Abstract. Background: Ameloblastin (AMBN) gene expresses an important protein that acts as a cell adhesion molecule. This protein plays an important role in maintaining the ameloblast secretory stage of differentiation by binding to them and inhibiting their proliferation. Due to the relationship of this protein in the differentiation and proliferation of odontogenic cells, here, we investigated this gene in different types of odontogenic tumors. Materials and Methods: Sequencing of the all encoding region of AMBN gene was carried out in four frozen cases of odontogenic tumors: one case of calcifying epithelial odontogenic tumor (CEOT), two calcifying odontogenic cysts (COC) and one ameloblastic fibroma (AF). Results: DNA sequencing was modified in an important domain of the AMBN only in the CEOT. Conclusion: The present study suggests that AMBN gene alterations may be relevant to the pathogenesis of CEOT.

The presence of odontogenic epithelium and/or odontogenic ectomesenchyme is important in the classification of odontogenic tumors. The calcifying epithelial odontogenic tumor (CEOT) is a benign epithelial odontogenic tumor characterized by a locally invasive behavior and affects individuals ranging from 30 to 50 years of age (1). However, the molecular mechanisms associated with its development have not been established.

Ameloblastic fibroma (AF) and calcifying odontogenic cyst (COC, calcifying cystic odontogenic tumor according to WHO’s classification of 2005) are tumors that contain odontogenic epithelium and odontogenic ectomesenchyme (2). AF occurs mainly in the first and second decades of life, affecting mandible rather than the maxilla (3). COC is a cystic odontogenic tumor with an epithelial lining containing ghost cells which may undergo calcification, closely resembling an ameloblastoma (4). Although the underlying genetic alterations are poorly understood in AF, mutations in the β catenin gene were described in COC (5).

Some proteins of the enamel matrix have important roles in initiating cytodifferentiation of the dental tissue (6). Ameloblastin (AMBN) is the most important protein involved in these processes and is highly expressed during the differentiation of inner enamel epithelium (6, 7). The AMBN protein is a cell adhesion molecule essential for amelogenesis. It is localized near the cell surface and helps maintaining the ameloblast secretory stage of differentiation by binding to them and inhibiting their proliferation (8). The human AMBN gene has been cloned and comprises a single-copy gene containing 13 exons with an open reading frame of 1,341 bp that encodes 447 amino acid 11 and maps to chromosome 4q21 (9).

We have previously demonstrated that AMBN gene mutations are associated with the development of ameloblastoma, adenomatoid odontogenic tumor and squamous odontogenic tumor (10). As AMBN protein has an important role in the differentiation of ameloblasts and epithelium-mesenchyme signaling during odontogenesis, we prompted to investigate this gene in CEOT, COC and AF.

Materials and Methods

In this report, we studied one CEOT, two COC and one AF. Fragments of these tumors were obtained during surgical removal procedure, after an informed written consent was signed by all patients. The University Ethics Committee approved this work. One fragment was fixed in 10% formalin buffer and paraffin-embedded tissue blocks were used for routine histological staining to confirm the clinical diagnosis. All four tumors studied showed typical histological features described in the literature (2, 4, 11). Specimen samples were immediately stored at –70°C for subsequent molecular analyses. In addition, contra-lateral oral

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mucosa swabs were taken from each patient for DNA extraction followed by DNA sequencing. DNA extraction was carried out as previously described and all coding regions of the AMBN gene studied using specific PCR reaction conditions as previously described (10, 12). Briefly, PCR reactions were performed in a final volume of 50 μl containing 100 ng of template DNA, 200 μmol/l dNTPs, 10 pmol/l of each primer and 1.25 U of proofreading taq DNA polymerase (Platinum® Taq DNA Polymerase High Fidelity; Invitrogen, USA). Thirty-five cycles of amplification were performed in a PTC-100-60 thermocycler (MJ Research, Watertown, MA, USA) with the appropriate parameters. DNA sequencing reactions were carried out using the BigDye terminator kit and DNA Sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing was performed in duplicate, both strands, from different amplification products. Mutation nomenclature follows published guidelines (13). Numbering of nucleotide and aminoacid refer to the complete cDNA sequence (GenBank: NP 057603 and NM 0165194).

Results

Sequencing analyses demonstrated that the AMBN gene was altered in only one of the four tumors investigated. The CEOT presented a heterozygous mutation, with the exchange of two base pairs in exon 6 of the AMBN (Figure 1). These alterations were somatic in origin because of their absence in the matching germline DNA obtained from the contralateral oral mucosa. The mutation was characterized by two consecutive changes of nucleotide, a transversion (338A>T) and a transition (339G>A) according to GenBank: NM 0165194. These mutations cause the exchange of the glutamine to leucine (Q113L) in an important and conservative phosphorylation site PPLPSQPSL (Figure 2).

To predict whether the amino acid substitution will affect protein function, we used the algorithm Sorting Intolerant from Tolerant (SIFT) (http://blocks.fhcrc.org/sift/SIFT.html). A SIFT score less than 0.05 is considered deleterious. In the present study, the amino acid substitutions Q113L showed a SIFT score of 0.03. The SIFT scores showed that the amino acid substitutions in the AMB protein were deleterious, affecting protein function.

Discussion

COC represents about 1% of jaw cysts and although it may occur in soft tissue, it presents more commonly within the bone (14). Both the intra- and extraosseous forms occur with equal frequency in the maxilla and mandible, mainly in the incisor and canine areas (15). To date, available data show that β-catenin mutation is the main molecular alteration in COC (5). AF is a benign neoplasm of the odontogenic apparatus that occurs mainly in the first and second decades of life (3). Whilst the majority of AFs are true neoplasms with a potential to recur, some of them could represent the primitive stage of a developing odontoma (16). In the present study, none of these tumor types demonstrated molecular alterations of the AMBN gene. Although COC and AF present some histological similarities to ameloblastoma, such as the presence of epithelial basal layer of palisade columnar cells and loosely arranged cells resembling stellate reticulum, AMBN gene mutations are restricted to the ameloblastoma (10).

Considering that AMBN has an important role in ameloblast maturation to the secretory phase, we speculate that the alteration of this protein in ameloblastoma, not found in COC and AF, could be related to the absence of an inductive effect on the former. This inductive effect is sometimes reported in COC and AF and is characterized by dentinoid formation and juxtaepithelial hyalinization, respectively (17).

Many case reports of CEOT have been described. This tumor is characterized by the presence of islands and sheets of polygonal cells with distinct cellular bridges (4). Although the CEOT shows eosinophilic amyloid-like material, no dentin formation is found. In the present study, we demonstrated a heterozygous mutation in exon 6 of the AMBN in the single CEOT studied. The mutation found led to the exchange of CAG to CTA. The alteration of the DNA modifies the mRNA and AMBN protein, leading to an exchange of glutamine, a neutral and polar hydrophilic amino acid, to leucine, a nonpolar and therefore a hydrophobic amino acid (Q113L). This mutation occurred in an important phosphorylation site and in a proline-rich region PPLPSQPSL. However, functional
studies of these mutations have not been performed, their association with disease, their location in areas of the protein highly conserved between species (Figure 2) and, most important, by their absence in normal mucosa suggest that they are pathogenic.

Areas of eosinophilic cells with prominent intercellular bridges similar to those observed in CEOT have been described in a few cases of adenomatoid odontogenic tumors (4). In a previous study, we demonstrated the presence of AMBN gene mutations in adenomatoid odontogenic tumor suggesting that both lesions share at least some common genetic pathways (10). Additional genetic or epigenetic damage in CEOT could explain its distinct local invasiveness growth.

Up-to-date molecular biology features have not been used in the classification of odontogenic tumors. The development and progression of odontogenic tumors are affected by alterations of many genes and molecules that have been recently reviewed (18). Understanding the underlying molecular mechanisms will help to predict the course of odontogenic tumors and the development of new therapeutic targets for these lesions (18). It is important to note that until now, all odontogenic tumors that had AMBN gene mutations, i.e. CEOT, ameloblastoma, adenomatoid odontogenic tumor and squamous odontogenic tumor, belong to the group of odontogenic epithelium with a mature, fibrous stroma, and without odontogenic ectomesenchyme. On the other hand, tumors with odontogenic epithelium and odontogenic ectomesenchyme (COC and AF) studied in the present investigation, albeit in reduced numbers, did not show AMBN gene alterations.

In conclusion, the present study together with the literature show that odontogenic tumors with a mature, fibrous stroma, and without odontogenic ectomesenchyme present AMBN gene alteration. Further studies are necessary to demonstrate the impact of this alteration on the tumorigenesis of this group of lesions.

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