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ROLE OF MOLECULAR MARKERS IN UNDERSTANDING THE TUMOUR INVASIVENESS OF AMELOBLASTOMA

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Abstract

The aim of the present article was to review the current new knowledge on the molecular markers of tumor invasion in ameloblastoma. In this review, tumor molecular markers were identified and allocated to the following six groups according to their functions: (I) Markers involved in extracellular matrix degradation, (II) Molecular markers involved in cell adhesion lost, (III) Molecular markers involved in bone remodeling, (IV) Cytokines involved in angiogenesis, (V) Molecular markers related to the function of tumor stromal cells on the invasion of ameloblastoma, and (VI) Molecular markers involved in cell proliferation related with invasion. In general, the location of markers within the tumor and not their quantitative assessments as such is emphasized. Data showed that the correlation among molecular markers of invasive relevance is still not quite clear. Results on markers of tumor invasion and metastatic potential appeared to be too premature for a statement regarding the instinct invasive nature of ameloblastoma. The unraveling of specific new details concerning these mechanisms, whereby the expression and relationships among the molecules are mediated, may provide an opportunity to afford efficient prevention and develop new treatment therapies.

Keywords: Ameloblastoma, metastatic invasion, molecular invasion

Introduction

Ameloblastoma (AM), the most frequently encountered tumor arising from odontogenic epithelium, is characterized by a benign but locally invasive behavior with a high risk of recurrence. According to the 2005 Histological Classification of Tumors of the World Health Organization, ameloblastomas are classified into the following four variants: solid/multicystic, extraosseous/ peripheral, desmoplastic, and uni cystic. [1] The invasion of surrounding healthy tissues by tumor cells is one of the essential steps in tumor progression. Identification of invasive activities in ameloblastomas may be useful to predict their biological behavior. Many studies have been done in an attempt to clarify the invasion phenomenon in ameloblastomas. However, the exact molecular mechanism of invasion in ameloblastomas has not been well elucidated. In our study, tumor

molecular markers were allocated to the following six groups according to their functions: (I) Markers involved in extracellular matrix (ECM) degradation, (II) Molecular markers involved in loss of cell adhesion, (III) Molecular markers involved in bone remodeling, (IV) Cytokines involved in angiogenesis, (V) Molecular markers related to the function of tumor stromal cells on the invasion of ameloblastoma, and (VI) Molecular markers involved in cell proliferation related with invasion.

Markers in extracellular matrix degeneration

ECM provides an essential framework upon which cells grow, migrate, and differentiate. It is essential that ECM undergoes remodeling during the developmental processes. ECM degradation that occurs during developmental processes, tissue repair, inflammatory diseases, and tumor progression requires the action of a number of proteolytic enzymes. Thus, many researchers tried to detect the role of markers of ECM degradation in the invasion of ameloblastoma.

Tumor cell invasion depends on the coordinated and temporal expression of proteolytic enzymes to degrade the surrounding ECM and adhesion molecules to remodel cell-cell and/or cell-matrix attachments. Matrix metalloproteinases (MMPs) are zinc metalloenzymes that are involved in ECM remodeling. Over expression of these MMP genes, which degrade various ECM components, plays an important role in organogenesis, tissue remodeling, and tumor invasion. It has been proven that expression of MMP-2 can be found in ameloblastoma, [2] and MMP-2 showed close correlation with the growth and invasion of ameloblastoma. [3],[4] Additionally, the results by Wang *et al.* indicate that inhibition of MMP-2 activity suppresses the local invasiveness of ameloblastoma cells, which may serve as a novel therapeutic target in ameloblastomas. [5] Reversion-inducing-cysteine-rich protein with Kazal motifs (RECK), the key action of which is to inhibit MMPs, especially MMP-2 and MMP-9, may participate in the invasion, recurrence, and malignant transformation of ameloblastoma by regulating MMP-2 at the post-transcriptional level. Lower or no expression of RECK and increased expression of MMP-2 may be associated with worse clinical outcomes in ameloblastoma, and RECK may help modify the behavior of ameloblastoma by regulating MMP-2 at the post-transcriptional level. [6] To date, studies showed that a high expression of MMP-2, TIMP-2, and MMP-14mRNA levels may contribute to the local invasive capacity of ameloblastoma, whereas the local invasive characteristics of ameloblastoma are more likely to be related to high transcriptional levels of TIMP-2 and MMP-14. [7]

Moreover, osteonectin/secreted protein acidic and rich in cysteine (SPARC), a major noncollagenous constituent of bovine and human bone, occurs in response to inflammation, tissue injury, tumor growth, and metastasis. The understanding of the interaction of SPARC and MMPs in ameloblastoma may enhance the knowledge of the locally aggressive behavior of this odontogenic tumor. It has been suggested that there is a putative association between SPARC, and MMPs (especially MMP-9) in ameloblastoma to regulate tumor invasion. [8]

CD147, also known as ECM metalloproteinase inducer (EMMPRIN), tumor collagenase stimulatory factor, M6 antigen, basigin, and neurothelin, was initially characterized as a factor on the surface of neoplastic cells that induces MMP production in fibroblasts. Upregulation of EMMPRIN in lung, bladder and breast carcinomas suggested that EMMPRIN may play a role in promoting MMP-dependent tumor aggression. EMMPRIN expression has been detected in some ameloblastomas,

indicating a collagenase-stimulating effect. [9] The study of immunoreactivity for EMMPRIN in ameloblastomas and malignant ameloblastic tumors suggested that EMMPRIN might participate in tumor cell progression of these epithelial odontogenic tumors by inducing MMP in stromal cells. No distinctive differences in EMMPRIN immunoreactivity were found among tumor subtypes. [10] Taken together, the precise mechanism responsible for all the proteases and factors in ameloblastoma should be investigated further, focusing on the correlative regulation passageway and mechanisms in this tumor.
Heparanase, an endo glucuronidase enzyme, is an important modulator of ECM and related to invasion of tumor cells. It can specifically cleave heparan sulfate and play an important role in degradation and remodeling of ECM in normal condition and dissemination and invasion of cells associated with inflammation and cancer metastasis. Through the study by Siar, [11] heparanase presented increased frequency in ameloblastoma at the mRNA as well as the protein level, which may predict heparanase as one of the important determinants of its local invasiveness.

Markers involved in cell adhesion and cell migration

Cellular invasion requires disintegration of the basement membrane and surrounding ECM, followed by the growth and proliferation of cells. Thus, decreased intercellular adhesion and changes in basal membrane composition influence the growth of malignant neoplasias. Regarding that cell-to-cell adhesion must be indispensable for the regulation of cellular behavior, a number of cell adhesion molecules have been identified in ameloblastoma.
Syndecan-1 (SDC1), a transmembrane heparan sulfate proteoglycan, also known as CD138, regulates many biological processes, including cytoskeletal organization, growth factor signaling, cell-cell adhesion, and ECM attachment. The Wingless-type 1 glycoprotein, belonging to a large family of 19 secreted signaling transducers, promotes cell proliferation. The loss expression of SDC1 in malignant epithelial neoplasms is associated with tissue invasion, metastasis, and poor prognosis. A statistically significant correlation was found between the percentage of intraosseous ameloblastoma-bearing SDC1-positive stromal cells and ECM and the percentage of intraosseous ameloblastoma-bearing Wnt1-positive epithelial cells. SDC1 immunostaining strongly depicted stromal cells, ECM, and basement membranes of ameloblastomas. It also showed in epithelial tumor cells in the acanthomatous and plexiform subtypes, and it often occurred in stellate reticulum cells and basal ameloblasts of tooth buds. Parallel Wnt1 expression occurred in ameloblastoma Tous epithelial cells, but it was common in basal cells of tooth buds too. Thus, SDC1 is conceivable as a critical factor for Wnt-induced carcinogenesis in the odontogenic epithelium. This heparan sulfate proteoglycan might be involved in promoting local invasiveness of some intraosseous ameloblastoma subtypes, depending on its expression by tumor epithelial cells and subsequent shifting to stromal cells and ECM. [12]
Based on the previous research, Bologna-Molina *et al.* further research demonstrated the decreased expression of SDC1 in solid ameloblastoma and supported the view that this subtype of ameloblastoma has a more aggressive biological behavior than the uni cystic ameloblastoma. [13]
Integrins, transmembrane receptors, can modulate cell-cell and cell matrix binding. They are implicated in growth, adhesion, migration, proliferation, apoptosis, and cellular morphology. Integrin α5β1 is the classic receptor for fibronectin, a protein that plays an important role in the epithelial-mesenchymal interactions observed in odontogenic tumors. Stronger labeling of α5β1 integrin in the neoplastic cells of

ameloblastomas may be associated with a greater migration capacity of these cells because large amounts of fibronectin have been detected in the stroma of these tumors. [14] Another role attributed to α5β1 integrin in the mechanism of tumor invasion is that its binding to fibronectin increases the secretion and expression of metalloproteinases. The findings by Emanuel demonstrated the participation of integrins in the mechanism of invasion of ameloblastomas, with α5β1 integrin apparently playing a greater role. [15]

In addition to the molecules discussed above, studies on gene expressions on tumor invasion in ameloblastoma also provided some valuable information. High-throughput cDNA microarray technologies and tumor array technologies are allowing the expressions of literally thousands of genes and proteins to be analyzed at one time. Hence, Heikinheimo *et al*. [16] analyzed gene expression in fresh-frozen ameloblastomas and human fetal tooth germs, using a cDNA microarray. Many of the genes found to be under-expressed in the study were involved in the regulation of cell adhesion, cell shape, and angiogenesis. For instance, cadherins (CDHs), Keratin7 (KRT7), Notch, and transforming growth factor-ß1 (TGF-β1) may be involved in disturbances of cell-to-cell adherence junctions and cell-to-cell communication. This indicated that gap-junction communication may be low and there was cell adhesion lost in ameloblastomas, as described for many types of neoplasia. Such alterations in the cell-membrane environment could also increase the locally aggressive growth potential of ameloblastoma. The study by Gonzαlez-Alva *et al.* suggests a role of podoplanin, a type-1 transmembrane sialomucin-like glycoprotein consisting of 162 amino acids in tumor invasiveness through collective cell migration in which the cadherin switch or epithelial-mesenchymal transition may not be involved. [17] Overexpression of WNT5A drastically increased enamel epithelium cell migration and actin reorganization when compared with controls. Suppression of endogenous WNT5A in enamel epithelium cells greatly impaired their migration and the cells failed to form significant actin reorganization, and membrane protrusion was rarely seen. The data indicate that WNT5A signaling is important in modulating tumorigenic behaviors of enamel epithelium cells in ameloblastomas. [18]

Molecular markers involved in bone remodeling

Ameloblastoma, a tumor located in bone, can perforate the bone and, ultimately, spread into the soft tissues. A number of cytokines, including interleukin -1α, interleukin -1β, interleukin -6, and tumor necrosis factor alpha (TNF-α) have osteolytic activity and can also stimulate cell growth. The activities of these cytokines were consistent with their roles in both ameloblastoma growth and intraosseous expansion. In addition, the immunocytochemical localization of IL-1α and IL-6 in ameloblastoma was confirmed by cytokine mRNA hybridization, thus supporting the hypothesis that the osteolytic expansion in the invasion of ameloblastomas may be due to the action of IL-1α and IL-6, with the former being the principal osteolytic factor. [19]

Osteopontin (OPN), an ECM protein of mineralized tissue with RGD (Arg-Gly-Asp) tripeptide, is associated with bone remodeling and plays an inductive role in mineralization process. OPN can also increase cell adhesion and migration. It is well acknowledged that the tumor nests of conventional ameloblastoma tend to infiltrate among cancellous bone trabeculae at the tumor margin. The molecular mechanism that results in local aggressive behavior and the osteolytic ability of ameloblastomas could be related. In the study of OPN in ameloblastomas, both ameloblast-like and stellate reticulum-like cells exhibited a high expression of OPN

and CD44v6. There was also a strong integrin αv immunostaining on the cell membrane of osteoclasts. Binding of OPN to osteoclast cell membrane receptor integrin αv can activate the osteoclast and increase its osteolytic activity. In addition, binding of OPN to ameloblastoma tumor cell membrane receptor CD44v6 can enhance tumor cell migration, invasion, and spread. [20]

It has been reported that abnormalities of the osteoclast differentiation factor (ODF)/receptor activator of nuclear factor-κB ligand (RANKL)-osteoclastogenesis inhibitory factor (OCIF)/osteoprotegerin (OPG) system, namely osteoclastogenesis regulators, have been implicated in the pathogenesis of various bone tumors. [21],[22],[23],[24],[25] Parathyroid hormone related protein (PTHrP), identified in the 1980s as a tumor product, is able to activate parathyroid hormone receptors and cause hypercalcemia. The local production of PTHrP by metastatic tumor cells in bone has been linked to bone destruction and tumor growth. Hence, the study presented that benign and malignant ameloblastomas expressed ODF/RANKL and OCIF/OPG predominantly in stromal cells rather than tumor cells, which suggested that these molecules might have a role in the regulation of local bone metabolism through parenchymal-stromal interactions in ameloblastomas. In addition, tumor cells showed slightly higher expression of ODF/RANKL and PTHrP in plexiform ameloblastoma than in follicular ameloblastomas, suggesting that these molecules were involved in tissue structuring of ameloblastomas. Therefore, PTHrP, DF/RANKL, and OCIF/OPG were considered to function as local regulating factors for bone resorption and tumor progression in these epithelial odontogenic tumors. Also, an interesting finding in the study was that the non-ameloblastic lining epithelium of the dentigerous cyst samples did not express PTHRP. Oligonucleotide microarray analysis of ameloblastoma compared with dentigerous cyst with semiquantitative RT-PCR showed that PTHrP were over expressed. It was suggested that PTHrP may play a significant role in local bone resorption, offering at least partial explanation for the infiltrative growth and destructive behavior of ameloblastoma. [26],[27] Twist, a mesoderm-determining factor, is a highly conserved basic helix loop helix transcription protein essential for embryological morphogenesis and mainly expressed in a subset of adult mesodermal cells. Recently, there was a research demonstrating that high expression of Twist in tumor cells might promote bone metastasis by modulating bone remodeling or by enhancing osteomimicry. The salient finding was that expression of Twist was related to the histological subtype of tumors, as there was a higher expression in solid ameloblastoma as compared with uni cystic ameloblastoma. Both nuclei and cytoplasm positivities were detected in positive cases, whereas cytoplasmic staining was diffused and predominant. Cases rich in stromal cells showed a higher percentage of positive cells than those with less stroma. The results suggest that Twist expression might be associated with invasion in ameloblastoma variants, and stromal cells might play a regulatory role during tumor development. [28]

Molecular markers involved in angiogenesis

Angiogenesis is an active process, regulated by a large number of proangiogenic and antiangiogenic molecules. The role of angiogenesis in ameloblastoma is also an area of research that has been increasingly focused on these years.

Growth factors and their receptors play a key role in the growth of normal tissues and the development and progression of human neoplasms. Myoken *et al*. [29] established a serum-free culture system for ameloblastoma cells and demonstrated the addition of fibroblast growth factor-1 (FGF-

1) and fibroblast growth factor-2 (FGF-2), which are mitogenic polypeptides that may contribute to neoplastic cell proliferation, and enhance cell growth in a dose-dependent manner. Ameloblast-like cells and stellate reticulum-like cells presented a high expression of FGF-1, whereas FGF-2 was identified mainly in the basement membrane. These results imply distinct roles for both molecules. FGF-1 might be associated with an autocrine mechanism of tumor growth, while FGF-2 would be involved not only in growth but also in the invasion process through the induction of proteases. Therefore, further studies are needed to confirm the specific roles of both FGF-1 and FGF-2 in ameloblastoma, as controversies regarding this matter are still apparent in the literature.

Molecular markers related to the function of tumor stromal cells on the invasion of ameloblastoma

It has been clearly shown that the mean number of myofibroblasts (MF) in solid ameloblastomas was high and did not differ significantly from that in squamous cell carcinoma. The study by Fregnani *et al.*[30] confirmed that MFs are the main source of MMP-2 in the stroma of ameloblastoma. It was also demonstrated that abundant presence of MFs of tumors and expression of MMP-2 in the neoplastic or stromal cells led to a more aggressive behavior, such as rupture of the osseous cortical. This has been considered an important prognostic marker of ameloblastoma aggressiveness.

The proliferation of stromal cells is commonly seen when cancer cells invade and metastasize. Another recent approach to the mechanism of invasion of ameloblastoma was focused on CD10, which is a cell surface zinc-dependent metalloprotease glycoprotein with endopeptidase activity and is present on the surface of many cell types. CD10 is associated with differentiation and growth of neoplastic cells, and CD10 expression is found to be increased with the increase of tumor dysplasia. The study reported that solid ameloblastoma with a high risk of recurrence was correlated with a high immunoreactivity for CD10 of the peritumoral stromal cells which showed a significantly higher percentage of peritumoral stromal CD10-positive cells than the uni cystic ameloblastoma and peripheral ameloblastoma variants. A strong intensity of immunostaining was observed only in solid ameloblastoma, suggesting that CD10 expression in stromal cells is associated with local tumor invasion and that the proliferation of CD10-positive stromal cells is part of the mechanism of invasive growth in ameloblastoma variants. CD10 immunostaining may be useful to identify areas with locally aggressive behavior also in low-risk ameloblastoma. [31]

Stromal and tumor cell interaction and their subsequent role in bone invasion resulting in tumor progression have not yet been reported in ameloblastoma. In the study by Sathi *et al.*, [32] stroma does not act only in bone resorption, but also in the suppression of new bone formation, in which sFRP-2 is the main factor for impaired bone formation. Tumor cells create a favorable environment for impaired bone formation by secreting sFRP-2 as well as bone resorption, by secreting RANKL and interleukin-6. The expression of markers related to osteoclastogenesis and suppression of osteoblast formation is higher in myxoid-type than in fibrous-type stroma.

In early stages of tumor progression, TGF-β1, a potent epithelial growth inhibitor, inhibits not only the growth but also the invasiveness of the tumor. But, when the tumor cells become metastatic, their ability of invasion may be stimulated by TGF-β1. TGF β1 also has a role in oncogenesis because TGF-β genes were found to be under expressed in all tumors. Several mechanisms might explain the tumor-promoting effects mediated by TGF-β: first

of all, a loss of the growth-inhibitory response to TGF-β; second, an increased expression and/or activation of the ligand; and third, a change in the signal transduction pathway, with the acquisition of a more invasive phenotype. From the study by Iezzi, [33] TGF-β showed increased expression in ameloblastomas with a high risk of recurrence. The interesting finding could be explained by the fact that although TGF-β acts as a potent tumor suppressor in the early stages of tumor progression, later it seems to enhance the invasive phenotype of the tumor. In AMs, the mechanisms underlying the increased stromal cell expression of TGF-β in tumors with a high risk of recurrence is presently not known. Thus, further investigations are needed to elucidate the mechanisms.

Markers involved in cell proliferation related to invasion:

The p27 protein binds to and inhibits a number of cyclin-CDK complexes and thus plays an important role in the regulation of the cell cycle in the normal cell. In resting cells, the level of p27 provides an inhibitory threshold above which G1 cyclins D/E/CDK accumulate before activation, while in cycling cells, it is well established that p21 mediates the growth inhibitory effects of p53 in response to DNA damage and other stresses. It is suggested that the genesis and invasion of ameloblastoma are associated with cell proliferation and in differentiation and regulated by the higher expression of cyclin E and the lower expression of $p21^{WAF1}$ and $p27^{KIP1}$. [34]

Conclusions

The mechanism of tumor invasion in ameloblastoma is very complex, involving a variety of adhesion molecules, MMPs, cytokines, and associated genetic changes. Studies concerning this area are not few. Taken together with the data in the new English literature, the mechanism underlying the local invasiveness of ameloblastoma remains unknown. Further studies, namely, the quantitative assessment and molecular studies, should be encouraged to clarify the essence of the molecules of tumor invasion in ameloblastoma and establish the relationship. Moreover, mechanisms underlying the local invasiveness of four variants of ameloblastomas should be further studied. Particularly, the analysis of the invasive front of the tumor with regard to the occurrence of molecules is supposed to be of great importance. It is likely that further investigations on the regulation of molecular markers involved in tumor invasion in ameloblastomas, as well as the coordination among them may provide a better understanding of the process, leading to the development of more efficient prevention, diagnosis, and treatment approach.

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