Expression of midkine in ameloblastomas and its correlation with clinicopathologic parameters

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Objective. Midkine (MK) is a heparin-binding growth factor that is overexpressed in various human cancers. The aim of this study was to investigate the expression of MK in ameloblastomas and correlate the results with clinicopathologic parameters.

Study Design. Cases of ameloblastoma seen between 1999 and 2010 were identified. Clinical information was collected regarding age, gender, race, and location of tumor. Cases were classified as solid/multicystic, unicystic, and peripheral. The expression of midkine was assessed using immunohistochemistry. A significant difference was considered present at $P < 0.05$.

Results. A total of 34 cases of ameloblastoma and 4 cases of ameloblastic carcinomas were identified. MK was expressed in 67% of lesions (23.5% weak expression; 14.7% moderate expression; 29.4% strong expression). A significant difference was seen between solid/multicystic and unicystic lesions.

Conclusions. MK is expressed in the majority of ameloblastomas, suggesting a role of the protein in the tumor's development, progression, and behavior. (Oral Surg Oral Med Oral Pathol Oral Radiol 2012;114:497-502)

Ameloblastoma is a locally aggressive, benign neoplasm with a tendency to invade the surrounding tissues and characterized by a relatively high risk of recurrence.[1] It is the most frequent clinically significant odontogenic tumor identified, with studies from different regions of the world indicating that it accounts for 9% to 88% of all odontogenic neoplasms. In the United States, ameloblastomas represent nearly 11% of all odontogenic neoplasms.[2]

Ameloblastomas are clinically and radiographically divided into solid/multicystic, unicystic, and peripheral. Solid/multicystic is the most common and aggressive type.[3] Unicystic and peripheral ameloblastomas represent less common lesions, with reports indicating a less aggressive behavior.[4,5] Microscopically, ameloblastomas show a wide variety of patterns, without major influence on the behavior of the tumor.[5] The treatment for ameloblastoma ranges from conservative enucleation to radical resection of the jaw, depending on the size, location, and subtype. The recurrence rate for solid/multicystic tumors after conservative treatment varies from 36% to 93% and from 8% to 21% with aggressive radical treatment.[6]

Midkine (MK) is a 13-kDa heparin-binding growth factor[7,8] that was found as the product of a retinoic acid gene located at chromosome 11p11.2.[9] MK is highly

Statement of Clinical Relevance

Our findings show that immunohistochemical analysis of midkine may be useful in evaluating cases in which the hematoxylin–eosin characteristics are not easily recognizable as ameloblastomas and also demonstrate that the protein could possibly serve as a molecular-based therapeutic target for the treatment of ameloblastomas.

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expressed in the midgestational period during embryogenesis and is involved in lung, kidney, bone, and tooth development.[10] Although its expression is highly restricted in the normal tissues of adults, studies have demonstrated that this protein is overexpressed in various human cancers.[7,8] Indeed, this protein has various biological activities that can contribute to tumor progression, including positive effects on angiogenesis and cell proliferation, as well as antiapoptotic properties.[10-14]

Previous studies suggest that MK may be involved in the development of ameloblastomas[15,16] and that the protein may give a growth advantage to ameloblasts through the upregulation of the MAPK and Akt pathways.[15] However, these studies did not address whether levels of MK expression correlated with clinical and histopathologic patterns. Because we have previously shown that the PTEN/Akt/mTOR pathway is altered in ameloblastomas,[17] the purpose of this study was to immunohistochemically investigate the expression profile of MK in the tumor and to correlate the results with clinical and histologic parameters.

MATERIAL AND METHODS

Patients diagnosed with ameloblastoma and ameloblastic carcinoma of the maxilla, mandible, or gingiva and treated by primary tumor resection at the University of Maryland, Federal University of Goiás, and Chulalongkorn University between 1999 and 2010 were identified. Clinical records for the ameloblastoma patients were reviewed and information was gathered regarding age, gender, race, and location of the tumor. The microscopic features were reviewed based on a single 5-μm hematoxylin and eosin–stained section for each case. Based on clinical, radiographic, and pathologic criteria, ameloblastoma cases were classified as solid/multicystic, unicystic, or peripheral. A total of 15 dental follicles were used as a control. Internal Review Board exemption was received from the University of Maryland, Baltimore.

Paraffin-embedded tissues were sectioned (3 μm) and serially collected on glass slides coated with 2% 3-aminopropyltriethoxysilane (Sigma–Aldrich, St. Louis, MO). Following deparaffinization by immersion in xylene, the sections were immersed in alcohol and incubated with 3% hydrogen peroxide for 40 minutes. For antigen retrieval, the sections were immersed in citrate buffer (pH 6.0) for 20 minutes. Subsequently, the sections were incubated for 20 minutes with 3% normal goat serum at room temperature. The slides were incubated at 4°C overnight with monoclonal mouse antihuman MK primary antibody (clone A-9, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:50. After being washed with Tris-buffered saline, the sections were labeled with streptavidin–biotin (LSAB kit K0492; Dako, Carpinteria, CA) and then incubated with 3,3′-diaminobenzidine (K3468, Dako) for 2 to 5 minutes at room temperature. Finally, the sections were stained with Mayer's hematoxylin and covered. Negative controls were obtained by omission of the primary antibodies, which were substituted with 1% phosphate-buffered saline/bovine serum albumin.

Two independent individuals with advanced education in oral pathology reviewed the immunostains. Immunohistochemical reactivity for all stains were graded in a semiquantitative manner according to the percentage of positive tumor cells: (0) 0%, (1) <20%, (2) 20% to 50%, and (3) >50%, as well as the intensity of staining: (—) no staining, (w) weak, (m) moderate, or (s) strong. The values for the quality (w = 1, m = 2, s = 3) and quantity (0–3) of staining were added to give a single number for each tumor, which was used in the final evaluation. Staining was considered weak if the total score was <3, moderate if the score was between 3 and 5 points, and strong if the total score was 6 points.

Correlation between MK expression and gender, age (<50 vs >50 years), race, histologic type, and pattern of staining was performed. Last, we also reviewed the staining results for PTEN, p-PTEN, Akt, p-thr Akt, p-ser Akt, Erk, and p-S6K from our previous study[17] to search for a correlation with the MK staining findings. For all measurements where applicable, a Student *t* test was used to assess the statistical significance, along with standard error. A significant difference was considered present at $P < 0.05$.

RESULTS

A total of 34 cases of ameloblastoma and 4 cases of ameloblastic carcinomas were identified. For ameloblastomas, gender equated to 22 males (66%) and 11 females (34%) or 2:1, respectively, with a mean age of 48.8 years (range 16-86). Gender information was unavailable in 1 case. There were 20 white (58%), 13 black (38%), and 1 Asian (2%) patient. Thirty lesions were located in the mandible, 2 in the maxilla, and 1 involving the gingiva (peripherally). Based on clinicopathologic criteria, the 34 ameloblastomas were classified as solid/multicystic (24), unicystic (9), and peripheral (1). The average age of patients with solid/multicystic ameloblastomas was 54.8 years (range 22-86), with a 2.8:1 male-to-female ratio and a 1.1:1 white-to-black ratio. Most of these cases (22) were in the mandible (91%), with only 2 (19%) in the maxilla. Further, most solid/multicystic ameloblastomas appeared histologically with a follicular pattern (50%), followed by a plexiform configuration (34%), mixed follicular/plexiform pattern (8%), and acanthomatous

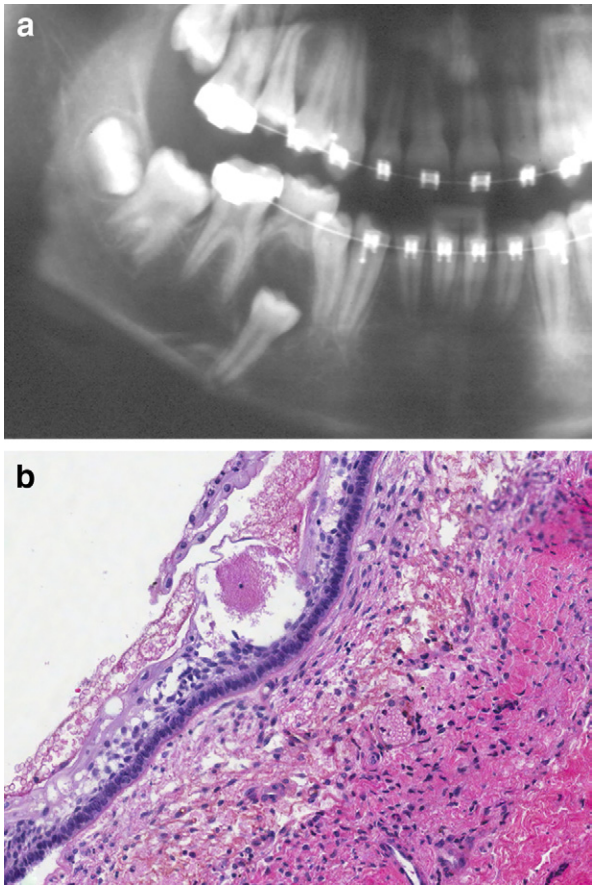

Fig. 1. (a) Radiographic and (b) histopathologic aspects of a unicystic ameloblastoma.

pattern (8%). The average age of patients with unicystic ameloblastomas was 36.7 years (range 11-70), with a 1.25:1 female-to-male ratio and a 3.5:1 white-to-black ratio. Eight lesions were found in the mandible and 1 in the maxilla. Figure 1 shows the radiographic and histopathologic aspects of a select case of unicystic ameloblastoma. The single case of peripheral ameloblastoma affected the gingiva of a 48-year-old white male. Histologically, the peripheral lesion presented with a follicular pattern (Table I). Finally, the average age of patients with ameloblastic carcinomas was 52.7 years (range 38-71), with a 3:1 male-to-female ratio. One lesion affected a white individual, 2 were seen in black patients, and race was unavailable in 1 case. All 4 ameloblastic carcinomas were found in the mandible. Follow-up information was not available for the cases.

MK was expressed in 67% (23 of 34 cases) of the ameloblastomas (weak expression in 23.5%; moderate expression in 14.7%; strong expression in 29.4%). The overall staining score was 3.05 when considering all lesions (positive and negative) and 4.52 when considering positive lesions only. Figure 2 shows representative images of MK immunohistochemistry. Regarding dental follicles, 6 of 15 showed MK positivity (1 case strong, 3 cases moderate, and 1 case weak expression), with an average staining score of 1.5. The average MK staining score was statistically higher in ameloblastomas than in dental follicles ($P = 0.03$). When stratified according to histologic subtype, solid/multicystic tumors showed an average score of 3.96, compared with a score of 1.00 for unicystic lesions ($P = 0.001$). The most common staining pattern was in the stellate reticulum–like cells only (56%), followed by staining observed in both the stellate reticulum–like cells and the peripheral columnar ameloblast–like cells (39% of cases; Figure 2). In 1 case, only the columnar ameloblast–like cells stained. Strong MK expression was found in 2 of 4 cases of ameloblastic carcinomas, whereas 1 case showed moderate staining (average score of 4.3). Figure 3 shows representative images of MK expression in a dental follicle, solid/multicystic ameloblastoma, unicystic ameloblastoma, and ameloblastic carcinoma. No significant differences in MK scores were observed in relation to age, gender, race, or pattern of staining.

Of the 34 cases stained for MK, 15 had been utilized in our previous study[17] where we assessed the expression of other proteins also involved in the PI3K/Akt pathway, namely PTEN, p-PTEN, Akt, p-thr Akt, p-ser Akt, Erk, and p-S6K. The average staining score for these proteins was 2.46, 0.60, 2.60, 2.94, 2.00, 2.13, and 3.13, respectively. In the ameloblastomas that were positive to MK (12 of 15), total PTEN was present in 75% of cases, whereas p-PTEN was present in only 25%. Total AKT was upregulated in 66.7% of cases of ameloblastoma, whereas p-thr AKT and p-ser AKT were overexpressed in 100% and 66.7% of cases, respectively. Active p-S6K expression (the terminal effector of the Akt/mTor pathway) was upregulated in 91.6% cases. Finally, ERK, a signaling molecule that cross-talks with AKT and can also activate mTOR and p-S6K, was also overexpressed in 66.7% of cases.

DISCUSSION

MK is a heparin-binding cytokine that promotes growth, survival, migration, and other activities of target cells.[18] To date, only 2 studies have assessed the expression of MK in ameloblastomas, an aggressive neoplasm that exhibits the disruption of various molecular pathways involved with cell proliferation and survival, including the sonic hedgehog and PI3K/Akt/mTor pathways.[19] Sandra et al.[15] examined, immunohistochemically, the expression of MK in 37 ameloblastomas. The protein was expressed in 70% of cases, mostly in the outer layer of ameloblast-like cells. Later, Fujita et al.[16] examined the expression of MK in various human odontogenic tumors, including 55 amelo-

Table I. Demographic data*

	Age (years)		Gender		Race			Location			Histologic typing			
	Range	Average	Male	Female	White	Black	Asian	Mandible	Maxilla	Gingiva	Follicular	Plexiform	Mixed	Acanthomatous
M/S (24)	22-86	54.8	17	6	12	11	1	22	2	—	12	8	2	2
U (9)	11-70	36.7	4	5	7	2	—	8	1	—	—	—	—	—
P (1)	—	48	1	—	1	—	—	—	—	1	1	—	—	—
AC (4)	38-71	52.7	3	1	1	2		4	—	—	—	—	—	—

AC, ameloblastic carcinoma; *M/S*, multicystic/solid; *P*, peripheral; *U*, unicystic.
*Gender was unavailable in 1 case of M/S and race was unavailable in 1 case of AC.

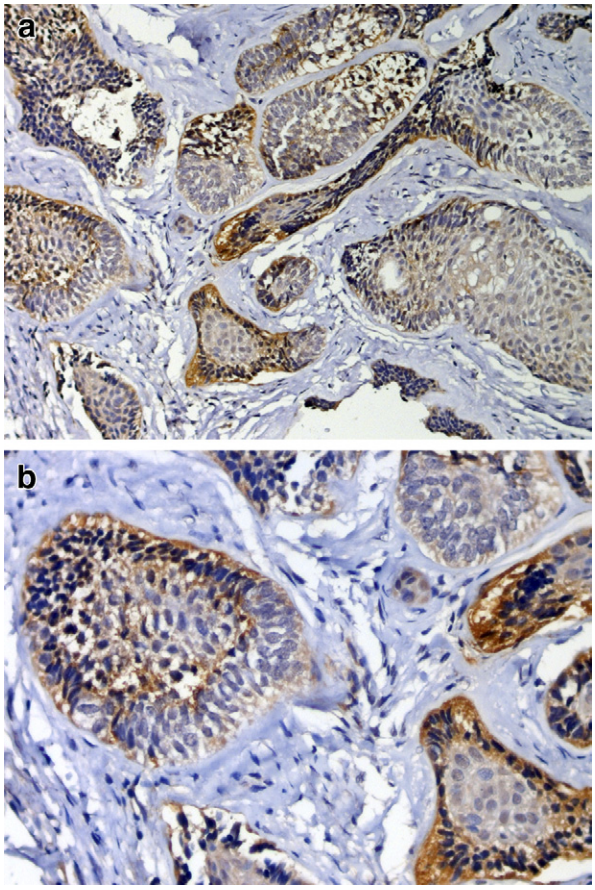

Fig. 2. Strong expression of midkine (brown stain) by stellate reticulum–like cells and peripheral columnar ameloblast–like cells in the solid/multicystic ameloblastoma. (b) A close-up view of (a). Immunohistochemical staining; original magnification: ×200 (a) and ×400 (b).

blastomas. MK localization in this study was found in 30 of 55 (54.5%) ameloblastomas, mostly confined to the columnar ameloblast–like cell cytoplasm. In our study, we found that 67% of the ameloblastomas stained for MK, a finding in between that of previous reports. A more significant difference was observed in the pattern of staining in relation to previous studies. Whereas previous studies showed that MK was expressed in the ameloblast-like cells, most of our cases showed staining of the stellate reticulum–like cells only or combined staining of both stellate reticulum–like and ameloblast-like cells. Only 1 of our cases showed staining of ameloblast-like cells alone. Importantly, previous studies did not assess whether a correlation existed between MK expression and clinicopathologic parameters. Thus, we also sought to investigate whether MK expression correlated with clinical and pathologic aspects of ameloblastomas and to determine whether the protein could be involved with distinct clinical behavior. Here, we observed that MK expression was significantly stronger in solid/multicystic lesions compared with unicystic tumors, suggesting that the protein may be involved in more aggressive behavior. Indeed, other studies have indicated that certain signaling pathways, such as the Notch pathway, may play roles in the acquisition of different ameloblastoma phenotypes.[20] A limitation of our study was the lack of follow-up data, which prevented us from investigating the correlation between MK expression and tumor biological aggressiveness.

The protein (serine/threonine) kinase Akt integrates a plethora of extracellular signals that lead to cell proliferation and survival.[21] Genetic alterations leading to an overactivation of the Akt pathway are frequent in a wide variety of human cancers.[22,23] Indeed, the Akt pathway is the second most frequently mutated pathway in cancer, after mutations of the tumor suppressor protein p53.[21] In this context, in vitro studies show that MK induces the phosphorylation (at Ser473 and Thr308) and activation of Akt and that upon pretreatment of cells with LY294002 (a PI3K/Akt inhibitor), MK-stimulated phosphorylation of Akt and MK-stimulated growth of cells was inhibited.[15] Fifteen of the 27 samples in our studies had been employed in a previous study where we analyzed the Akt pathway in ameloblastomas.[17] In the current study, we found that all MK-positive samples also showed activation of Akt at Thr308, whereas 66.7% showed upregulation of p-Akt at Ser473, in agreement with previous studies. However, we also observed that 25% of the samples lacked PTEN and 75% lacked p-PTEN, which could also account for the increased Akt activation seen in our study.

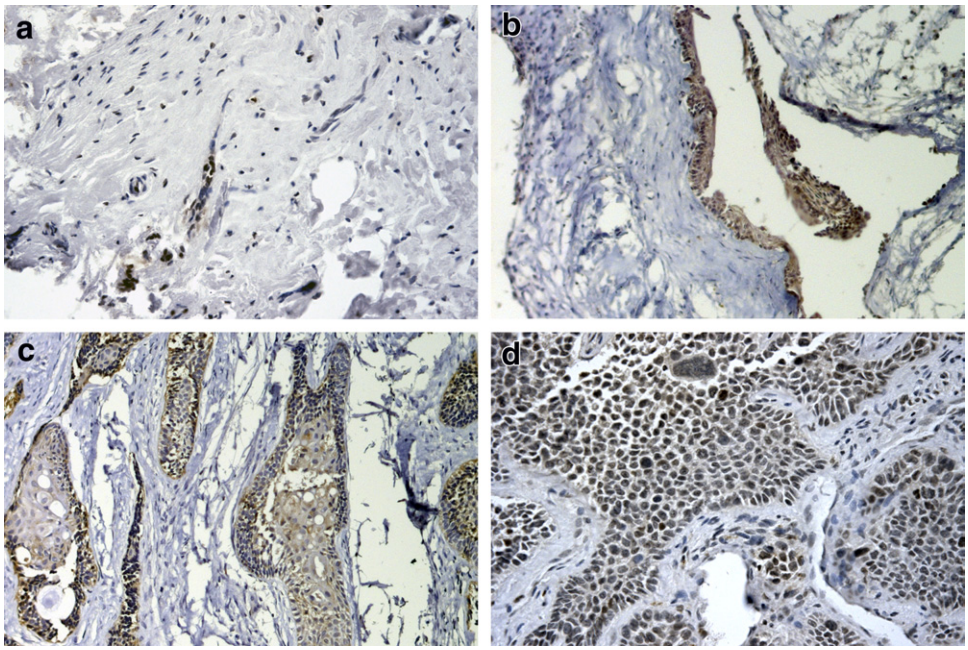

Fig. 3. Representative expression of midkine (brown stain) in dental follicle (a), unicystic ameloblastoma (b), solid/muticystic ameloblastoma (c), and ameloblastic carcinoma (d). Immunohistochemical staining; original magnification: ×00.

One of the most studied target factors of the PI3K/AKT pathway is mTOR, which is critical for cell proliferation, survival, and tumorigenesis.[24] In our study, p-S6K, which is the terminal effector of the mTOR pathway, was overexpressed in 86.6% of cases that were positive for MK, suggesting that the latter participates in the growth and progression of ameloblastomas through the Akt/mTOR pathway. Alternatively, MK may activate the mTOR pathway through Akt-independent pathways; indeed, we observed that 66.7% of the MK-positive samples also showed activation of Erk, a signaling molecule that can also activate p-S6K and mTOR. Together, these results indicate that MK may be an important therapeutic target in ameloblastomas, with the potential to block 2 important downstream pathways that participate in cell proliferation and survival (Akt/mTOR and Erk/mTOR).

Ameloblastic carcinoma is a rare, malignant, odontogenic tumor that arises de novo or from a preexisting ameloblastoma.[25] The microscopic features favoring the malignant transformation include the presence of sheets, islands, or trabeculae of epithelium; the absence of stellate reticulum–like structures; and round-to-spindled epithelial cells with little or no differentiation toward the columnar cell morphology of ameloblastoma.[26] Clinically, Yoon et al.[25] found that ameloblastic carcinoma showed a much higher mean age, a higher rate of occurrence in men, and a relatively higher proportion of maxillary lesions compared with ameloblastoma. Similarly, our 4 cases also showed high mean age and were more frequent in men; however, all cases were seen in the mandible. Because of its rarity, there are few immunoprofile studies of ameloblastic carcinoma and few comparative studies of ameloblastic carcinoma and ameloblastoma.[25] Thus, in the current study we also stained ameloblastic carcinomas with MK and found that three quarters of the tumors (75%) were moderately to strongly positive to the protein, with scores higher than that seen for ameloblastomas. Our results are in agreement with the study by Fujita et al.,[16] who found that two thirds (66%) of ameloblastic carcinomas were positive to MK (compared with 54.5% of ameloblastomas).

In conclusion, our results show that MK is expressed in the majority of ameloblastomas, particularly solid/multicystic lesions, suggesting a role of the protein in the development, progression, and behavior of the tumor. The immunohistochemical analysis of MK may be useful in evaluating cases where the hematoxylin–eosin characteristics are not easily recognizable as ameloblastomas. In addition, MK may represent a molecular-based target for the treatment of ameloblastomas. Further studies are warranted to confirm our observations.

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