Expression of Autocrine Motility Factor Correlates with the Angiogenic Phenotype of and Poor Prognosis for Human Gastric Cancer

Weida Gong,1 Yixing Jiang,2 Liwei Wang,2 Daoyan Wei,2 James Yao,2 Suyun Huang,1,3 Shengyun Fang,4 and Keping Xie2,3

Abstract

Autocrine motility factor (AMF) is a cytokine known to regulate tumor cell motility. Recent studies have extended its role to many other aspects of cancer biology. In the present study, we examined the level of AMF expression and its relationship with vascular endothelial growth factor (VEGF) expression and the angiogenic phenotype in human gastric cancer and their effect on survival. The AMF and VEGF expression level and tumor microvessel density (MVD) status in archived tissue specimens from 86 resected gastric cancer cases were determined. AMF expression was significantly higher in both primary tumors and lymph node metastases than in adjacent normal gastric mucosa and normal gastric mucosa from individuals without gastric cancer. In univariate survival analyses, strong AMF expression was associated with inferior survival ($P = 0.028$). In a Cox proportional hazards model, strong AMF expression ($P = 0.019$) was independently prognostic of poor survival. Strong AMF expression in the lymph node metastases was associated with poor survival ($P = 0.011$). Furthermore, AMF expression in the primary tumors was directly correlated with VEGF expression and MVD status. We found the first clinical evidence that AMF expression is directly correlated with VEGF expression and MVD status and predicts clinical outcome in patients with gastric cancer, supporting the hypothesis that the AMF/AMF receptor pathway plays an important role in multiple aspects of cancer biology.

Cell motility (i.e., movement) is an essential component of normal development, inflammation, tissue repair, and angiogenesis (1). Tumor cell motility is required for tumor invasion and metastasis. The locomotory machinery of cells is regulated by a complicated linkage between cell surface receptors or sensors and the internal cytoskeleton. Many lines of evidence have shown that tumor cells respond in a motile fashion to a variety of external ligands, including autocrine motility factors (AMF), growth factors, and components of the extracellular matrix (2). Recent studies have shown a group of motility-stimulating proteins that do not seem to fit into any of these categories, including scatter factor, AMF, and migration-stimulating proteins (3). AMF expression is detected in various types of normal fibroblast and epithelium as well as tumor cells (5–7). As a specific motility modifier, AMF was originally identified because of its ability to induce migration of cells and has been implicated to play a role in stimulating motility during invasion and metastasis (5). AMF stimulates random and directed cell motility via a receptor-mediated signaling pathway (5). Signal transduction following binding of AMF to its receptor (AMFR), a cell surface glycoprotein with a molecular mass of 78 kDa (gp78) that is homologous to p53, is mediated by a pertussis toxin-sensitive G protein, production of inositol phosphate, and phosphorylation of gp78 (3, 4). Binding of AMF to its receptor induces signal transduction in a manner similar to chemotactic stimulation of neutrophil mobility as well as internalization and transport of its receptor to the leading edge, stimulating pseudopodial protrusion and cell motility (6, 7). Because autonomous motility of tumor cells plays an important role in recurrent disease (5, 8), detection of AMF likely will provide a new tool for cancer diagnosis. This is strongly supported by several recent studies demonstrating that...
increased expression of AMFR is strongly correlated with a high incidence of recurrence and decreased survival in patients with colorectal, bladder, esophageal, or gastric cancer (9–12). AMF secreted by tumor cells up-regulates vascular endothelial growth factor (VEGF) receptor (FLT-1) expression in endothelial cells (13). Further characterization of AMF may yield valuable insights for gastric cancer prognosis and treatment. In particular, immobilizing tumor cells may be a crucial step in inhibiting metastasis (1, 3). However, whether AMF overexpression confers an elevated angiogenic phenotype to gastric cancer cells, at least in part through elevated VEGF expression, and whether AMF is an independent prognostic marker in gastric cancer are not known.

In the present study, we examined the level of AMF expression and its relationship with VEGF expression and the angiogenic phenotype in tumor tissue specimens obtained from patients with resected gastric cancer and their impact on survival duration. We found that elevated AMF expression strongly correlated with VEGF expression and microvessel density (MVD) status but inversely correlated with survival. Therefore, abnormally expressed AMF overexpression may contribute to angiogenesis and overall aggressive biology of gastric cancer and is a potential molecular marker for poor prognosis and potential therapeutic target in patients with gastric cancer.

Materials and Methods

**Human tissue specimens and patient information.** We used human gastric cancer tissue specimens preserved in the Gastric Cancer Tissue Bank and obtained information about the respective patients from the bank’s comprehensive database at University of Texas M. D. Anderson Cancer Center. These patients underwent diagnosis and treatment of their primary gastric cancer at M. D. Anderson Cancer Center from 1985 to 1998. The patients had well-documented clinical histories and follow-up information. None of them underwent preoperative chemotherapy and/or radiation therapy. We selected 86 cases to represent all of the stages and histologic types of malignant gastric cancer for the present study. All of the patients had undergone gastrectomy with lymph node dissection and had been observed at M. D. Anderson through the end of 1999. The median follow-up duration was 25.7 months. At the last follow-up examination, 30 patients were still alive, whereas 56 had died. We included 86 primary tumor specimens, 53 lymph node metastasis specimens, 32 cases of adjacent normal gastric tissue, and 57 normal gastric tissue specimens obtained from patients without gastric cancer in the present study. The patients consisted of 56 men and 30 women, and their mean age was 62 years. Twenty cases had proximal cancer localization. Fifty-three patients had an intestinal-type Lauren’s histopathologic classification, whereas 33 had a diffuse-type classification. Table 1 contains these and other patient characteristics.

**Immunohistochemistry.** Sections (5 μm thick) of formalin-fixed, paraffin-embedded tumor specimens were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was done with 0.05% saponin for 30 minutes at room temperature. Endogenous peroxidase was blocked by using 3% hydrogen peroxide in PBS for 12 minutes. The specimens were incubated with a protein-blocking solution consisting of PBS (pH 7.5) containing 5% normal horse serum and 1% normal goat serum for 20 minutes at room temperature and then incubated at 4°C in a 1:500 dilution of a rabbit polyclonal antibody against human AMF or 1:100 dilution of a rabbit polyclonal antibody against human VEGF (clone A-20; Santa Cruz Biotechnology, Santa Cruz, CA). The rabbit anti-human AMF polyclonal antibody was generated using glutathione S-transferase fusion of the full-length AMF. The anti–glutathione S-transferase antibodies were removed by passing the serum from glutathione S-transferase on glutathione beads. The specimens were then rinsed and incubated with peroxidase-conjugated anti-rabbit IgG for 1 hour at room temperature. Next, slides were rinsed with PBS and incubated for 5 minutes with diaminobenzidine (Research Genetics, Huntsville, AL). The sections were washed thrice with distilled water, counterstained with Mayer’s hematoxylin (Biogenex Laboratories, San Ramon, CA), and washed once each with distilled water and PBS. Afterward, the slides were mounted by using a universal mount (Research Genetics) and examined under a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm. Depending on the percentage of positive cells and staining intensity, AMF and VEGF staining were classified into three groups: negative, weak, and strong expression. Specifically, the percentage of positive cells was divided into five grades (percentage scores): 0 (<10%), 1 (10-25%), 2 (26-50%), 3 (51-75%), and 4 (>75%). The staining intensity was divided into four grades (intensity scores): 0 (no staining), 1 (light brown), 2 (brown), and 3 (dark brown). AMF and VEGF staining positivity was determined using the following formula: overall score = percentage score × intensity score. An overall score of ≤3, >3 to ≤6, and >6 was defined as negative, weak positive, and strong positive, respectively (14). Two independent investigators scored the sections without knowledge of the patients’ outcome (double-blinded). A mean value of the two scores was presented in the present study.

**Western blot analysis.** Fresh gastric cancer and matched adjacent noncancerous gastric tissues were obtained from patients who underwent gastrectomy at M. D. Anderson Cancer Center. The cancerous and noncancerous portions were macroscopically identified and excised by experienced pathologists and further confirmed later by histopathologic examination. All fresh tissues used in this study were flash frozen in liquid nitrogen and whole-cell lysates were extracted as described previously (15). Four paired normal gastric and gastric tumor tissue specimens from the patients with known levels of expression of AMF and VEGF as confirmed by immunostaining as well as similar percentages of tumor epithelial cells present relative to stroma were selected. Standard Western blotting was done with a polyclonal rabbit antibody against human AMF, a polyclonal rabbit antibody against human VEGF, and anti-rabbit IgG, a horseradish peroxidase-linked F(ab’)_2 fragment obtained from a donkey (Amersham Life Sciences, Chicago, IL). The兔 polyclonal antibody against human VEGF and anti-rabbit IgG, a horseradish peroxidase-linked F(ab’)_2 fragment obtained from a donkey (Amersham Life Sciences, Chicago, IL).

<table>
<thead>
<tr>
<th>Parameter</th>
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<tr>
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<tr>
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<td></td>
<td>28</td>
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<td>III</td>
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<td>30</td>
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<tr>
<td>IV</td>
<td></td>
<td>14</td>
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<tr>
<td>Tumor location</td>
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<td>Cardia</td>
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<td>66</td>
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<tr>
<td>Gastric body and antrum</td>
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<td>Completeness of resection</td>
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<tr>
<td>R0</td>
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<td>53</td>
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<td>R1, R2</td>
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<td>33</td>
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<tr>
<td>Lauren’s classification</td>
<td></td>
<td>33</td>
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NOTE: Pearson’s χ² test was done to determine the statistical significance of the relationship of AMF expression with various parameters.
Arlington Heights, IL). Equal protein-sample loading was monitored by incubating the same membrane filter with an anti–β-actin antibody (14). The probe proteins were detected by using the Amersham enhanced chemiluminescence system according to the instructions of the manufacturer.

Quantification of tumor micro vessel density. For CD34 staining, tissue sections were processed and stained with a 1:100 dilution of a monoclonal goat anti-CD34 antibody (PECAM1-M20; Santa Cruz Biotechnology) and peroxidase-conjugated anti-goat IgG and then counterstained with Mayer's hematoxylin (Biogenex Laboratories). Slides were mounted with the sections and examined under a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm. For quantification of tumor MVD, highly vascular areas were initially identified by scanning tumor sections under a light microscope at low power. Vessels were counted in areas of the tumor specimens containing the highest numbers of capillaries and small venules based on the criteria described by Weidner et al. (16). Vessels in five high-power fields (×200 magnification, ×10 ocular) were counted by two independent investigators without knowledge of the patients’ outcome (double-blinded). A mean value of the two scores was presented. The MVD was classified into three groups: low (<50 vessels per five high-power fields), moderate (50-100 vessels), and high (>100 vessels).

Statistical analysis. The two-tailed χ2 test was done to determine the significance of the difference between the covariates. Survival durations were calculated by using the Kaplan-Meier method. The log-rank test was used to compare the cumulative survival durations in the patient groups. Also, the Cox proportional hazards model was used to compute univariate and multivariate hazards ratios for the study parameters. The patients’ level of AMF and VEGF expression, MVD status, age, gender, Lauren’s histologic classification, disease stage (American Joint Committee on Cancer), and completeness of surgical resection (R0 versus R1) were included in the model. In all of the tests, a P value < 0.05 was defined as statistically significant. The SPSS software program (version 11.05; SPSS Inc., Chicago, IL) was used for the analyses.

Results

Autocrine motility factor expression in normal human gastric mucosa, tumor tissue, and metastatic lymph nodes. AMF expression was evaluated in the primary tumor tissue of all 86 patients by using immunohistochemistry. AMF expression was classified as strong, weak, and negative in 32 (37%), 41 (48%), and 13 (15%) cases, respectively (Table 1). No significant differences in the distribution of the patients according to gender, race/ethnicity, type of resection, residual disease status, extent of lymphadenectomy, or Lauren’s histologic classification were detected in the three AMF expression categories. When AMF expression was compared in tumors located in proximal (cardia) and nonproximal (gastric body and antrum) regions, there was no significant difference (Table 1). When AMF expressions in normal gastric mucosa, primary gastric tumors, and lymph node metastases were compared, significantly higher AMF expression was found in both primary tumors and metastases when compared with that in adjacent normal gastric tissue and normal mucosa obtained from individuals without gastric cancer (Fig. 1A). Representative pictures of AMF expression in the normal mucosa, primary tumors, and metastases are shown in Fig. 1B. The samples with known AMF expression were further selected for confirmation of AMF protein expression by using Western blot analysis. As shown in Fig. 1C, AMF protein expression was higher in the primary tumors than in the matched adjacent normal mucosa.

Association of strong autocrine motility factor expression with poor survival. The median survival duration in patients who had a tumor with negative, weak, and strong AMF expression was 53, 59, and 37 months, respectively. Strong AMF expression was associated with an inferior survival duration.
(P = 0.028). The patients' AMF expression level, disease stage, completeness of resection, age, and Lauren's histologic classification were entered into a Cox proportional hazards model for multivariate analysis. After adjustment for the effect of covariates, strong AMF expression (P = 0.019) was found to be an independent predictor of poor survival (Fig. 2A). The hazard ratio in the patients with strong AMF expression (3.6; 95% confidence interval, 1.23-10.49) was significantly higher than that in the patients with negative AMF expression (reference). Additionally, there was a trend toward worse survival in patients with weak AMF expression. The odds ratio in the patients with weak AMF expression was 1.6, which was higher than that in the patients with negative AMF expression. However, the difference was not statistically significant (P = 0.331). The patients' age at diagnosis, completeness of resection, and Lauren's histologic classification did not have a statistically significant effect on survival in the multivariate analyses.

**Association of strong autocrine motility factor expression in lymph node metastases with poor survival.** Next, we examined AMF expression in the lymph node metastases. Of the 53 lymph node metastasis specimens we examined, 25 (47%), 19 (36%), and 9 (17%) had strong, weak, and negative expression of AMF, respectively. The median survival duration in patients who had a lymph node metastasis with negative, weak, and strong AMF expression was 83, 49, and 14 months, respectively. Strong AMF expression was associated with an inferior survival duration (P = 0.011; Fig. 2B). Strong AMF expression in lymph node metastases was an independent predictor of poor survival.

**Direct correlation of autocrine motility factor expression with vascular endothelial growth factor expression and microvessel density status.** In a previous report, recombinant AMF up-regulated VEGF expression in tumor cells (13). In the present study, we sought to determine the relationship of AMF expression with VEGF expression and MVD status in the primary tumors as described previously (14, 17, 18). We found that AMF expression was highly correlated with VEGF expression (P < 0.001). Similarly, AMF expression was significantly correlated with the tumor MVD status (P < 0.001; Table 2). These findings were further confirmed by analysis of consecutive gastric tumor tissue sections, in which we found that the pattern of AMF expression was consistent with that of VEGF expression and the tumor MVD (Fig. 3). These data provided clinical evidence that aberrant AMF expression is associated with VEGF expression and increased angiogenesis.

**Association of autocrine motility factor expression in primary tumors with that in lymph node metastasis.** Finally, we determined the relationship of AMF expression in primary tumor and lymph node metastasis. The levels of expression of AMF in lymph node metastases were correlated with those in the primary tumors; this association was statistically significant (Pearson's correlation coefficient, +0.532; P < 0.001; Table 3). These data suggested that AMF overexpression in both primary tumor and lymph node metastasis consistently predicted poor prognosis of gastric cancer patients.

**Discussion**

In the present study, we examined the level of AMF expression in tissue specimens obtained from patients with resected gastric cancer. Specifically, we studied its relationship with VEGF expression in and the angiogenic phenotype of the tissue specimens and their impact on survival. We found that elevated AMF expression was strongly correlated with VEGF expression and MVD status but inversely correlated with survival. Therefore, the level of expression of AMF in both the primary tumors and lymph node metastases significantly impacted clinical outcome. Our clinical evidence also suggested two potential mechanisms for the overall aggressive biology of gastric cancer due to abnormally expressed AMF: elevated angiogenesis and increased risk of lymph node metastasis.

Survival in patients with resected gastric cancer remains suboptimal in the United States. Of the numerous tumor prognostic factors identified thus far, angiogenesis, which is
essential for tumor growth and metastasis (19) and quantitated according to the MVD, has been considered the most important in predicting overall survival (19–24). MVD is an indicator of poor prognosis (25–28). Specifically, high MVDs have been significantly and positively associated with the depth of tumor invasion and distant metastasis. Moreover, overall survival in patients with a high MVD have been found to be significantly lower than those in patients with a low MVD (28–31). In addition, a recent study showed a statistically significant correlation between MVD and the two main histologic parameters: tumor grade and Lauren's histologic classification (32). In well and moderately differentiated tumors, the MVD was significantly lower than that in poorly differentiated tumors. Also, the MVD was higher in diffuse-type gastric tumors than in intestinal-type tumors. In the present study, patients with strong AMF expression had a shorter survival duration than did patients with negative or weak AMF expression. Our multivariate analysis revealed that AMF was an independent prognostic factor that predicted poor survival. Because of the close association between AMF expression and MVD, our findings suggest that altered AMF expression directly impacts the angiogenic potential of human gastric cancer. This was clearly supported by a previous report suggesting that AMF has a role in regulation of VEGF expression in tumor cells and VEGF receptor (FLT-1) expression in endothelial cells (13). Of the many angiogenesis-regulating factors, VEGF has been shown to be a critical one (7–11, 33). This was further supported by our present work showing for the first time that AMF expression is directly correlated with VEGF expression in human gastric cancer. Clearly, further studies are needed to determine the casual relationship between AMF expression and tumor angiogenesis and its cellular and molecular basis.

Table 2. VEGF expression and MVD status versus AMF expression in primary gastric cancer

<table>
<thead>
<tr>
<th>AMF expression</th>
<th>P</th>
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<tr>
<td>VEGF expression</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
</tr>
<tr>
<td>Weak</td>
<td>8</td>
</tr>
<tr>
<td>Strong</td>
<td>0</td>
</tr>
<tr>
<td>MVD status</td>
<td></td>
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<tr>
<td>Low</td>
<td>4</td>
</tr>
<tr>
<td>Moderate</td>
<td>9</td>
</tr>
<tr>
<td>High</td>
<td>0</td>
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NOTE: Pearson’s χ² test was used to determine the statistical significance of the relationship of AMF expression with VEGF expression and MVD status.

Table 3. Association of AMF expression in primary tumor and lymph node metastases

<table>
<thead>
<tr>
<th>AMF expression in primary tumor</th>
<th>AMF expression in lymph node metastases</th>
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</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Negative 4 (7.6)</td>
</tr>
<tr>
<td>Weak</td>
<td>Weak 4 (7.6)</td>
</tr>
<tr>
<td>Strong</td>
<td>Strong 1 (1.9)</td>
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</table>

NOTE: Pearson’s χ² test was used to determine the statistical significance of the relationship between AMF expression in the primary tumors and lymph node metastases (P < 0.001).

Fig. 3. Association of AMF expression with VEGF expression and MVD status. Two sets of consecutive tissue sections with strong and negative AMF expressions, respectively, were prepared from formalin-fixed, paraffin-embedded gastric tumor specimens. Immunohistochemical staining was done with specific antibodies against AMF, VEGF, and CD34. Representative photographs taken at same magnifications (×100) were presented. Of note is that strong AMF expression was directly correlated with increased VEGF expression and MVD status.
This was strongly supported by several recent studies demonstrating that increased expression of AMFR is strongly correlated with a high incidence of recurrence and decreased survival in patients with colorectal, bladder, esophageal, or gastric cancer (5, 8–12). In the present study, we found the first clinical evidence that overexpression of AMF is associated with increased incidence of lymph node metastasis. Thus, the AMF/AMFR pathway significantly contributes to tumor progression and metastasis. Further characterization of AMF may yield valuable insights into gastric cancer prognosis and treatment. In particular, immobilizing tumor cells may be a crucial step in inhibiting metastasis (1, 3).

Finally, although some tumor samples were classified as “negative,” those tumor tissues, like the majority of normal tissues, express AMF, but at very low levels. This is supported by Western blot analysis, showing that tumor tissues tend to express higher levels of AMF compared with matched adjacent normal tissues. Whether there is a complete loss of AMF expression in tumor tissues is not clear and requires further genetic and epigenetic analyses. Additionally, we have not observed obvious uniqueness of those samples, such as the relationship with tumor stages, histologic classifications, and locations. However, the close correlation of AMF with VEGF may suggest that AMF expression relate to the degree of hypoxia in the tumors (i.e., those low, or “negative,” AMF tumors may be less hypoxic; ref. 33–35). Therefore, molecular mechanisms underlying altered AMF expression warrant further investigation.

In summary, we discovered clinical evidence that AMF overexpression is closely associated with an elevated angiogenic phenotype. Therefore, AMF, like its receptor, AMFR, is a potential molecular marker for poor prognosis and therapeutic target in these patients. A better understanding of the molecular basis for regulation of angiogenesis and metastasis by the AMF/AMFR pathway may aid the design of more effective antiangiogenesis and antimetastasis therapy for gastric cancer.

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References

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