Title
Midkine expression in malignant salivary gland tumors and its role in tumor angiogenesis

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SUMMARY

The aims of this study were to investigate midkine (MK) expression patterns in salivary gland tumors (SGTs) and to evaluate the correlation between MK expression and the degree of malignancy. We performed immunohistochemistry to examine MK expression in specimens of adenoid cystic carcinoma (ACC), mucoepidermoid carcinoma (MEC), and pleomorphic adenoma (PA). In addition, we performed immunohistochemistry for CD31 and measured microvessel density (MVD), which is an indicator of angiogenesis. Immunohistochemistry showed that MK protein expression was significantly higher in specimens of malignant SGTs (ACC [$P < 0.01$] and MEC [$P < 0.001$]) than in benign SGT (PA) samples. Furthermore, MVD values tended to be higher in cases that exhibited high expression of MK, which indicated a significant correlation between the degree of MK expression and MVD ($P < 0.001$). These results suggest that MK may play important roles in malignant transformation and tumor angiogenesis in SGTs.

Keywords

Midkine, Head and neck cancer, Salivary gland tumor, Adenoid cystic carcinoma, Mucoepidermoid carcinoma, Pleomorphic adenoma, Pathologic angiogenesis, CD31 antigen, Immunohistochemistry
Introduction

Salivary gland tumors (SGTs) have been documented to make up 1-4% of all human neoplasms.\textsuperscript{1, 2} The majority of SGTs have an epithelial origin (90%), with nearly 70% being pleomorphic adenomas (PAs).\textsuperscript{3} Of all malignant SGTs, specifically carcinomas, mucoepidermoid carcinoma (MEC; 35-45%) and adenoid cystic carcinoma (ACC; 20-30%) are most common.\textsuperscript{4, 5} SGTs show various morphological features that complicate making a precise diagnosis. Furthermore, the diversity of salivary gland malignancies in terms of morphology and clinical course makes defining risk factors for these tumors difficult.

The molecular development of SGTs remains unclear. Although malignant SGTs are slow-growing carcinomas characterized by wide local infiltration, ACCs and high-grade MECs have a comparatively poor long-term prognosis with regard to local recurrence, perineural invasion, and distant metastasis. In those types of malignant SGTs, no clear indicator exists within or around the tumors to allow a precise diagnosis or prediction of prognosis. For this reason, discovering new molecules that can be utilized for efficient diagnosis and therapy of SGTs is an important challenge.

Midkine (MK), which is a heparin-binding growth factor that was initially found as the product of a retinoic acid-induced gene, participates in various biological
activities, including neuronal survival, angiogenesis, promotion of fibroblast growth, suppression of apoptosis, inflammatory cell migration, and mitogenesis. Thus, increased MK expression was found in patients with ischemic nephritis, rheumatoid arthritis, cerebral infarct, and Alzheimer’s disease. In addition, MK has been reported to play important roles in carcinogenesis and tumor progression and was expressed in higher concentrations in different malignant tumors, including breast, lung, prostate, colorectal, and oral squamous cell carcinomas. MK protein expression was reportedly correlated with poor prognosis in patients with neuroblastomas, pancreatic head carcinomas, and oral squamous cell carcinomas. Moreover, previous studies revealed that MK protein expression was significantly correlated with angiogenesis in esophageal squamous cell, pancreatic head, and oral squamous cell carcinomas. However, no studies have focused on MK expression in SGTs.

Here, we investigated the expression pattern of MK in various types of SGTs in 45 patients (15 with ACCs; 15 with MECs; 15 with PAs). We used immunohistochemistry to compare the expression of MK protein in these SGTs and to evaluate its correlation with microvessel density (MVD) in each SGT.
Materials and methods

Materials

The primary antibodies used were monoclonal MK antibody (IP-14; Cellmid Limited, Sydney, Australia) and monoclonal CD31 antibody (JA70A; DakoCytomation, Glostrup, Denmark). Anti-mouse labeled polymer (EnVision+ System-HRP) from DakoCytomation served as the secondary antibody.

Patients and tissue specimens

Tissue specimens were obtained from 45 patients with SGTs who underwent surgery at the Department of Oral and Maxillofacial Surgery (Kumamoto University Hospital) between 1995 and 2008. Fifteen ACC specimens, 15 MEC specimens, and 15 PA specimens were collected. Patients included 11 males and 34 females, with a mean age of 57.6 ± 14.47 years (median 60; range 16-90). Of the ACC specimens, 10 had a cribriform appearance, 1 was a solid type, and 4 showed a tubular pattern. Of the MEC specimens, 12 were low-grade tumors and 3 were high-grade tumors. All subjects gave informed consent for their participation in this investigation.

Immunohistochemistry

We performed immunohistochemical analysis with primary antibodies: IP-14 to identify MK and JA70A to identify CD31. Paraffin-embedded 4-µm-thick sections were
prepared, deparaffinized in xylene, and rehydrated in a graded series of alcohol. Endogenous peroxidase activity was blocked by immersing the sections in 0.3% hydrogen peroxide in methanol for 30 min, and antigens were retrieved by using a microwave (MK: 15min; CD31: 30 min) with the sections in a citrate buffer (pH 6.0). After sections were incubated with Protein Block Serum-Free (Dako), they were incubated overnight at 4 °C with primary antibodies (MK: dilution 1:100; CD31: dilution 1:50). After incubation of the sections with anti-mouse labeled polymer (EnVision+ System-HRP) for 30 min at room temperature, 3,3′-diaminobenzidine was used as the chromogen. Sections were immunohistochemically stained and were then counterstained with hematoxylin to enhance nuclear detection.

Two independent observers interpreted the immunohistochemical data in a blinded fashion. For each specimen, one score was assigned according to the percentage of positive cells: <5% of the cells: 1 point; 6-35% of the cells: 2 points; 36-70% of the cells: 3 points; and >71% of the cells: 4 points. Another score was assigned according to the intensity of staining, with negative staining equal to 1 point; weak staining, 2 points; moderate staining, 3 points; and strong staining, 4 points. MK expression score was then calculated by multiplying the two scores just described. If the MK expression score was ≥4, the tumor was considered to be positive.
Evaluation of CD31 positivity was restricted to intratumor microvessels or microvessels at the tumor invasion front. Five areas containing high numbers of microvessels were randomly selected by using a microscope field at 200× magnification to count the number of microvessels. The MVD, which is a common indicator of the extent of tumor angiogenesis, was then determined via the method previously described by Weidner et al.²⁵ The MVD was defined as the mean value of the microvessel density in the five selected areas of the tumor.

**Statistical analyses**

MK immunoreactivity data were evaluated and analyzed with Student’s t test to assess differences among the three groups—ACC, MEC, and PA. Analyses were performed with JMP IN (version 5.1; SAS Institute Japan, Tokyo, Japan). The correlation between MK expression and MVD was calculated by using Pearson product-moment correlation coefficient. P values of less than 0.05 were regarded as statistically significant.

**Results**

**MK protein levels in SGTs**

We performed an immunohistochemical analysis for MK (Fig. 1a-g) and
quantitatively evaluated the degree of MK expression score (Fig. 1h). In malignant SGTs specimens, MK protein immunoreactivity was localized mainly in the cell cytoplasm and rarely in the cell nuclei (Fig. 1a-e). In 13 of 15 ACC samples, all three typical ACC histological tissue types—cribriform, solid, and tubular—showed positive MK expression (Fig. 1a-c). Similar patterns of MK protein expression were also observed in each histological type. The two negative ACC specimens were cribriform. All 15 MEC specimens demonstrated positive MK protein immunoreactivity in mucous cells, intermediate cells, and epidermoid cells (Fig. 1d and e). MK reactivity was localized mainly in the cytoplasm of mucous cells, whereas in intermediate cells and epidermoid cells, it was found in both nuclei and cytoplasm. MK expression in PA tissues (Fig. 1f and g) was lower than that in malignant tissues, however: 10 of 15 benign PA specimens were negative for MK expression.

Immunoreactivity of MK protein among the three different histological types of SGTs was also quantitated. MK immunoreactivity of specimens of two malignant tumors (ACC and MEC) was significantly higher than that of benign tumor (PA) (Fig. 1h). The level of statistical significance between ACC and PA was $P < 0.01$, and that between MEC and PA was $P < 0.001$. 
Correlation between MK expression and MVD in SGTs

A recent report documented that the angiogenic action of MK in tumors accelerated tumor growth and increased tumor vascularity in nude mice. To determine whether angiogenesis may be involved in MK metabolism, we analyzed the correlation between MK expression and MVD in SGTs. Figure 2a shows a typical tumor sample with a high MVD. The MVD value tended to be higher in samples with high expression of MK. A statistically significant correlation was observed between MK expression and MVD ($P < 0.001, r = 0.70$) (Fig. 2b).

Discussion

In this study, we demonstrated two new important findings. First, malignant SGT specimens (ACC and MEC) evidenced higher MK expression than did in benign SGT (PA) tissues. We also confirmed, via Western blotting analysis, stronger results for ACC and MEC specimens than in PA samples (Fig. S1).

MK has recently become a topic of great interest in cancer research. High MK expression levels were reported in various cancer specimens including breast, lung, prostate, colorectal, and oral squamous cell carcinomas. No studies, however, examined MK expression in SGTs. Our results here suggest that MK may play an
important role in malignant transformation in SGTs.

Our study also determined that immunoreactivity for MK protein was located in both cell nuclei and cytoplasm in epidermoid and intermediate cells of MEC tissues (Fig. 1d and e). High-grade MEC samples showed larger numbers of epidermoid and intermediate cells compared with low-grade MEC specimens. A previous study showed that full MK activity required nuclear targeting during promotion of cell survival. For this reason, our observations suggest that a tendency toward malignant formation may be correlated with MK protein expression.

In addition, our study revealed that MK expression was significantly correlated with MVD. High MVD occurred more often in tumors with high MK expression, which reflects a positive effect of MK on tumor vascularity in SGTs. This finding suggests that MK may have an important function in tumor angiogenesis in SGTs (Fig. 2b). However, the mechanism of this function is still unclear. Previous studies found that MK protein expression was significantly correlated with expression of vascular endothelial growth factor (VEGF) in esophageal squamous cell carcinomas, pancreatic head carcinomas, and oral squamous cell carcinomas. However, another recent study reported that MK down-regulated VEGF-A-induced neovascularization and vascular permeability. Moreover, additional studies reported that, in some cases, high MVD was seen in
tumors with high MK expression despite low levels of VEGF expression. Further investigations are necessary to clarify the correlation between the role of MK in tumor angiogenesis and VEGF.

Another interesting finding from a clinical point of view is that our present results may also suggest a correlation between MK expression and difficulties encountered in treating malignant SGTs. ACCs and high-grade MECs have been well documented to show resistance to chemotherapy and radiotherapy. For this reason, treatment options are limited, with the consequence being a high recurrence rate and poor prognosis.

Previous studies reported that MK protein rescued Wilms’ tumor cells from cisplatin-induced apoptosis by up-regulation of Bcl-2 and that increased MK expression exerted a significant cytoprotective effect against doxorubicin in drug-sensitive cells. These results suggested that MK indirectly mediates acquired drug resistance to protect neighboring drug-sensitive cells and contributes to development of resistance to chemotherapeutics. Another previous study also demonstrated that combined therapy utilizing MK small interfering RNA and paclitaxel significantly enhanced anticancer activity or maintained the effective anticancer activity of paclitaxel. Inhibiting production of MK may be a novel tool against drug-resistant
cancers such as malignant SGTs.

In conclusion, we established that MK expression was higher in malignant SGTs (ACC and MEC) than in benign SGTs (PA) and that MK expression was significantly correlated with MVD. These results suggest that MK expression levels in tissues may be correlated with malignancy and tumor angiogenesis in SGTs. Further investigations utilizing molecular genetics methods are required to support our new findings.
Conflict of Interest Statement

None declared.

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References


Captions

Fig. 1. Immunohistochemistry of MK in SGTs and evaluation of MK expression in malignant SGTs (ACC and MEC) and benign SGTs (PA).

ACCs: (a) cribriform, (b) solid, (c) tubular. MEC: (d) area with mucous cells, (e) area with intermediate cells and epidermoid cells. PA: (f) cellular PA, (g) representative PA. (h) Comparison of MK immunoreactivity of three different histological types of SGTs. The mean (± SD) MK expression scores from ACC, MEC, and PA tissues were 5.79 ± 1.76, 6.71 ± 1.07, and 3.64 ± 1.65, respectively. MK immunoreactivity in the two malignant tumor specimens (ACC and MEC) was significantly higher than that in the benign tumor (PA), with the significant values via Student’s t test being $P < 0.01$ for ACC versus PA and $P < 0.001$ for MEC versus PA. *$P < 0.01$; **$P < 0.001$.

Fig. 2. Quantitation of MVD after immunostaining of CD31 and evaluation of the correlation between MK expression and MVD in SGTs.

(a) Representative tumor specimen with high MVD. A microscope field at 200× magnification was used to select five areas for counting the number of microvessels. The MVD was defined as the mean value of the number of microvessels in these areas.

(b) Relationship between MK expression and MVD in SGTs. The mean MVD value (± SD) in SGTs was 31.29 ± 12.65 (range per field, 8.7–59.6). The MVD values (± SD) for ACC, MEC, and PA tissues were 37.20 ± 12.10, 35.21 ± 8.10, and 22.12 ± 11.96,
respectively. Microvessels in ACC and MEC were much denser than those in of PA ($r = 0.70$, $P < 0.001$, $y = 4.5949x + 6.5057$, Pearson product-moment correlation coefficient).

Fig. S1. Western blotting analysis for MK protein in SGTs.

Western blotting to identify MK was performed with anti-MK antibody (EP1143Y) as a primary antibody.

Lanes 1-3: adenoid cystic carcinoma (ACC); lanes 4-6: mucoepidermoid carcinoma (MEC); and lanes 7-9: pleomorphic adenoma (PA). MK protein expression was higher in the malignant tumor specimens—ACC (3/3) and MEC (2/3)—than in the benign tumor, PA (1/3).