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# Immunohistochemical assessment of hypoxia-inducible factor-1α (HIF-1α) and carbonic anhydrase IX (CA IX) in ameloblastomas

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ABSTRACT

*Objective:* To investigate roles of hypoxia-related proteins in odontogenic tumors, we analyzed the immunohistochemical expression of hypoxia-inducible factor-1α (HIF-1α) and carbonic anhydrase IX (CA IX) and compared with angiogenesis.
*Methods:* 10 dental follicles and 67 ameloblastomas were immunohistochemically examined with antibodies against HIF-1α, CA IX, and CD34.
*Results:* Immunohistochemical reactivity for HIF-1α and CA IX was detected in odontogenic epithelial cells, as well as in several macrophages and fibroblasts, in dental follicles and ameloblastomas. HIF-1α was positive in 8 of 10 dental follicles, 45 of 48 primary ameloblastomas, and all 19 recurrent ameloblastomas. Increased HIF-1α reactivity was often found in keratinizing cells in acanthomatous ameloblastomas. Immunoreactivity for CA IX was detected in all samples of dental follicles and primary and recurrent ameloblastomas. CA IX reactivity was significantly higher in ameloblastomas than in dental follicles, in solid ameloblastomas than in unicystic ameloblastomas, and in follicular ameloblastomas than in plexiform ameloblastomas. Acanthomatous ameloblastomas showed increased CA IX reactivity around keratinizing areas, while granular cell ameloblastomas showed increased reactivity in peripheral non-granular cells. Microvessel density (MVD) of CD34-positive capillaries in solid ameloblastomas was significantly higher than in unicystic ameloblastomas. MVD tended to be greater in follicular ameloblastomas than in plexiform ameloblastomas, in granular cell ameloblastomas than in other subtypes of ameloblastomas, and in mural unicystic ameloblastomas than in other types of unicystic ameloblastomas.
*Conclusions:* Our data suggest that hypoxia-related molecules and angiogenesis play a role in tumorigenesis, tissue structuring, cell differentiation, and prognosis of ameloblastomas in the intraosseous microenvironment.
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## 1. Introduction

Ameloblastoma is the most frequently encountered epithelial odontogenic tumor arising in the jawbones, and is characterized by benign but locally invasive behavior with a high rate of recurrence. Therefore, clinical long-term follow-up is essential, and the required treatment includes excision with an adequate margin of uninvolved tissues, similar to that for malignant tumors [1,2]. This tumor is usually solid and unicystic. Histologically, solid ameloblastomas show follicular and plexiform proliferation patterns with or without acanthomatous and granular cell changes, and unicystic ameloblastomas have luminal, intraluminal, and mural variants [1]. In some respects, these epithelial odontogenic tumors histologically resemble physiological structures, such as enamel organ or dental lamina; however, the detailed mechanisms of oncogenesis and cytodifferentiation remain unknown.

Hypoxia is thought to trigger a more aggressive tumor phenotype by inducing genomic instability, loss of apoptotic potential, and angiogenesis, which is the sprouting of new capillaries from a pre-existing vascular bed [3–5]. It plays a significant role in tumor recurrence, metastasis, and poor response to treatment, including radiotherapy, chemotherapy, and antiangiogenic treatment [5,6].

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Recently, molecules involved in the hypoxic response of tumor cells were identified as endogenous hypoxia markers using non-invasive and cost-effective methods to determine the condition of tumor hypoxia [7–10]. Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric protein consisting of an alpha and beta subset, in which the α subset mediates HIF-1 function as a transcription factor in response to cellular hypoxia [11]. Expression of HIF-1α correlates with advanced tumor stage and metastasis, leading to poor outcomes of common human malignancies, such as carcinomas of the breast, brain, lung, prostate, and head and neck [8–10,12–16].

Carbonic anhydrase IX (CA IX) is one of the HIF-1α-dependent enzymes most consistently upregulated under hypoxic conditions. Since there is an oxygen-dependent step that inhibits HIF-1α transactivation of target genes, it would be anticipated that CA IX protein levels, like other target gene products, would show a better correlation with low oxygen levels than would HIF-1α itself [4,7,17]. The effect of CA IX on the tumor microenvironment is characterized by the regulation of pH [18]. Recently, some human tumors have been reported to express CA IX, which is proposed to influence the clinical features of these tumors [4,5,7–9,16]. Thus, HIF-1α and CA IX play a role in angiogenesis, apoptosis, invasion, and metastasis of malignant tumors [8,9].

Our previous studies have confirmed the presence of angiogenic factors, vascular endothelial growth factor (VEGF), thymidine phosphorylase, and angiopoietins, and apoptosis-inducing molecules, p53, Bax, BH3-only proteins, and TNF family molecules, in ameloblastic tumors [19–24]. However, hypoxia-related factors in epithelial odontogenic tumors remains poorly understood. In the present study, HIF-1α and CA IX expression, as well as CD34 as a marker of microvessel density (MVD), were immunohistochemically examined in ameloblastomas as well as in dental follicles to clarify the role of these hypoxic agents in epithelial odontogenic tumors.

## 2. Material and methods

The study protocol was reviewed and approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry (No. 2014-1-854).

### 2.1. Tissue preparation

Specimens were surgically removed from 63 patients with ameloblastoma at Tohoku University Hospital and affiliated hospitals. The specimens were fixed in 10% buffered formalin for one to several days and were embedded in paraffin. The tissue blocks were sliced into 3 μm-thick sections for routine histological and subsequent immunohistochemical examinations. Tissue sections were stained with hematoxylin and eosin for histological diagnosis according to the World Health Organization histological classification of odontogenic tumors [1]. The specimens consisted of 48 primary ameloblastomas and 19 recurrent ameloblastomas. Of the primary ameloblastomas, 37 were solid, and 11 were unicystic. The solid ameloblastomas were divided into 19 follicular and 18 plexiform types, including 11 acanthomatous and three granular cell subtypes. The unicystic ameloblastomas were divided into five luminal, three intraluminal, and three mural types. All recurrent ameloblastomas were solid and were divided into 13 follicular and 6 plexiform types, including 10 acanthomatous and 1 granular cell subtypes. Specimens of 10 dental follicles of the mandibular third molars were similarly prepared and compared with the ameloblastomas.

### 2.2. Immunohistochemistry for HIF-1α, CA IX, and CD34 expression

Tissue sections were deparaffinized and immersed in methanol with 0.3% hydrogen peroxide. Sections were heated in 0.01 M citrate buffer (pH 6.0; for HIF-1α) or 1 mM EDTA buffer (pH 9.0; for CA IX) for 10 min by autoclaving (121 °C, 2 atm). Next, the sections were incubated with primary antibodies at 4 °C overnight. The antibodies used were as follows: rabbit anti-HIF-1α monoclonal antibody (Abcam, Cambridge, UK; isotype IgG; diluted at 1:100), rabbit anti-CA IX polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; purified IgG; diluted at 1:50), and mouse anti-CD34 monoclonal antibody (Nichirei, Tokyo, Japan; isotype IgG1; prediluted). The sections were allowed to react with peroxidase-conjugated anti-rabbit IgG (for HIF-1α and CA IX) or anti-mouse IgG (for CD34) polyclonal antibodies (Histofine Simple Stain MAX-PO; Nichirei, Tokyo, Japan) for 45 min, and reaction products were visualized by immersing the sections in 0.03% diaminobenzidine solution containing 2 mM hydrogen peroxide for 3–5 min. Nuclei were lightly stained with Mayer's hematoxylin. For control studies of the antibodies, the serial sections were treated with phosphate-buffered saline, normal rabbit IgG, and mouse anti-desmin monoclonal antibody (Nichirei; isotype IgG1) instead of the primary antibodies; these were confirmed to be unstained.

### 2.3. Evaluation of immunostaining and statistical analysis

Immunohistochemical reactivity for HIF-1α was evaluated and classified into three groups: (−) negative in epithelial or tumor cells, (±) weakly positive (less than 30% of epithelial or tumor cells), and (+) moderately to strongly positive (more than 30% of epithelial or tumor cells) [14]. Immunohistochemical reactivity for CA IX was evaluated and classified into two groups: (+) diffusely positive in most epithelial or tumor cells, and (++) diffusely positive in most epithelial or tumor cells with increased reactivity in those epithelial or tumor cells near the basement membrane.

Immunoreactivity for CD34 was conducted to evaluate MVD in the dental follicles and ameloblastomas. After scanning 5 areas showing the highest neovascularization (vascular hotspots) in normal mesenchymal tissues or tumor stromal tissues at 20-fold magnification, CD34-positive vessels were counted in the hotspots at 200-fold magnification, and the average count was recorded as MVD for each case [19,25,26]. Based on the criteria of Weidner et al. [27], a highlighted endothelial cell or a cell cluster clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements was regarded as a distinct countable microvessel. A lumen was not required, nor was the presence of red blood cells. Single cell sprouts were included in the counts.

The statistical significance of the differences in the percentage of cases with various HIF-1α and CA IX reactivity levels, as well as mean MVD, was determined by the Mann-Whitney *U*-test for differences between two groups or the Kruskal-Wallis test for differences among three or more groups. *P*-values <0.05 were considered to indicate statistical significance.

## 3. Results

The results of immunohistochemical studies of HIF-1α and CA IX expression and MVD in dental follicles and ameloblastomas are summarized in Table 1. Immunohistochemical reactivity for HIF-1α was detected in the cytoplasm and nuclei of odontogenic epithelial cells as well as in several macrophages and fibroblasts in the dental follicles and ameloblastomas (Fig. 1). Dental lamina showed HIF-1α reactivity in 8 of 10 dental follicles (Fig. 1A, Table 1). In primary ameloblastomas, tumor cells showed HIF-1α

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