall lined by ameloblastomatous epithelium characterized by basal cuboidal/low columnar pre-ameloblast-like cells and suprabasal stellate reticulum-like cells with scattered clusters of ghost









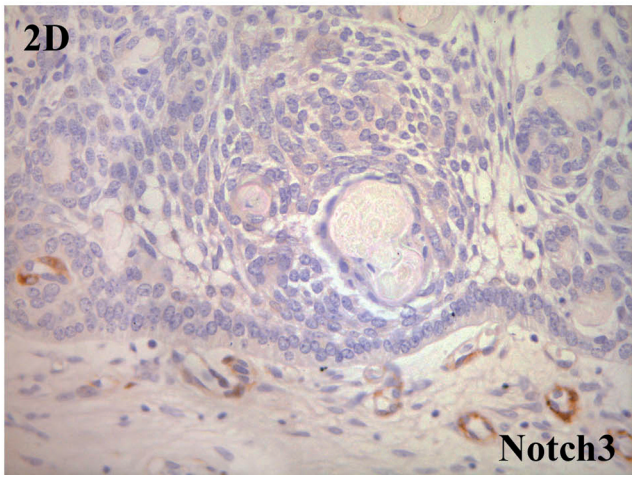


Fig. 1. Microscopic features of the calcifying cystic odontogenic tumor showing fibrous connective tissue wall lined by ameloblastomatous epithelium characterized by basal low columnar/cuboidal cells and suprabasal stellate reticulum-like cells with interspersed clusters of ghost cells (Hematoxylin & eosin, x100).

Fig. 2. Photomicrograph of CCOT lining epithelium and ghost cells demonstrating Notch receptor proteins: A and B, Notch1: positive expression was observed in both ghost cells and tumoral epithelium; C, Notch2: all lesional components were negative; D, Notch3: weak to negative labeling of tumoral tissues. Note strong Notch3 positivity in stromal blood vessels. (A, x100, B-D, x200).

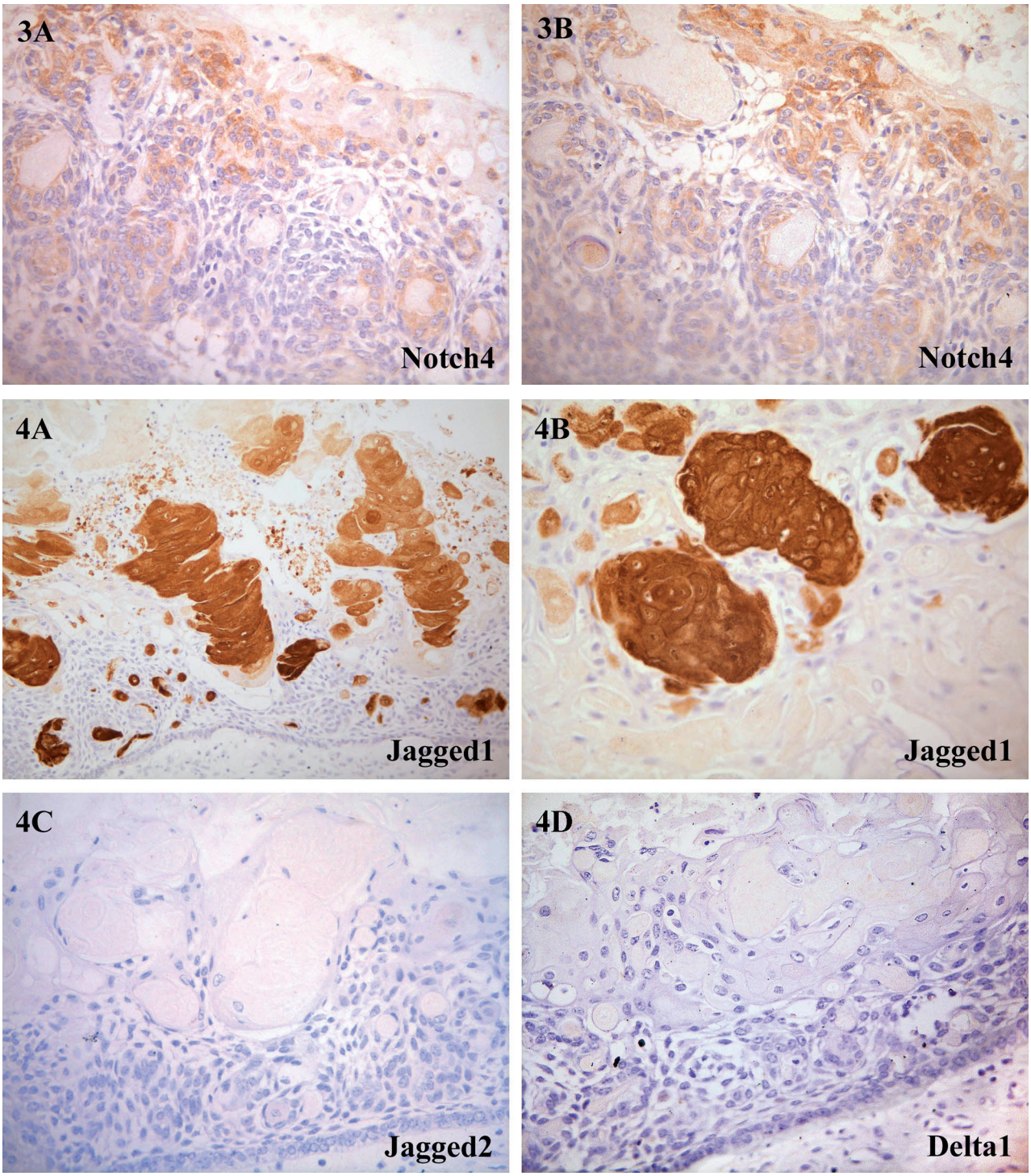


Fig. 3. Notch4: A and B, positive expression in CCOT epithelial and ghost cells; (A, x100; B, x200).

Fig. 4. Photomicrograph of CCOT lining epithelium and ghost cells demonstrating distribution of Notch ligand proteins: A and B, Jagged1: strong expression in CCOT ghost cells and mildly in squamous transitory cells and tumoral epithelium (A, x100; B, x200); C, Jagged2: no immunoreactivity was detected (x200); D, Delta1: no immunoreactivity was detected (x200).

cells and squamous transitory cells (Fig. 1). Foci of ghost cells with calcifications were also observed. Dentinoid and odontoma formation were not evident.

Immunohistochemical Findings

Results on the semiquantitative analysis of distribution and level of positivity for Notch receptors and ligands for all five cases of CCOT are summarized in Table 2 and illustrated in Figs. 2-4. Tumoral epithelium demonstrated mild positivity for Notch1 (Fig. 2A-B), Notch4 (Fig. 3A) and Jagged1 (Fig. 3B-C) but was nonreactive for Notch2 (Fig. 2C), Notch3 (Fig. 2D), Jagged2 (Fig. 4C), and Delta1 (Fig. 4D). In those immunoreactive cells, Notch receptor and ligand protein localization was cytoplasmic and/or membranous.

Ghost cells showed moderate labelling for Notch1 (Fig. 2A-B), mild expression for Notch4 (Fig. 3A-B) and overexpression for Jagged1 (Fig. 4A-B). Protein localization was also cytoplasmic and/or membranous. Transitory squamous cells were mildly positive for Notch1 (Fig. 2A-B), Notch4 (Fig. 3A-B) and Jagged1 (Fig. 4A-B). Both ghost cells and transitory squamous cells were not immunoreactive for Notch2 (Fig. 2C), Notch3 (Fig. 2D), Jagged2 (Fig. 4C) and Delta1 (Fig. 4D). Dentinoid and odontome were not present in all five cases of CCOT, and therefore Notch receptor and ligand distribution in these structures remained unknown. Stromal fibroblasts and blood vessels were stained variably positive.

Discussion

The calcifying odontogenic cyst is a developmental odontogenic lesion first recognized as a likely analogue of the calcifying epithelioma of Malherbe (pilomatrixoma) by Gorlin et al. in 1962 [8]. It is rare and accounts for 1-2% of all jaw cysts. Since its initial description, it became clear that the calcifying odontogenic cyst consists of two basic pathobiologic subsets: a cystic neoplasm and a solid tumor. In 2005, the World Health Organization updated its classification of odontogenic tumors [10], and reclassified the calcifying odontogenic cyst subdividing it into three distinct entities: calcifying cystic odontogenic tumor (CCOT), dentinogenic ghost cell tumor (DGCT) and ghost cell odontogenic carcinoma (GCOC) [10]. CCOT is a benign cystic odontogenic neoplasm, characterized by local invasiveness and rare recurrence following enucleation. DGCT resembles an ameloblastoma clinically and histopathologically except that large numbers of ghost cells and dysplastic dentin are present in DGCT. GCOC is a malignant odontogenic epithelial tumor characterized by a highly aggressive and recurrent biologic behavior. All five cases examined in this study belong to the CCOT category.

Other than our single CCOT case study that examined for aberrant Notch1 signaling [23], a search of the English language literature disclosed that the expression patterns of other Notch family members and their ligands in ghost cells remain unknown. The distinct histological appearance of the ghost cells in the CCOT and the critical role of Notch signaling pathway in odontogenesis led us to investigate further whether Notch signaling modulates the fate of ghost cells in this neoplasm.

Notch activation in cell fate decisions is known to operate via three distinct mechanisms: lateral inhibition, binary cell fate, and lateral induction. In the classic Notch 'lateral inhibition' cells with a given fate inhibit their neighboring cells from adopting the same fate. This means that signaling between Notch ligand and Notch receptor on an adjacent cell inhibits ligand production in the receiving cell through a negative feedback loop [24, 25]. In contrast, during binary cell fate decisions, distinct cell fates are determined by asymmetric distribution of Notch pathway components, such as the cytoplasmic Notch inhibitor, Numb. Finally, in lateral induction, which is the least well described mode of Notch action, signaling between Notch ligand and Notch receptor on adjacent cells results in a positive feedback, which promotes ligand expression and activation of Notch on both cells. This mechanism has been suggested to propagate Notch signals through a cell-to-cell relay mechanism [25, 26]. These various modes of signaling allow Notch to perform different functions within the same tissue in a spatially and temporally regulated manner. It is believed that the role of Notch is to block differentiation by maintaining the competence of undifferentiated cells [1]. This implies that Notch expression would preferentially be found in cell types that are not terminally differentiated. During odontogenesis, the determination of cell fates in the enamel organ occurs via inhibitory interactions between adjacent dental epithelial cells. Initially, cells of the forming dental epithelium appear to constitute a developmental equivalent group in which inner enamel epithelial cells suppress differentiation in their immediate neighbours through lateral inhibition [27]. In our study, we observed that ghost cells demonstrated co-expression for Notch1 and Jagged1 indicating that there is a likelihood that signaling might occur via lateral induction between Notch ligand and Notch receptor on adjacent ghost cells resulting in a positive feedback, which promotes ligand expression and activation of Notch on both these cells [25]. On the other hand, the adjacent tumoral epithelium only weakly expressed these molecules, and these observations suggest that probably Notch signal activation in ghost cells exerts a lateral inhibitory effect on the neigboring tumoral epithelium blocking them from adopting the same cell fate. Absence of Notch members (Notch2, and 3) and ligands (Jagged2 and Delta1) in both the ghost cells and tumoral epithelium implies that these molecules most likely play little or no role in the cytological differentiation and proliferation of this neoplasm.Notch3 positivity observed in the stromal blood vessels of CCOT indicate that Notch receptors and ligands are generated by different inductive mechanisms.

In summary, this study investigated the role of aberrant Notch signaling in ghost cell fate in five cases of CCOT. Our results suggest that Notch may play an oncogenic role in the tumorigenesis of CCOT where Notch1 signal activation induced by Jagged1 is necessary for specification of ghost cell fate.

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