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Disclosure of conflict of interests

The authors state that they have no conflict of interests.

Abstract

Aims: Ameloblastoma (AME) is a benign tumor characterized by local invasiveness, high recurrence rates and diverse histological patterns. Oxygen concentration is reduced in specific areas of the tumor microenvironment, which leads to intratumoral hypoxia. Crosstalk between NOTCH1, ADAM-12, HIF-1α and HB-EGF under hypoxic conditions has been implicated in invadopodia formation, tumor invasiveness and metastasis development. The objective of this

study was to analyze the expression of these proteins to further elucidate mechanisms underlying AME invasiveness.

Methods and results: A total of 20 cases of AME, 8 cases of calcifying cystic odontogenic tumors (CCOTs) and 10 samples of dental follicle were used to investigate the expression of these proteins by immunohistochemistry using the primary antibodies anti-NOTCH1, anti-ADAM-12, anti-HIF-1α and anti-HB-EGF. Immunostaining results were expressed as the percentage of stained area in images acquired in an AxioScope microscope equipped with an AxioCamHRc camera and a 40× objective. The results showed that immunoexpression of all proteins were higher in the AME samples than in the CCOT and dental follicle samples ($p<0.05$).

Conclusions: AME exhibited increased presence of proteins associated with tumor invasiveness, which indicates a possible role of these proteins in the biological behavior of this tumor.

Keywords: Ameloblastoma, Invadopodia, Cellular Hypoxia, NOTCH1, ADAM-12, HIF-1α, HB-EGF

INTRODUCTION

Ameloblastoma (AME) is a common benign odontogenic tumor with local aggressive and invasive behavior, with high rates of recurrence and morbidity.[1-3] Studies have been conducted to elucidate the mechanisms associated with cellular invasiveness and to characterize the tumor microenvironment of this neoplasia.[4-6]

During AME progression, mitogens are randomly released and promote the secretion of metalloproteinases (MMPs) that induce localized proteolysis of the extracellular matrix (ECM).[4,5,7] Recent studies revealed that localized proteolysis is mediated by invadopodia,[8,9] which are finger-like cellular protrusions that have intrinsic proteolytic activity and are associated with early tumor invasion and activation of MMPs.[10,11] Cortactin and membrane type-1 matrix metalloproteinase (MT1-MMP) have been described in ameloblastoma.[6]

It was recently reported that low-oxygen microenvironments favor the formation and activity of invadopodia.[12-14] During tumor progression, oxygen concentration in the microenvironment of tumor cells is reduced, which leads to intratumoral hypoxia.[13] Hypoxia is

associated with metastasis development, cell invasion[15] and potentializing the formation of invadopodia in tumor epithelia.[12]

Some proteins and transcription factors are related to autocrine and paracrine signaling pathways induced by hypoxia, such as the hypoxia-inducible factor 1-alpha (HIF-1α). HIF-1α activation has an essential role in the invasive process by regulating specific genes involved in cell motility, adhesion, and invasion.[16,17]

A disintegrin and metalloproteinase 12 (ADAM-12) is a protein that is involved in the formation of invadopodia under hypoxic conditions. An extensive number of studies have addressed the relationship between ADAM-12 and specific malignant tumors,[18-20] which indicates a direct correlation with the pathogenicity of these tumors. One study reinforced that this protein is involved in invadopodia formation in hypoxic microenvironments.[12]

Heparin-binding epidermal growth factor (HB-EGF) is also likely to have a role in promoting the aggressiveness of AME. HB-EGF is a potent mitogen and chemotactic for different cell types.[21,22] HB-HGF participates in the formation of invadopodia.[12] In addition, ADAM-12 is responsible for HB-EGF shedding.[23] Cellular stress caused by inflammatory cytokines, reactive oxygen species and osmotic shock can also induce the release of HB-EGF.[24]

Another key protein involved in the mechanism of invadopodia formation is the signaling protein NOTCH1. The result of NOTCH1 signaling determines whether cell differentiation is promoted or inhibited and largely depends on the tumor microenvironment and crosstalk with other signaling pathways.[25] The NOTCH1 signaling pathway can be activated by hypoxia, because under this conditions, transcription factor HIF-1α is activated and allows stabilization of its intracellular response.[26-28,12]

The tumor microenvironment is an important regulator of invasive behavior and may help reveal associated molecular mechanisms and thereby improve AME diagnosis and treatment approaches. Therefore, the objective of this study was to analyze the expression of the proteins NOTCH1, HIF-1α, ADAM-12, and HB-EGF in AME to further elucidate the early phase of the invasion cascade and its relationship with hypoxia.

MATERIAL AND METHODS

Samples

Tissue microarrays (TMA) with 5-µm thick sections from 20 cases of AME (Biomax Inc., Rockville, MD, USA), 8 cases of calcifying cystic odontogenic tumors (CCOTs) and 10 samples of

dental follicle obtained from the records of the Department of Oral Pathology of the Odontology Faculty at the University Center of Pará (CESUPA, Belém-PA, Brazil) were used in this study. The CCOT cases were included,as controls because this is an odontogenic tumor with a favorable prognosis and few reported cases of relapse.[1] Ten samples of dental follicles, a normal odontogenic tissue, were included as additional control.

This study was approved by the Human Research Ethics Committee of the Institute of Health Sciences at the Federal University of Pará (CAAE: 36572414.7.0000.0018, No. 877.322/2014).

Immunohistochemistry

The 5-µm histological sections obtained from the CCOT samples were mounted on glass slides treated with 3-aminopropyltriethoxysilane (Sigma Chemical Corp, St. Louis, MO, USA). The sections from the AME (TMA) and CCOT samples were deparaffinized in xylol and hydrated in decreasing concentrations of ethanol. The slides were then immersed in 20% H_2O_2 and methanol at a 1:1 ratio for 20 min to inhibit endogenous peroxidase activity. Subsequently, antigen recovery was performed in a citrate buffer (pH 6.0) in a Pascal pressure chamber (Dako, Carpinteria, CA, USA) for 30 s. Nonspecific binding sites were blocked with 1 % bovine serum albumin (BSA, Sigma Chemical Corp) in phosphate-buffered saline (PBS) for 1 h. The slides were incubated with the primary antibodies anti-NOTCH1 (1:100, Merck Millipore, Darmstadt, Germany), anti-HIF-1α (1:25, Merck Millipore), anti-ADAM-12 (1:150, Abcam, Cambridge, MA, USA) and anti-HB-EGF (1:7.5, Merck Millipore) for 1 h. Subsequently, the sections were incubated for 30 min with the EnVision Plus (Dako) detection system**.** Diaminobenzidine (Dako) was used as a chromogen. The sections were then counterstained with Mayer's hematoxylin (Sigma Chemical Corp) and mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA). Non-immune serum was used as a negative control.

Immunoastaining assessment

Immunostaining was assessed by measuring the area (µm) and the area fraction (%) of NOTCH1, HIF-1α, ADAM-12 and HB-EGF staining in AME and CCOT. Bright field images of at least six arbitrarily selected areas in each sample were acquired using an AxioScope microscope equipped with an AxioCam HRC color CCD camera (Carl Zeiss, Oberkochen, Germany), using a 40× objective. The areas stained with diaminobenzidine were separated and segmented using the color deconvolution plug-in (Gabriel Landini,

http://www.mecourse.com/landinig/software/software.html) of ImageJ (NIMH, NIH, Bethesda, MD, USA, http://rsbweb. nih.gov/ij/).

After image segmentation, the area and percentage of total staining were measured. The differences in immunoexpression between AME, CCOT and dental follicle were then analyzed.

Statistical analysis

Data obtained from the experiments were analyzed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). The non-parametric Mann-Whitney test was used after analysis of normality to assess the differences between the three groups.

RESULTS

Ameloblastoma expressed NOTCH1, HIF-1α, ADAM-12 and HB-EGF

All ameloblastoma samples expressed NOTCH1, HIF-1α, ADAM-12 and HB-EGF. NOTCH1 staining was intense in the cytoplasm and weak in the nucleus of tumor parenchyma cells (Figure 1A–B, arrows). Eleven follicular, 7 plexiform and 2 acanthomatous subtypes of ameloblastoma were studied. NOTCH1, HIF-1α, ADAM-12 and HB-EGF were present in all ameloblastoma subtypes. There was strong HIF-1α staining, predominantly in the nucleus (Figure 2A, arrow). Strong cytoplasmic and nuclear staining was observed in the central cells of the islands formed by the tumor epithelium (Figure 2B, arrow) and in the cells around the cysts (Figure 2C–D, arrows). Both ADAM-12 and HB-EGF exhibited cytoplasmic and cell membrane staining (Figures 3A–B and 4A–B, arrows).

All proteins exhibited weak staining in tumor stromal cells (Figures 1A–B, 2B, 3A–B, and 4A–B, asterisks). CCOT and dental follicle samples exhibited weak staining. (Figures 1C–D, 2E–F, 3C and 4C–D). Dental follicle sample showed a weak or a negative staining for ADAM12 (Figure 3 D).

Expression of NOTCH1, HIF-1α, ADAM-12 and HB-EGF was higher in AME than CCOT and dental follicle samples

The results showed that expression of NOTCH1, HIF-1α, ADAM-12 and HB-EGF was higher in AME tumor cells than in CCOT and dental follicle cells ($p<0.01$; Figure 5A). The expression of NOTCH1, ADAM-12 and HB-EGF was higher in CCOT tumor cells than in dental follicle cells ($p<0.001$; Figure 5A). There was no significant difference in HIF-1α expression

between CCOT and dental follicle (Figure 5A). Moreover, their expression was higher in tumor cells than in AME stromal cells ($p<0.001$; Figure 5B).

DISCUSSION

Immunochemistry results showed that the AME, CCOT and dental follicle samples expressed NOTCH1, HIF-1α, ADAM-12 and HB-EGF proteins. In addition, immunoexpression in AME samples was higher compared with that in CCOT, which is a noninvasive odontogenic tumor that has an ameloblastomatous epithelium, and dental follicle. CCOT, in turn, showed a higher expression of NOTCH1, ADAM-12 and HB-EGF than dental follicle. This was expected, since these proteins are associated with neoplastic behavior, which is not the case of dental follicle epithelia.

The results indicate that these proteins are involved in the local invasiveness of AME. The main focus of this study was to correlate these proteins with hypoxia, since low-oxygen microenvironments correlate with agressiveness of malignant tumors.[12,15]

AME exhibited increased NOTCH1 compared to controls. Staining was predominantly cytoplasmic, although some nuclear staining was observed. When the extracellular receptor of NOTCH1 is activated by one of its ligands, the intracellular domain of NOTCH (NICD) is cleaved and translocated to the nucleus.[25,29] This would explain the cytoplasmic and nuclear distribution of NOTCH, allowing transcription of several genes.[25,29-31] NOTCH1 has a role in the invasive process of some malignant tumors[32-34] and is required for the formation of more invadopodia, thus promoting cellular invasion.[12]

HIF-1α is a factor responsible for stabilization of the intracellular response triggered by NOTCH1.[26-28] HIF-1α is a transcription factor induced by hypoxia.[35,36] Once activated, it is localized in the nucleus,[37] which promotes the transcription of hypoxia-responsive genes. [26-28] In this study, we found increased immunoexpression of HIF-1α in AME, mostly at the central area of the tumor parenchyma and at the epithelium adjacent to cystic formations.

In tumorigenesis, hypoxic conditions are crucial for the development and adaptation of tumor cells.[15] Hypoxia has been recently shown using endogenous biomarkers.[38] Among these, HIF-1α has been described as the main biomarker. HIF-1α

the transcription of genes responsible for the synthesis of other biomarkers, such as CA-IX and GLUT-1.[39] The presence of HIF-1α in AME is crucial to demonstrating the presence of intratumoral hypoxia in specific areas, such as the central area of the tumor parenchyma and the epithelium adjacent to cystic masses.

The AME samples exhibited high levels of HIF-1α, which means that NOTCH1 signaling stabilized. This triggers the transcription of several genes, including the ADAM-12 gene.[40] Our results showed increased expression of ADAM-12 in ameloblastoma . The presence of this enzyme may be related with release of mitogens in this neoplasm. During tumor progression, growth factors are randomly released in ameloblastoma, probably through MMP acitivy.[4,5] ADAM-12 is also a metalloprotease. Taken together our results suggest that ADAM-12 and MMPs may contribute to mitogens release, thus increasing local aggressiveness and morbidity rate of this tumor.

ADAM-12 is essential for shedding certain ectodomain growth factors, such as HB-EGF, promoting events that are fundamental for cellular invasion, such as cell proliferation, differentiation, and migration.[41] This explains why HB-EGF and ADAM-12 exhibited similar immunostaining patterns. Thus ADAM-12 would contribute to HB-EGF cleavage, thereby increasing HB-EGF immunoexpression.

Moreover, ADAM-12 can generate signaling through its cytoplasmic domain, thus allowing interaction with substrates of the Src family such as TKs-5, a major invadopodia player.[42] Although there are no reports of TKs-5 expression in AME, we have successfully found other key invadopodia proteins, such as cortactin and MT1–MMP.[6] We may speculate that increased ADAM-12 expression would influence invadopodia activity through Tks5, cortactin and MT1-MMP, with subsequent increase in ameloblastoma local invasiveness.

In conclusion, we suggest that NOTCH1, HIF-1α, ADAM-12, and HB-EGF are directly associated with the invasiveness mechanism of AME. The expression of these proteins in AME increases intratumoral hypoxia; these proteins are probably members of the same cell signaling pathway during hypoxia and facilitate increased invadopodia formation and tumor invasiveness.

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Natacha Malu Miranda da Costa contributed to the research conception, design, data collection, analysis, interpretation, and manuscript preparation.

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Carolina Carmine Proietti contributed to data collection and analysis.

Maria Sueli da Silva Kataoka contributed to the research conception and design, and critical review of the manuscript for intellectual content.

Ruy Gastaldoni Jaeger contributed to the critical review of the manuscript for intellectual content and approved the final version for publication.

Sérgio Melo de Alves-Júnior contributed to the critical review of the manuscript for intellectual content.

João de Jesus Viana Pinheiro contributed to the research conception, design, data collection and interpretation, manuscript preparation, critical review of the manuscript for intellectual content, and approved the final version for publication.

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FIGURE LEGENDS

Figure 1. Immunostaining of NOTCH1 in AME (A and B), CCOT (C) and dental follicle (D). Immunoperoxidase. Scale, 20 µm.

Figure 2. Immunostaining of HIF-1α in AME (A–D), CCOT (E) and dental follicle (F). Immunoperoxidase. Scale, 20 µm.

Figure 3. Immunostaining of ADAM-12 in AME (A and B), CCOT (C) and dental follicle (D). Immunoperoxidase. Scale, 20 µm.

Figure 4. Immunostaining of HB-EGF in AME (A and B), CCOT (C) and dental follicle (D). Immunoperoxidase. Scale, 20 μm.

Figure 5. Comparison of NOTCH1, HIF-1α, ADAM-12, and HB-EGF immunoexpression between AME, CCOT and dental follicle (A). In AME, expression of the proteins was higher in the tumor epithelium than in the tumor stroma (B). Significance: **p<0.01; ***p<0.001.

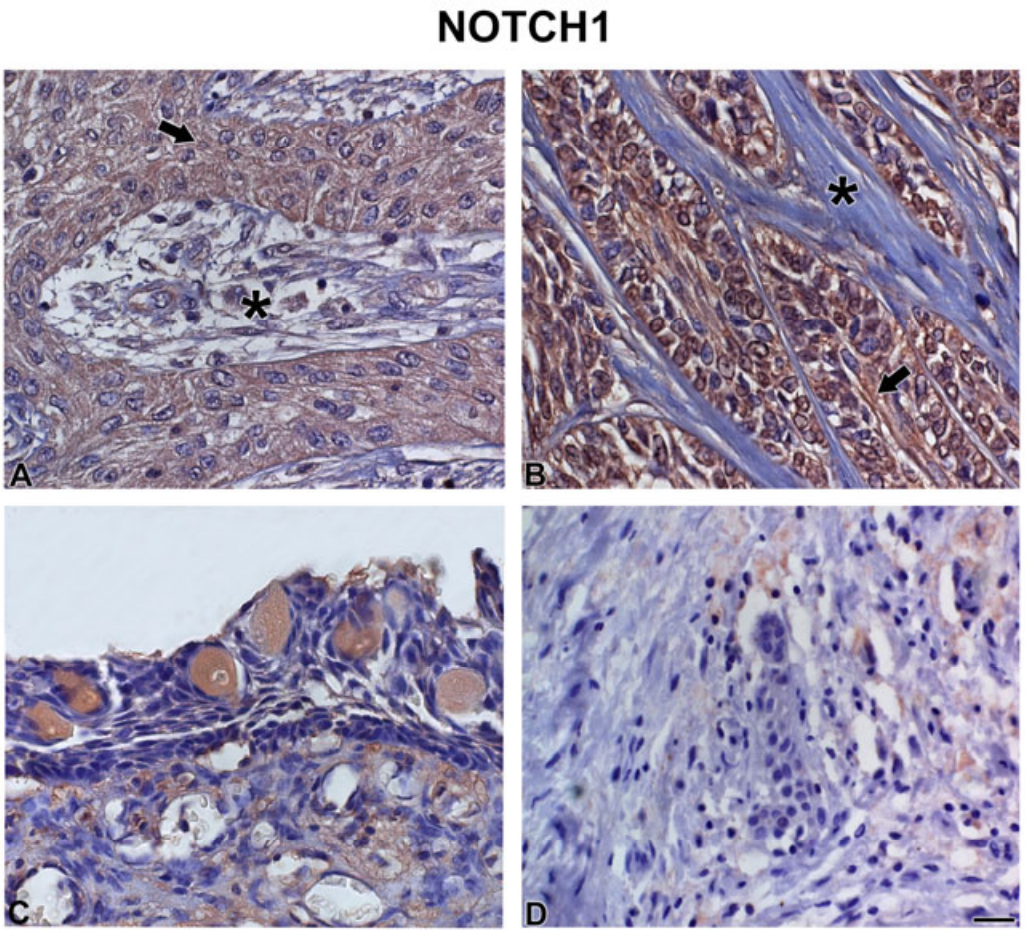

Figure 1

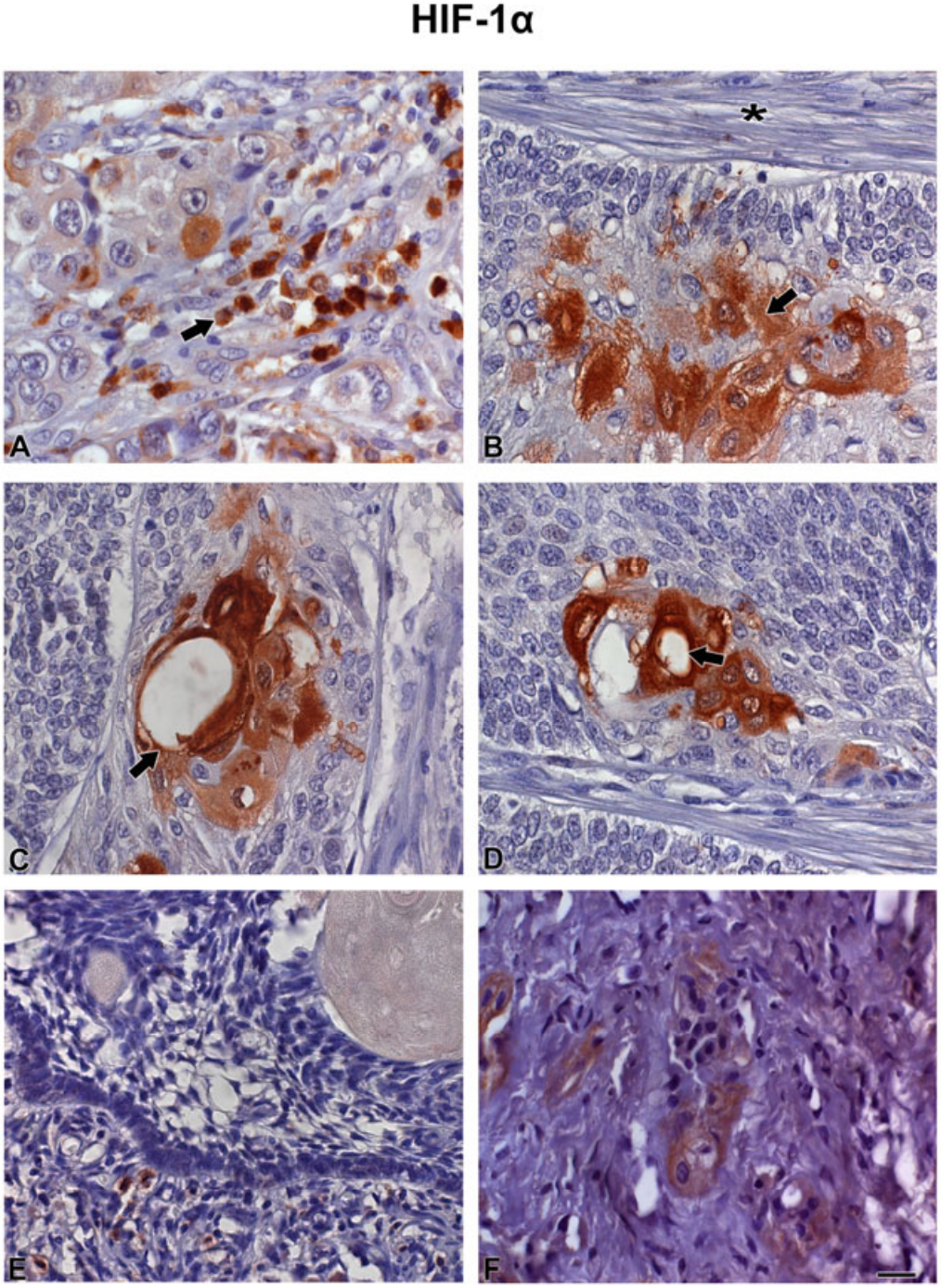

Figure 2

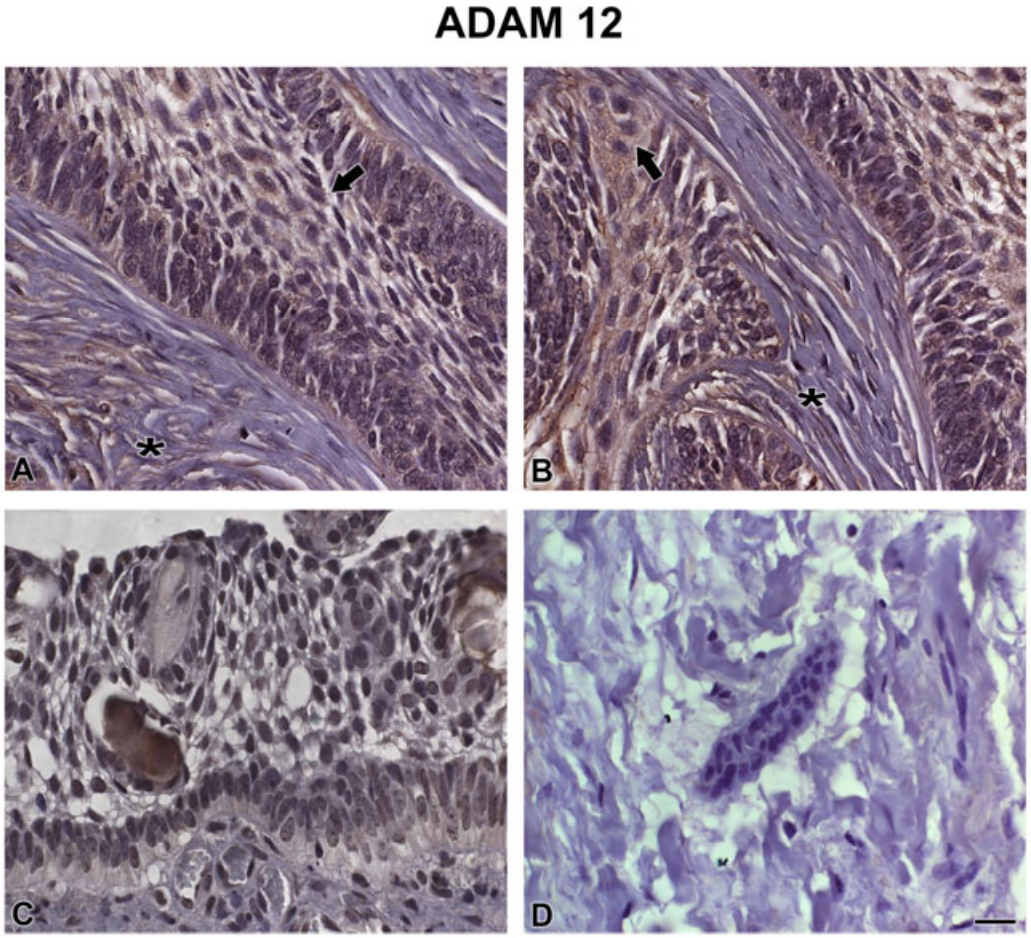

Figure 3

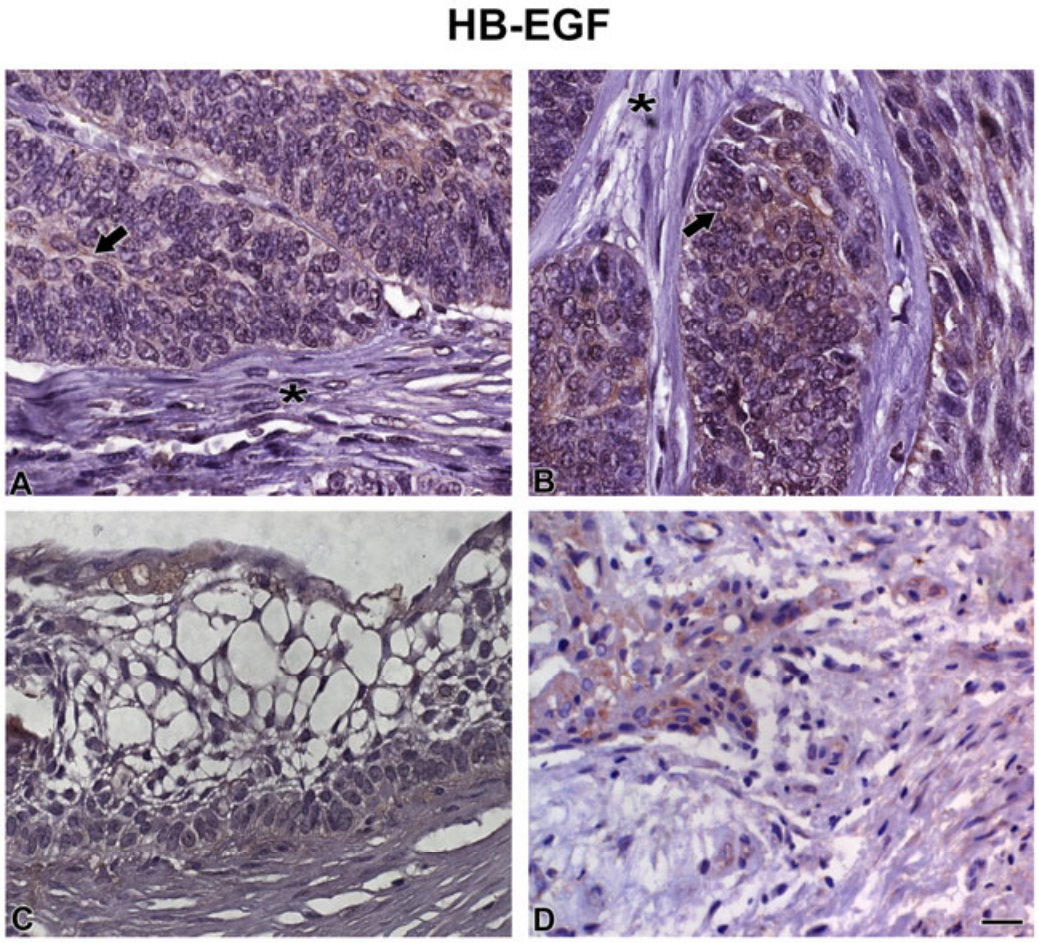

Figure 4

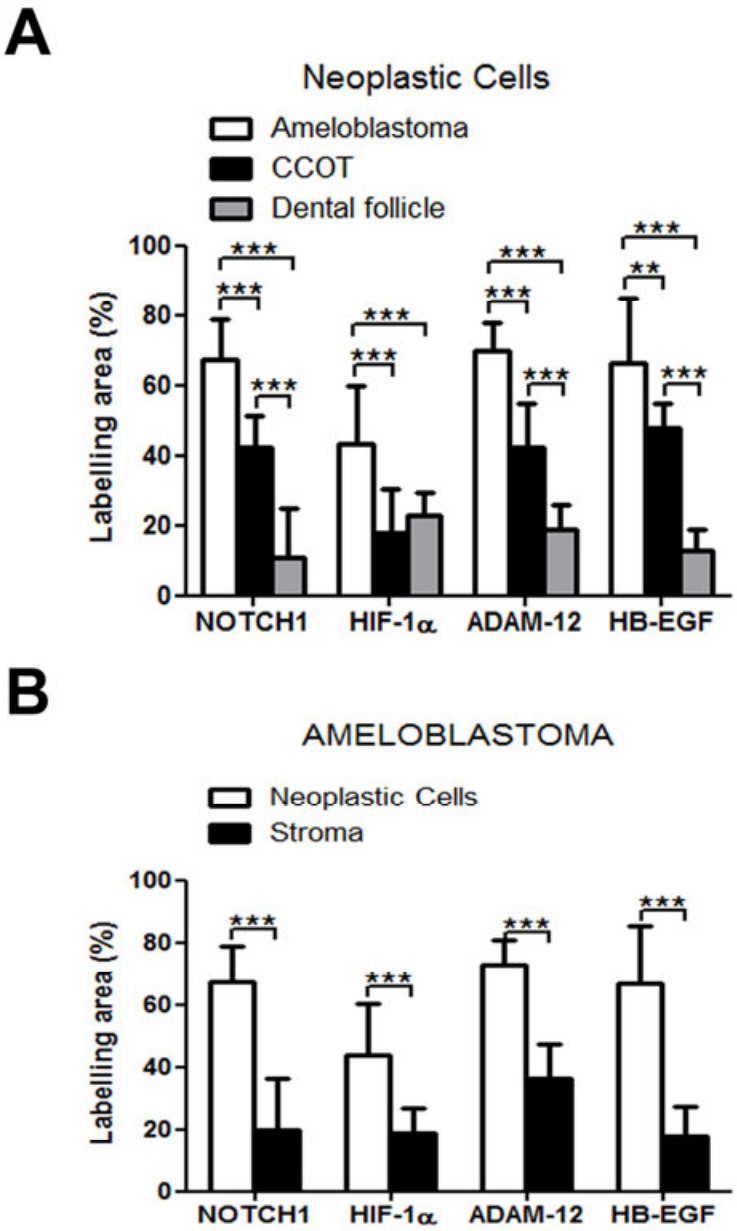

Figure 5