Bone morphogenetic protein-2/4 and bone morphogenetic protein receptor type IA expression in metastatic and nonmetastatic oral squamous cell carcinoma

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Abstract

Purpose: The study aimed to analyze the expression of bone morphogenetic protein-2/4 (BMP-2/4) and its receptor BMPR-IA (BMP receptor type IA) in metastatic and nonmetastatic oral squamous cell carcinoma (OSCC) and its implications for disease prognosis.

Materials and methods: The experimental group included 16 cases of OSCC without metastasis and 7 cases of OSCC with metastasis. The presence or absence of nodal metastasis was used as a parameter for the evaluation of disease prognosis. Ten cases of oral fibroepithelial hyperplasia were selected as the control group. The expression of BMP-2/4 and BMPR-IA was analyzed by immunohistochemistry.

Results: In the experimental group with metastasis, strong expression of BMP-2/4 was observed in most cases (71.4%), whereas BMPR-IA exhibited weak expression (85.7%). In the experimental group without metastasis, there was strong expression of BMP-2/4 (62.5%) and BMPR-IA (100%). A significant association was observed between the prognosis of OSCC and the intensity of BMP-2/4 staining ($P = .002$). Weak immunoreactivity to BMP-2/4 and BMPR-IA was observed in all control specimens.

Conclusions: The results suggest that strong expression of BMP-2/4, associated with low expression of BMPR-IA, observed in metastatic OSCC has a prognostic value, with the loss of responsiveness to BMPs through the loss of expression of their receptors being indicative of the development of metastasis.

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1. Introduction

Bone morphogenetic proteins (BMPs) are pleiotropic cytokines of the transforming growth factor β family [1]. These proteins are components of an evolutionarily conserved signaling system and are involved in the regulation of cell growth, differentiation, apoptosis, chemotaxis, angiogenesis, and matrix production both during embryo and postnatal animal development [2,3].

Studies in the areas of embryology, genetics, and carcinogenesis have demonstrated that disturbances in the BMP signaling pathway contribute to the development of neoplasms. The first evidence of this involvement arose from genetic studies on familial cancer syndromes such as familial juvenile polyposis, in which mutations in Smad-4 and BMPR-IA have been implicated in the origin of the disease. In addition, the BMP signaling pathway is found to be altered in sporadic human cancers. The action of these proteins during carcinogenesis is complex and involves both protumor and antitumor activities, depending on the stage of the disease [4].

During the initial phase of neoplastic development, BMPs inhibit the cell cycle by stimulating the overexpression of protein p21, a universal inhibitor of cyclin-dependent
kinases. Interaction of p21 with the cyclin–cyclin-dependent kinase complex results in the inhibition of the kinase activity of the latter and consequently paralyzes cell cycle progression [5]. The protumor activity of BMPs occurs in more advanced stages of neoplastic development and favors the dissemination of metastasis by inducing the expression of vascular endothelial growth factor (VEGF), thus exerting a proangiogenic effect. Therefore, elevated expression of these cytokines in tumors might be significantly associated with a poor prognosis [6-8].

The role of BMPs in the development of epithelial tumors is still uncertain, and various studies have investigated their mechanisms of action in different tissues of the human organism. In view of the scarcity of reports investigating these proteins in oral epithelial tumors, the present study analyzed the immunohistochemical expression of BMP-2/4 and its receptor BMPR-IA in metastatic and nonmetastatic oral squamous cell carcinoma (OSCC) and its implications for disease prognosis.

2. Materials and methods

The sample consisted of 23 squamous cell carcinoma specimens involving different sites in the oral cavity. The specimens were obtained from incisional biopsies stored in the archives of Hospital Dr Luís Antônio, Natal, Rio Grande do Norte, Brazil. These specimens were divided into 2 groups: a nonmetastatic group consisting of 16 OSCC cases without nodal or distant metastasis and a metastatic group consisting of 7 cases of OSCC with nodal and distant metastasis. The parameter presence or absence of nodal and distant metastasis was collected from the patient records based on the TNM staging system and was used for the selection of the OSCC cases and the evaluation of disease prognosis. For the control group, 10 specimens of fibroepithelial hyperplasia were selected among excisional biopsies involving different sites in the oral cavity. The specimens were obtained from the archives of the Pathological Anatomy Service, Discipline of Oral Pathology, Dentistry School, Federal University of Rio Grande do Norte (UFRN).

Histologic sections (3 μm thick) were obtained from the selected material and submitted to immunohistochemistry by the streptavidin-biotin method. Bone morphogenetic protein-2/4 and its receptor BMPR-IA were first reconstituted in 1 mL sterile phosphate-buffered saline each, pH 7.6, and immunohistochemistry was performed after 24 hours. For this, the paraffin-embedded sections were deparaffinized in xylene and rehydrated in a decreasing ethanol series. Endogenous tissue peroxidase was blocked by 2 baths of 5 minutes each in 10 volumes of 6% hydrogen peroxide in methanol (1:1, vol/vol). Antigens were retrieved with 0.1% trypsin and 0.1% CaCl₂ in Tris for 30 minutes at 37°C. After incubation in normal serum for 30 minutes, the sections were incubated with the following primary antibodies: BMP-2/4 (AF355) and BMPR-IA (AF346) (both from R&D Systems, Inc, Minneapolis, MN, USA, overnight at 4°C and 1:50). The LSAB peroxidase kit (Dako Corporation, Glostrup, Denmark) was used as secondary antibody and tertiary complex for the 2 proteins studied. The reaction was developed with 0.3% diaminobenzidine (Sigma Chemical Co, St Louis, MO, USA) diluted in Tris, pH 7.4, and activated with 600 mL 6% hydrogen peroxide in a dark chamber for 3 minutes. The sections were then washed under running water and in distilled water and counterstained with Mayer’s hematoxylin for 10 minutes. Human cartilage was used as positive control, and samples in which the primary antibody was omitted and replaced with buffered 1% bovine serum albumin were used as negative control. Positive and negative controls were processed as described above.

Immunoreactivity to BMP-2/4 and BMPR-IA was analyzed qualitatively in a double-blind fashion by 2 observers. Only the epithelial component of the selected specimens was examined. Scores of 1 (weak expression) and 2 (strong expression), adapted from Wakulich et al [9], were used for analysis.

Expression of the proteins studied, as well as its correlation with the prognosis of OSCC, was evaluated using the χ² test, with the level of significance set at 5% (α = .05). The study was approved by the ethics committee of UFRN (no. 65/06-2006).

3. Results

The immunoexpression of BMP-2/4 and of its receptor BMPR-IA showed a typical cytoplasmic location, with variations in the intensity of expression in the epithelial layer depending on the type of tumor studied.

In the experimental group without metastasis (n = 16), expression of BMP-2/4 was predominantly strong (n = 10, 62.5%) (Fig. 1, Table 1). Strong immunoreactivity to BMPR-...
IA was observed in all specimens (n = 16, 100%) (Fig. 2). In the experimental group with metastasis (n = 7), strong expression of BMP-2/4 was observed in most cases (n = 5, 71.4%) (Fig. 3, Table 1), whereas BMPR-IA exhibited weak expression in 87.5% of the specimens analyzed (Fig. 4). Weak expression of BMP-2/4 (Fig. 5, Table 1) and BMPR-IA (Fig. 6) was observed in all 10 control cases of fibroepithelial hyperplasia.

Statistical analysis showed a significant association between the prognosis of OSCC and the intensity of BMP-2/4 staining ($P = .002$) (Table 1). However, statistical analysis was not possible in the case of BMPR-IA and, therefore, the data were only analyzed descriptively.

4. Discussion

During neoplastic development, BMPs can act as oncogenes or as tumor suppressors depending on the stage of the disease and their physiological concentration in the tumor matrix [4]. In addition, the effects of BMPs are cell-specific and may therefore vary between different types of tumors, even those of the same cellular origin. Langenfeld et al [10] also reported that the culture conditions of in vitro studies, as well as the concentration of intra- and extracellular antagonists, interfere with the biological activity of BMPs.

Several investigators have demonstrated the expression of BMPs and their receptors in different neoplastic processes. In tumors of mesenchymal origin, BMPs are associated with the pathologic formation of bone, calcified masses, and chondroid tissue inside the tumor or at ectopic sites; in addition, BMPs have been implicated in tumor progression [11]. Kusafuka et al [12] investigated the expression of BMPs in chondroid areas of 15 pleomorphic adenoma specimens and observed frequent overexpression of BMP-2 in these regions. Jin et al [11] reported overexpression of BMPs 2/4 and 5 and their receptor BMPR-IA in malignant tumors of the oral epithelium. In addition, BMPs 2/4 and 5, but not their receptors, have been implicated in the development of metastasis in some cases of OSCC.

In the present study, expression of BMP-2/4 was weak in the 10 (100%) control specimens analyzed, indicating that the concentration of these proteins tends to be low in normal differentiated tissues and benign pathologic processes as previously reported [10,11,13,14]. Kusafuka et al [12] also found low immunoreactivity to BMPs 1, 2, 3, 4, and 7 in normal glandular epithelium. On the other hand, Kumamoto and Ooya [15] reported significant expression of BMPs 2, 4, and 7 and their receptors in normal odontogenic epithelium of tooth germs during the late bell and crown stage, a fact compatible with the active participation of BMPs in tooth formation during postnatal animals development. Hardwick et al [16] investigated the immunoeexpression of BMP-2 in

### Table 1

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<th>Group</th>
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Fig. 2. Overexpression of BMPR-IA in nonmetastatic OSCC (arrows) (original magnification ×400).

Fig. 3. Overexpression of BMP-2/4 in metastatic OSCC (arrows) (original magnification ×400).
intestinal colon specimens of patients with familial adenomatous polyposis and observed the loss of BMP-2 expression in dysplastic areas of the microadenoma, when compared to the superficial layer of normal epithelium adjacent to the tumor that exhibited significant immunoreactivity. Kim et al [17,18] observed elevated expression of BMP receptors in normal prostatic and bladder tissue. These studies suggest that the responsiveness to BMPs and their receptors may vary among different cell types, including those of the same embryonic origin.

In the present study, immunoreactivity to BMPR-IA was low in the 10 control specimens analyzed (100%). This finding can be explained by the same reasons stated above for BMPs.

In the experimental group, strong expression of BMP-2/4 was observed in both metastatic (n = 5, 71.4%) and nonmetastatic OSCC cases (n = 10, 62.5%). This finding suggests disturbances in the signaling pathway mediated by these proteins in the presence of malignant neoplastic processes. This result agrees with Jin et al [11], Langenfeld and Langenfeld [19], Kumamoto and Ooya [15], and Arnold et al [20] who also observed strong expression of BMPs 2, 3, 5, and 6, especially BMP-2, in highly and poorly metastatic breast cancer cell lines. Several studies have investigated the probable causes for this overexpression of BMPs in malignant neoplasms. According to Hsu et al [4], during the initial stages of the disease, neoplastic cells tend to increase the synthesis of BMPs in an attempt to inhibit cell growth because BMPs are known to function as tumor suppressors and therefore act directly on the cell cycle and induce the apoptosis of altered cells. In this respect, Kawamura et al [21] and Hjertner et al [22] investigated the antiproliferative effect of BMP-2 and BMP-4, respectively, on human multiple myeloma cells. The authors suggested that these BMPs are able to inactivate STAT3 protein, a signal transducer activated by interleukin 6, and to increase the expression of cell cycle inhibitors such as p21 and p27, with the consequent hypophosphorylation of pRb and blockade of cell replication.

Hardwick et al [16] used colorectal cancer cell lines to determine the role of BMP-2 in the development of this cancer. The authors observed that BMP-2 reduced cell growth, as demonstrated by the low expression levels of BMP receptors in normal prostatic and bladder tissue. These studies suggest that the responsiveness to BMPs and their receptors may vary among different cell types, including those of the same embryonic origin.

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PCNA (a marker of cell proliferation), and stimulated apoptosis and cell adhesion, as demonstrated by the high levels of cleaved caspase-3 (a marker of apoptosis) and β-catenin (an adhesion molecule). In an in vitro study, Wen et al [23] reported that gastric cancer cell lines incubated with BMP-2 for 4 to 5 days exhibited low levels of cell proliferation and morphologic alterations characterized by enlarged cells, an increase in intercellular contacts, and a decrease in the nucleus-cytoplasm proportion. Recent studies have suggested that the antiproliferative effect of BMPs occurs in 3 steps: blockage of the cell cycle, stimulation of apoptosis, and increase of cell adhesion.

In the present study, BMPR-IA showed variable expression in the experimental group. In metastatic OSCC specimens, expression tended to be weak in all cases analyzed. During carcinogenesis, especially in the more advanced stages of the disease, neoplastic cells commonly lose their responsiveness to BMPs, either due to the loss of expression of their receptors or to the increased synthesis of BMP inhibitors such as noggin [4,17,18,24]. The loss of expression of BMP receptors, especially BMPR-IA, is expected in many human cancers such as prostate, bladder, breast, and intestinal colon cancer. However, in the case of nonmetastatic OSCC, we observed a high expression of BMPR-IA in all specimens studied. This finding suggests the presence of responsiveness to BMPs and, consequently, a trend of neoplastic cells to inhibit their own growth, thus characterizing an antitumor effect of BMPs. According to Hardwick et al [16] and Kim et al [18], the loss of BMP receptor expression might be attributed to microsatellite instability and the presence of mutations in the receptors, or even to promoter suppression through methylation. In contrast, several investigators reported the overexpression of BMP receptors, a finding confirming that the function of BMPs and their receptors varies among different cell lines and tumors [11,15,20,23,25]. According to Hsu et al [4], these receptors might be overexpressed but are inactive because of the interaction with extracellular BMP antagonists, such as noggin, which are overexpressed in more advanced stages of tumor progression. Consequently, overexpressed BMPs no longer act through an autocrine pathway, associated with an antiproliferative effect, and start to act through a paracrine pathway associated with a proangiogenic effect.

Bone morphogenetic proteins possess various mechanisms to stimulate angiogenesis. Bone morphogenetic protein-2 acts as a potent chemotactic agent for monocytes, which differentiate into tissue macrophages and secrete proangiogenic cytokines such as VEGF. Bone morphogenetic protein-2 also stimulates undifferentiated mesenchymal cells to release P1GF (placental growth factor), a factor important for the recruitment of hematopoietic and undifferentiated endothelial cells under pathologic conditions such as ischemia, inflammation, healing, and cancer [19,26]. According to Langenfeld and Langenfeld [19] and Raida et al [26], BMPs act directly on endothelial cells by activating Id1 (inhibitor of differentiation) and Erk-1/2 (signal transducer in endothelial cells) triggered by the release of VEGF. In addition, protein Id delays senescence of endothelial cells.

In the present study, we observed a significant association between BMP-2/4 expression and disease prognosis (P = .002) (Table 1), whereas statistical analysis was not possible in the case of BMPR-IA; however, a weak expression was detected in most of cases of the metastatic group.

The data suggest that the strong expression of BMP-2/4, associated with the weak expression of BMPR-IA, in the metastatic group may have prognostic implications, with the loss of responsiveness to BMPs through the loss of expression of their receptors being indicative of the development of metastasis in OSCC. In addition, the high immunoreactivity to BMP-2/4 observed only in the experimental group agrees with the trend toward overexpression of these proteins in malignant neoplasms and consequent disturbance in the BMP-mediated signaling pathway.

Acknowledgments

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References


