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Notch Signaling in Benign and Malignant Ameloblastic Neoplasms

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Abstract

Background: In general, Notch is a representative signal which controls morphosis and differentiation of cells, but its role in human odontogenic neoplasms, especially in ameloblastoma and its malignant counterpart, ameloblastic carcinoma, is not known.

Methods: We examined Notch1 peptide and its gene (mRNA) in an ameloblastoma (case 1: 27-year-old female, right mandibular tumor) and an ameloblastic carcinoma (case 2: 93-year-old female, right mandibular tumor), using immunohistochemistry (IHC) and in situ hybridization (ISH) techniques.

Results: Notch1 intracellular domain (NICD) positive products were observed in the cells at the peripheral layer of most proliferating epithelial tumor nests in case 1. In case 2, positive products were similarly detected. In particular, small numbers of mitoses were identified in the nuclear region with intense NICD positive reaction.

Conclusions: Notch signaling plays some role in cytological differentiation or acquisition of tissue specific characteristics in neoplastic cells of odontogenic neoplasms, including ameloblastoma and ameloblastic carcinoma. Notch1 may also contribute to cell cycle arrest induced by Notch1 activation in ameloblastic carcinoma.

Key words: odontogenic; ameloblastoma; ameloblastic carcinoma; Notch; malignancy;

Introduction

Previously, we have reported examination results of immunohistochemical expression of some morphogenesis regulation factors, such as Notch1 and Runx2 collected from human neoplasm cases [1-3]. The results showed these morphogenesis regulation factors to be closely related to cytological differentiation in neoplastic cells. Furthermore, we have examined the expression of Notch1 and Jagged1 in a case of ameloblastoma, a typical tooth enamel organ-derived and well-differentiated benign neoplasm [4]. In general, Notch signaling is responsible for cytological regulation of cell fate, morphogenesis and/or development. To date, there has been no previous report on Notch1 signaling in ameloblastic carcinoma. The aim of the present study was to carry out detection of Notch1 peptides and genes (mRNA) using IHC and ISH in a case of ameloblastoma and a case of ameloblastic carcinoma, in order to obtain information and to speculate on the possible role of Notch signaling during odontogenic tumorigenesis. We hope to clarify whether these signaling molecules play important roles in cytological differentiation or acquisition of tissue specific characteristics in the neoplastic cells of these tooth enamel organ-derived neoplasms.

Materials and Methods

Materials

The examination materials were from the surgical pathology files of the Department of Oral Pathology, Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia. Histopathological diagnoses of the tumor entities were made and confirmed according to the WHO classification of odontogenic tumors [5, 6].

Case 1: A 27-year-old Malaysian female had noticed a swelling of her right lower jaw since 3 months ago. At the time of consultation, a multilocular radiolucent lesion associated with an embedded lower right third molar and involving the right ramus of mandible with extension anteriorly to the lower right first premolar region was observed. Examination of both the incisional biopsy and enucleated specimens led to the histopathological diagnosis of ameloblastoma.

Case 2: A 93-year-old Malaysian female complained of bleeding from the mouth since one month earlier. Pain was present at the same time. Extraoral examination revealed a hard swelling over the right mandible and a right submandibular lymphadenopathy was clinically evident. Intraoral examination showed an ulcerated lesion over the right buccal mucosa extending to the right floor of the mouth. Subsequent histopathological examination of the excised specimen led to a diagnosis of ameloblastic carcinoma.

Methods

Tissue preparation
Surgical materials were fixed in 10% neutral buffered formalin fixative. The materials were then dehydrated by passage through a series of ethanols and embedded in paraffin. Serial sections were made at 4μm, and examined by histopathology (stained by hematoxylin-eosin, HE), IHC and ISH techniques.

IHC
For IHC, deparaffinized sections were treated with methanol containing 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity. The sections were either autoclave heated in 10 mM of citrate buffer solution pH 3.3 for 10 min at 120 °C (for Ki-67) or pretreated with 0.1% pepsin solution at 37 °C for 15min (for NICD) for antigen retrieval. IHC examination was carried out using a DAKO EnVision™ + Kit-K4006 (Dako, Glostrup, Denmark) and 2 primary antibodies: anti-human Notch1 intracellular domain (NICD: 1/10) and anti-human Ki-67 (Ki-67: 1/200). The NICD monoclonal antibody developed by Atranvanis-Tsakonas [7, 8] was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development of the NIH and maintained by The University Iowa, Department of Biological Sciences, Iowa City, IA, USA. The Ki-67 monoclonal antibody was obtained from Lab Vision Corp (Fremont, CA, USA). DAB was applied for the visualization of IHC activity. For negative control, sections were incubated with omission of the primary antibody under the same protocol. Positive control sections were incubated for each antibody and internal staining controls, if present in the specimen, were checked for appropriate reactions with each antibody.

ISH
For ISH, deparaffinized sections were pretreated with 3mg/ml of proteinase K (Roche Diagnostics GmbH, Penzberg, Germany) in 10mM Tris-Hcl buffer (pH 8.0) and 1mM EDTA for 15 min at 37 °C. Acetylation of the sections was performed by incubation with 0.25% acidic anhydride in 0.1M triethanolamine-HCl buffer (pH 8.0) for 10 min at RT. Digoxigein (DIG)-labeled single-strand RNA probe were prepared using a DIG RNA Labeling Kit (Roche Diagnostics GmbH, Penzberg, Germany). For Notch1, single-strand RNA probe (base 5398-6160 of the total cDNA: Gene Bank Accession # NM_017617) was obtained. The hybridization solution contained 50% formamide, 10% dextran sulfate, 1x Dehardt's solution, 600mM Nacl, 0.25% SDS, 250 mg/ml of *E. coli* tRNA, 10mM dithiothreitol, and 0.1 to 2.0 mg/ml of DIG-UTP labeled RNA probe. The probe was place on the sections and incubated at 50 °C overnight in a moist chamber. Signal detection was carried out using DIG DNA Labeling and Detection Kit (Roche Diagnostic GmbH, Penzberg, Germany) according to the manufacturer's instructions. After hybridizaton, anti-DIG-AP antibody (1:1000) in DIG1 buffer was applied to the sections and incubated for 1hr at RT. NBT was applied for the visualization of RNA probe on the sections. The negative controls included hybridization with sense RNA probe.

Results

Histology

In case 1, the tumor nests consisted of islands of odontogenic epithelium with peripheral columnar ameloblast-like cells and central stellate reticulum-like cells disposed in a follicular growth pattern in the mature fibrous connective tissue stroma (Fig. 1). In some nests, parenchymal cyst formations or squamous metaplasia were observed. These histopathological features were compatible with follicular ameloblastoma.

In case 2, the tumor nests of cancer cells exhibited increased nuclear cytoplasmic ratio and prominent nuclei. Proliferating polyhedral neoplastic cells showed strong cellular atypism, such as mitoses and pleomorphism, especially in the peripheral layers of the nests (Fig. 2). These histopathological features were compatible with ameloblastic carcinoma.

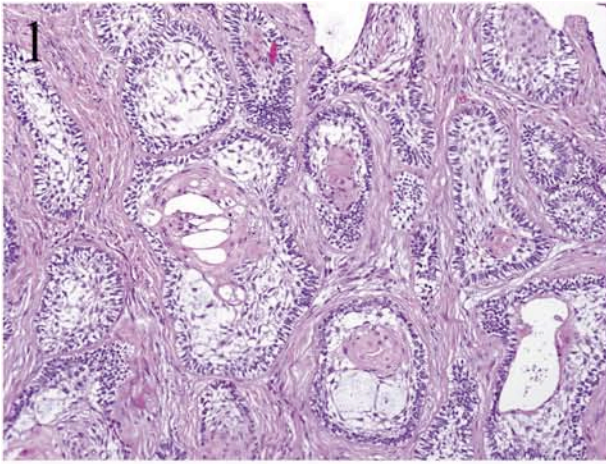
IHC
In case 1 of ameloblastoma, NICD-positive IHC reactions were detected in most proliferating odontogenic epithelial cells within the peripheral layer of the tumor nests (Fig. 3). In the budding areas of epithelial nests, positive reactions of NICD were especially strong (Fig. 3). These IHC positive reactions were observed in the cytoplasm and/or nucleus. In case 2 of ameloblastic carcinoma, NICD strong positive reactions were detected in most neoplastic cells. Positive reactions tended to be strong in the peripheral columnar or cuboidal cells and to be moderate in the central polyhedral cells (Fig. 4). Immunohistochemically, there were two different regions of stainability in the same tumor nests. One was the region the cytoplasm was densely stained (Fig. 4), the other was the nucleus was strongly stained (Fig. 5). In addition, large numbers of mitotic figures were detected in the former (Fig. 4), a small number of mitotic figures in the latter (Fig. 5).

In the case of ameloblastoma, a few Ki-67 positive cells were observed in proliferating tumor nests. In contrast, in the case of ameloblastic carcinoma, an increased number of Ki-67 positive cells were evident in almost all epithelial tumor nests (Fig. 6).

ISH
Notch1 gene expressions were detected in the cytoplasm of immnohistochemically NICD positive neoplastic cells (Figs. 7 and 8). In the case of ameloblastoma, Notch1 gene expression was mainly observed in the budding areas and in the peripheral cells of epithelial tumor nests (Fig. 7). In the case of ameloblastic carcinoma, Notch1 gene expression was detected in most neoplastic cells.

Discussion

Notch is a single-pass transmembrane receptor, activated by direct contact with membrane-bound ligands Delta 1 to 4 and Serrate/Jagged 1 and 2.9 Mammalian Notch gene encodes 300 kDa single pass transmembrane receptor, which are expressed on the surface of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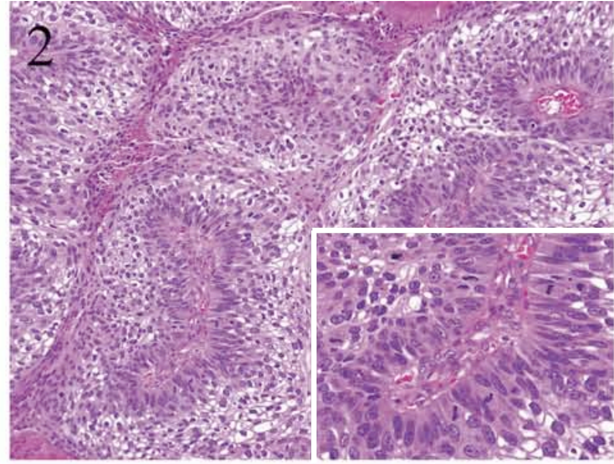
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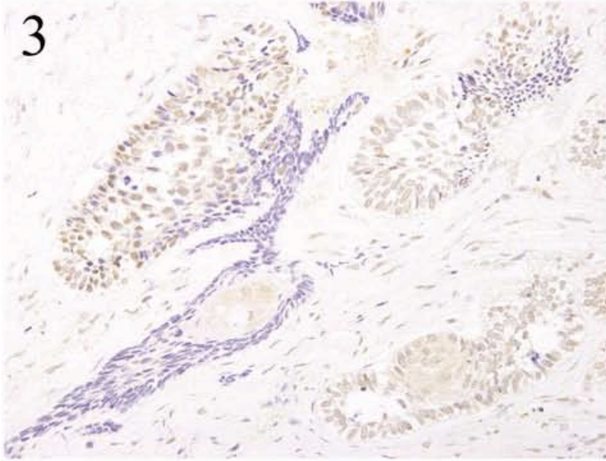
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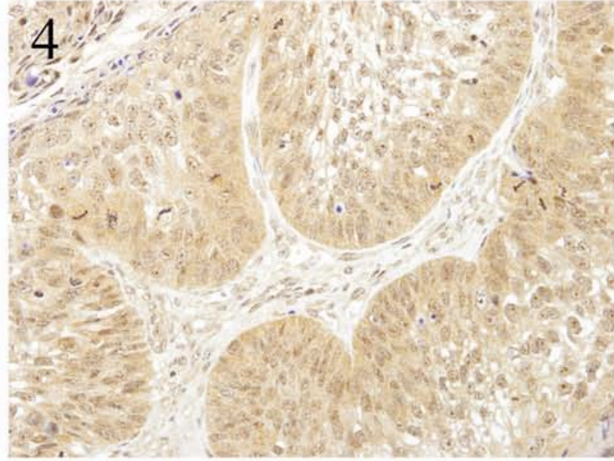
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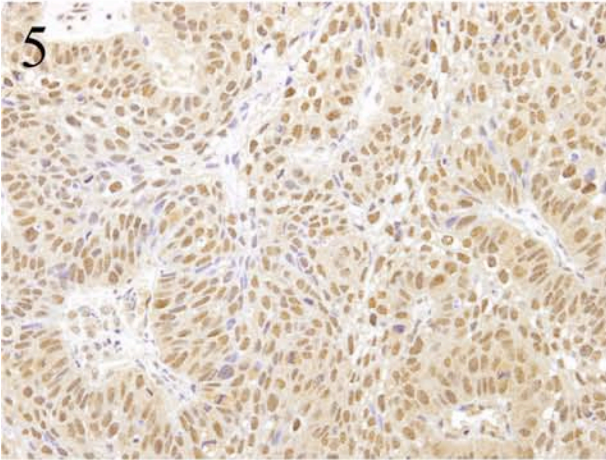
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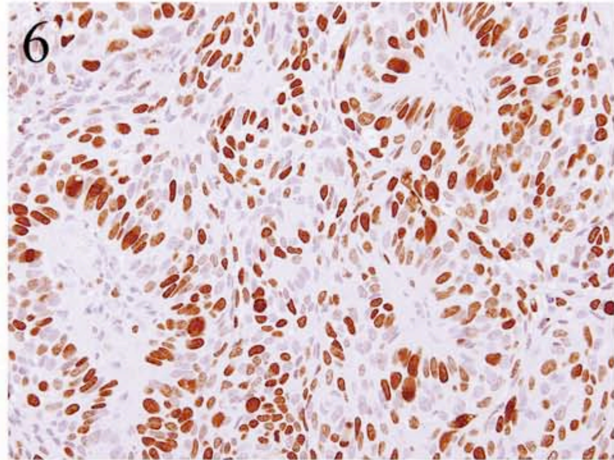
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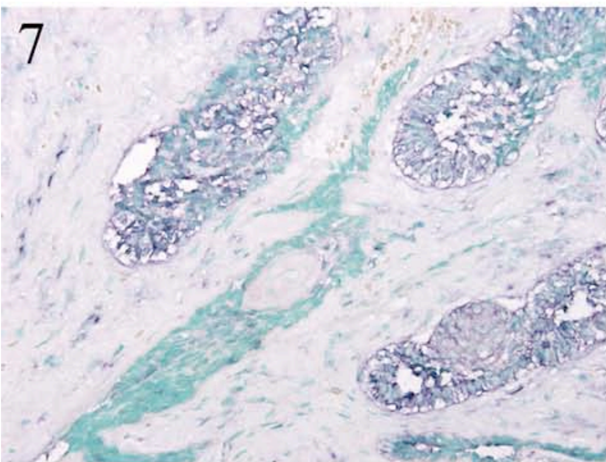
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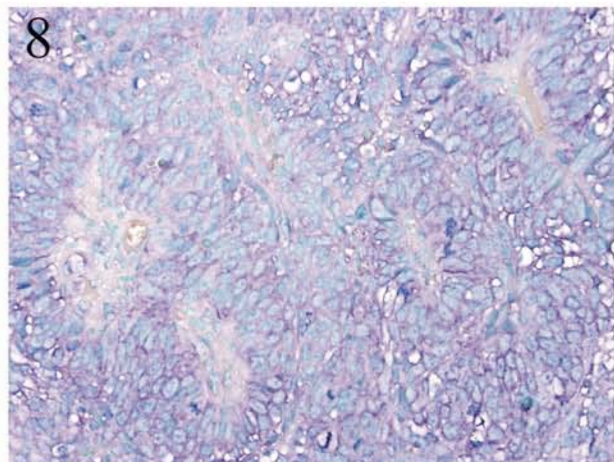
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neighboring cells [10, 11]. Receptor-ligand interaction is followed by cleavage events by the γ-secretase complex that contains the membrane proteins, presenilin and nicastrin. The cleavage event releases the soluble intracellular domain of Notch (NICD). After that, NICD translocates to the nucleus and binds to the transcriptional regulator CSL (for CBF-1, Su(H), and LAG-1), resulting in displacement of corepressors previously bound to CSL and recruitment of coactivators. Notch signaling is involved in a variety of cell functions such as specification, proliferation and apoptosis, which affect the development and function of many organs [12-14].

In the oral and craniofacial regions, Notch signaling plays an important role for early embryonic development of mandibular condylar cartilage formation. Furthermore, transdifferentiation of hypertrophic chondrocytes to osteoblasts is regulated [15]. Regulation of the Notch pathway is also important for maintaining the correct balance among cell proliferation, differentiation and apoptosis of embryonic tooth development [16]. During tooth development, Notch expression has been associated with the differentiation of odontogenic epithelial and mesenchymal tissues [17].

Ameloblastoma is one of the most frequently encountered tooth enamel organ-derived neoplasms. It is clinically characterized as a benign but locally invasive tumor with a high recurrence risk. The prognosis, in general, is good, but long-term follow up is essential, as recurrence has been noted to occur more than ten years after initial treatment [5]. Histopathologically, the follicular type of ameloblastoma is the most common, consisting of proliferating odontogenic epithelial islands and nests in the fibrous stromal tissues. Cellular modifications, such as squamous metaplasia, keratin pearl formation, parenchyma cell degeneration and cystic changes, may also occur. These histopathological features were likewise observed in our current case of ameloblastoma.

In case 1 of ameloblastoma, our IHC results demonstrated intense NICD positive reactions in the peripheral layer and budding areas of the tumor nests. It is known that during tooth development, Notch expression is regulated by odontogenic epithelial and ectomesenchymal interactions. However, ameloblastoma is an odontogenic tumor that consists of only odontogenic epithelial components without odontogenic ectomesenchyme [5]. It is thought that there are no interactions of odontogenic epithelium and ectomesenchyme in ameloblastoma. Therefore, our data indicate that Notch signaling is activated in the neoplastic epithelium of ameloblastoma in the absence of ectomesenchymal participation.

In the ameloblastoma, the columnar ameloblast-like cells were located in the peripheral regions of tumor nests. Budding areas of the tumor nests resembled tooth germ of the bud stage. These morphological characteristics closely resembled the tooth germ differentiation and development. Furthermore, NICD positive cells were localized in these areas. The data suggest that Notch1 may contribute to the acquisition of morphological characteristics of ameloblastoma.

Ameloblastic carcinoma is a rare malignant odontogenic tumor. This neoplasm combines the histological features of ameloblastoma with strong cytological atypism. The characteristics are tall columnar cellular morphology with pleomorphism, mitotic activity, focal necrosis and nuclear hyperchromatism. Atypical cells form nests and broad ribbons with focal areas of subtle necrosis to more obvious central, comedo necrosis-like areas. Ameloblastic carcinomas show a high proliferation index compared to ameloblastomas by virtue of an increased index of proliferating cell nuclear antigen in addition to higher levels of aneuploidy [6]. These histopathological features were observed in the current case of ameloblastic carcinoma.

There are some reports on the function of Notch signaling in malignant tumors. Notch signaling in cultured small cell lung cancer cells results in a profound G_1 cell cycle arrest induction, and induction of the downstream ras signaling pathway [18]. Over expression of active Notch1 inhibits the proliferation of various prostate cancer cells [19]. These published data suggest that Notch activation can also induce growth arrest and apparently reduce the neoplastic potential of tumors. However, there has been no report on the function of Notch in the odontogenic carcinoma. In our case of ameloblastic carcinoma, there were some regions in which the nucleus was strongly positive to NICD. This finding indicates NICD translocates to the nucleus. In these regions, a small number of mitotic figures were present. In addition, large numbers of Ki-67 positive cells were also observed. Ki-67 is a nuclear protein, which is expressed in proliferating cells (G_1, S, M and G_2-phase). In other words, there are small numbers of mitoses in spite of the presence of proliferating cells. Overall, these data suggest that the translocation of Notch to the nucleus may contribute to cell cycle arrest induced by Notch1 activation in ameloblastic carcinoma.

◀ *Fig. 1*. Follicular ameloblastoma, identified by nests of odontogenic epithelium with peripheral columnar ameloblast-like cells proliferating in the mature fibrous connective tissues (Case 1, HE, x 40).

Fig. 2. Neoplastic cells with increased N/C ratio and and polyhedral neoplastic in the proliferating tumor nests (Case 2, HE, x 40). Mitoses and strong atipism are shown in insert photo (Case 2, HE, x 100).

Fig. 3. Positive reactions in the budding and peripheral layer of epithelial nests (IHC, NICD, x 100).

Fig. 4. Positive products in the cytoplasm of neoplastic cells. Many mitotic figures are evident (IHC, NICD, x 100).

Fig. 5. Positive products in the nucleus of neoplastic cells (IHC, NICD, x 100).

Fig. 6. Ki-67 positive cells are evident in most epithelial tumor nests, nearly the same area shown in Fig. 5. (IHC, Ki-67, x 100).

Fig. 7. Gene expressions mainly in the budding areas and peripheral columnar cells of epithelial tumor nests, nearly the same area shown in Fig. 3. (ISH, Notch1, x 100).

Fig. 8. Gene expressions in most neoplastic cells, nearly the same area shown in Fig. 5. (ISH, Notch1, x 100).

Our results demonstrated that NICD was localized in tumor cells of both ameloblastoma and ameloblastic carcinama. Our data indicated that Notch 1 signaling is activated in the neoplastic epithelium, and this event is common to both ameloblastoma and ameloblastic carcinoma. However, there are functional differences of NICD in both tumors. In ameloblastoma, Notch1 signaling is mainly related to the acquisition of morphological characteristics of tumorigenesis. However in ameloblastic carcinoma, Notch1 may contribute to cell cycle arrest induced by Notch1 activation.

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