RESEARCH ARTICLE

Aberrant Expression of the Autocrine Motility Factor Receptor Correlates with Poor Prognosis and Promotes Metastasis in Gastric Carcinoma

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Abstract

AMFR, autocrine motility factor receptor, also called gp78, is a cell surface cytokine receptor which has a dual role as an E3 ubiquitin ligase in endoplasmic reticulum-associated degradation. AMFR expression is associated with tumor malignancy. We here investigated the clinical significance of AMFR and its role in metastasis and prognosis in gastric cancer. Expression of AMFR, E-cadherin and N-cadherin in cancer tissues and matched adjacent normal tissues from 122 gastric cancer (GC) patients undergoing surgical resection was assessed by immunohistochemistry. Levels of these molecules in 17 cases selected randomly were also analysed by Western blotting. AMFR expression was significantly increased in gastric cancer tissues, and associated with invasion depth and lymph node metastasis. Kaplan-Meier analysis showed AMFR expression correlated with poor overall survival and an increased risk of recurrence in the GC cases. Cox regression analysis suggested AMFR to be an independent predictor for overall and recurrence-free survival. E-cadherin expression was decreased in gastric cancer tissues; conversely, N-cadherin was increased. Expression of AMFR negatively correlated with E-cadherin expression, whereas N-cadherin expression showed a significant positive correlation with AMFR expression. AMFR might be involved in the regulation of epithelial-mesenchymal transition, with aberrant expression correlating with a poor prognosis and promoting invasion and metastasis in GCs.

Keywords: Gastric carcinoma - AMFR - prognosis - metastasis - epithelial-mesenchymal transition

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Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries (WHO, 2008), one in four deaths is due to cancer in American each year (Siegel et al., 2013). Although the incidence and mortality of gastric cancer (GC) is declining, it remains the fourth most common epithelial malignancy and the second leading cause of cancer-related mortality following lung carcinoma throughout the world over the past several decades. Over 70% of new cases and deaths occur in developing countries (Jemal et al., 2011). Clinically, early gastric cancer is often asymptomatic or causes non-specific symptoms; when the cancer has reached the advanced stage, though some typical symptoms occur, the patients with gastric cancer have poor prognosis due to primary tumor invasion and metastasis (Murray et al., 2008). Similar to other solid tumors, gastric cancer is characterized by local invasion, high regional lymph node metastasis and distant metastasis, which are serious clinical problems that lead to recurrence and poor prognosis (Saito et al., 2008). Therefore, in order to develop novel treatment options for this fatal disease, it is critical to understand the molecular mechanisms that regulate invasion and metastasis of gastric cancer.

Invasion and metastasis, the aggressive nature of gastric cancer, are often related to a number of molecular abnormalities, including microsatellite instability, inactivation of various tumor suppressor genes, activation of various oncogenes, and reactivation of telomerase (Tahara, 2000; Sud et al., 2001). These abnormalities affect the downstream signal transduction pathways involved in the control of cell growth and differentiation and confer a tremendous advantage of invasion and metastasis to gastric cancer cells (Wei et al., 2005). But the potential roles of these factors in the pathogenesis of gastric cancer remain unclear.

AMFR, autocrine motility factor receptor, also called gp78 (78 kDa glycoprotein), is a cell surface cytokine receptor for autocrine motility factor (AMF) (Fairbank et al., 2009), which involved in numerous physiological and pathological processes, including cell motility, signal transduction and protein ubiquitination (Cai et al., 2011).

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As an extracellular phospho hexose isomerase (PHI) and a specific ligand for AMFR, AMF is also identified as neuroleukin (NLK) or maturation factor (MF), that is secreted from malignant or neoplastic cells (Watanabe et al., 1996; Niinaka et al., 1998; Haga et al., 2000). AMF has a series of biochemical effects, including stimulation of tumor angiogenesis (Funasaka et al., 2001), apoptotic resistance (Haga et al., 2003; Romagnolia et al., 2003) and cell proliferation (Tsutsumi et al., 2003) as well as cell motility by acting in a cytokine-like manner via AMFR, which is a seven transmembrane glycoprotein (Shimizu et al., 1999). This phenotypic variation is connected to tumor progression and metastasis. Initially, AMFR is isolated from B16-F1 murine melanoma (Silletti et al., 1991) and HT-1080 human fibrosarcoma cell lines (Watanabe et al., 1991). Then, the gene encoding the human AMFR is cloned from HT-1080 fibrosarcoma cDNA library (Watanabe et al., 1991), which located in 16q21 chromosome. Furthermore, AMFR also has a dual function as an E3 ubiquitin ligase implicated in endoplasmic reticulum-associated degradation (ERAD) (Fairbank et al., 2009). Substrates of AMFR E3 ubiquitin ligase activity include CD3-δ, the T cell receptor subunits, apoB lipoprotein, hydroxymethylglutaryl-CoA reductase, cystic fibrosis transmembrane conductance regulator, and some metastasis suppressors (Fang et al., 2001; Liang et al., 2003; Zhong et al., 2004; Song et al., 2005; Morito et al., 2008). As a polytopic protein, AMFR has the structural features of an integral membrane protein, consisting of extracellular domain, transmembrane domain and cytoplasmic region; and AMFR N-terminally anchored to the ER-membrane with its intrinsic RING-finger Ub-ligase, Cue1-like, UBC7/Ube2g2-binding, substrate recognition and p97-binding regions all situated in the C-terminal domain in its extended cytoplasmic tail (Fang et al., 2001; Zhong et al., 2004; Chen et al., 2006; Kostova et al., 2007). Each of these is implicated in ubiquitylation and degradation of ERAD substrates. Recent researches show that AMFR overexpression is closely linked to tumor malignancy and human cancer and identified as one of the 189 most mutated genes in breast and colon cancers (Sjöblom et al., 2006). AMFR expression correlates with aggressive tumor biology and poor outcome for malignancies of the lung, tongue, esophagus, colon, rectum, liver, breast, and skin (Chiu et al., 2008). Notably, in bladder, colorectal, skin, and esophageal cancers, AMFR is either not expressed or expressed at significantly reduced levels in adjacent normal tissue.

Epithelial-mesenchymal transition (EMT) is a common biological process, which plays an important role in embryogenesis, chronic inflammation, tissue remodeling, fibrosis and cancer metastasis (Thiery et al., 2009; Yilmaz and Christofori, 2009). Generally, it refers to epithelial cells transform into cells with mesenchymal phenotype through a specific process. Currently, EMT has become prominently implicated as a means by which transformed epithelial cells can acquire the abilities to invade, to resist apoptosis, and to disseminate (Hanahan and Weinberg, 2011). And EMT endows tumor cells, especially those epithelium-derived cells with migratory and invasive properties during the malignant processes. As mentioned previously, overexpression of AMFR in tumors is well documented. However, it has yet to be determined whether AMFR expression contributes to gastric cancer progression and prognosis. Moreover, a clear link between AMFR and EMT in gastric cancer progression has not yet been demonstrated. Therefore, in the present study, we investigated the expression of AMFR in a series of GCs and its correlations with clinical features and prognosis. We also demonstrated a correlation between the levels of AMFR and the key molecules in EMT, E-cadherin and N-cadherin. We expected that these results would help to elucidate the mechanism of carcinogenesis and metastasis in gastric cancer.

Materials and Methods

GC patients and clinical samples

Fresh samples of GC tissues and matched adjacent normal tissues (5 cm away from the edge of carcinoma) were obtained from 122 patients ranged in age from 27 to 85 years (mean age: 57.5 years, 85 males) with primary GC who underwent standard D2 radical gastric resection or palliative gastrectomy between 2009 and 2011, in the Department of Gastrointestinal Surgery, the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). No patients underwent chemotherapy or radiotherapy prior to surgery. All specimens obtained from gastrectomy were confirmed by pathologic examination, and clinicopathologic parameters, such as clinical stage, histopathologic classification, invasion depth, lymph node metastasis and distant metastasis, were obtained. Tumor stage was classified according to the 7th TNM classification of the Union for International Cancer Control (UICC). The clinicopathological data are shown in Table 2.

Follow-up information about the postoperative clinical course of patients was available from outpatient medical records, telephone calls, or letters. Follow-up ranged from 3 months to 4 years was successfully completed in the patients. Overall survival was calculated from surgery until last contact or death. Recurrence-free survival was defined at the time of surgery to tumor recurrence. The study protocol was approved by the ethics committee of the Chongqing Medical University.

Immunohistochemistry (IHC)

Formalin-fixed and paraffin-embedded specimens were prepared, cut into 5-μm sections and mounted onto poly-L-lysine coated glass slides. The tissue sections to be stained with anti-AMFR (1:200 dilution, Abcam, Cambridge, UK), anti-E-cadherin (1:100 dilution, Proteintech, Chicago, IL, USA) or anti-N-cadherin (1:200 dilution, eBioscience, San Diego, CA, USA), were subjected to microwave antigen retrieval in citrate buffer (0.01 M citric acid, 0.01 M sodium citrate; pH 5.6) for 20 min at 95-98°C. The slides were cooled for 30 min, rinsed three times with phosphate-buffered saline (PBS), incubated with 0.3% H_2O_2 for 30 min, and subsequently incubated with 5% BSA for 30 min. Then, the sections were incubated with primary antibody at 4°C overnight. Next day, the slides were subjected to several washes with PBS and

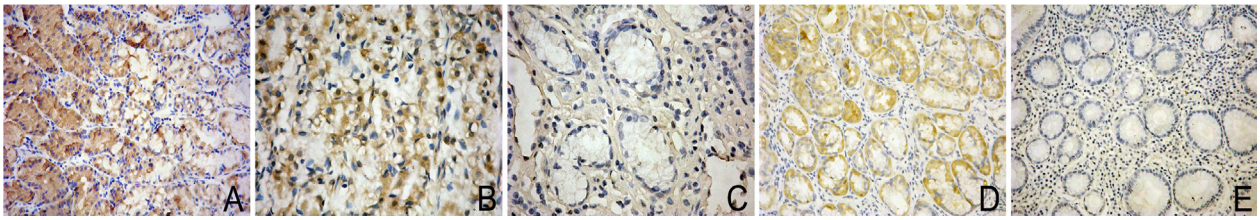

Figure 1. Expression of AMFR in Human Gastric Adenocarcinomas and Matched Adjacent Normal Tissues by Immunohistochemistry. (A) Strong positive staining (++) of AMFR in gastric adenocarcinoma. (B) Positive staining (+) of AMFR in gastric adenocarcinoma. (C) Negative staining (-) of AMFR in gastric adenocarcinoma. (D) Positive staining (+) of AMFR in matched adjacent normal tissue. (E) Negative staining (-) of AMFR in matched adjacent normal tissue. Original magnification, ×200

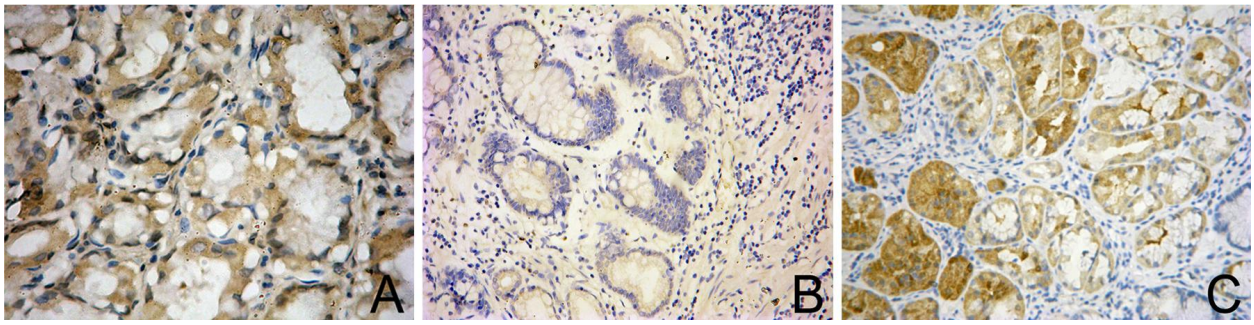

Figure 2. Expression of E-cadherin in Human Gastric Adenocarcinomas and Matched Adjacent Normal Tissues by Immunohistochemistry. (A) Positive staining (+) of E-cadherin in gastric adenocarcinoma. (B) Negative staining (-) of E-cadherin in gastric adenocarcinoma. (C) Positive staining (+) of E-cadherin in matched adjacent normal tissue. Original magnification, ×200

incubated with either anti-rabbit or anti-mouse biotin-conjugated secondary antibody (1:100 dilution, Boster, Wuhan, China) at 37°C for 1 h. The slides were incubated with strept avidin-biotin complex (SABC, Boster, Wuhan, China) at 37°C for 30 min and subsequently stained with DAB, counterstained with hematoxylin for 20 s, washed, xylene-cleaned, and mounted. Positive staining was a reddish-brown precipitate in the membrane or cytoplasm. Two independent investigators assessed the subcellular localisation and staining level for each sample according to the following grading system: staining intensity was categorized as negative (-), weak positive (+), moderate positive (++) or strong positive (+++). The percentage of staining was categorized as no staining (-), <10% of tumor cell stained (+), 10-40% (++), 40-70% (+++) and >70% (++++). To simultaneously gauge the staining intensity and uniformity, the average values for the intensity in each slice were multiplied by the average values for the percentage area stained in each slice to derive a composite histoscore (histoscore = intensity × area).

Protein isolation and western blot analysis

The specimens obtained from gastrectomy were immediately flash-frozen in liquid nitrogen and stored at −80°C for western blot analysis. Total protein of each tissue (50 mg) was isolated using RIPA Lysis Buffer (Beyotime, Jiangsu, China) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) for 30 min at ice, followed by centrifugation at 15,000 g for 15 min. The supernatant was measured for protein concentration with Enhanced BCA Protein Assay Kit (Beyotime, Jiangsu, China). The protein samples were mixed with SDS-PAGE Sample Loading Buffer (30% glycerol, 6% SDS, 62.5 mM Tris-HCl, pH 6.8, Beyotime, Jiangsu, China). For immunoblotting, equal amounts of proteins were separated on 8%-10% SDS-PAGE and electrophoretically transferred onto PVDF membranes (Millipore, Billerica, MA, USA), which were blocked in TBST containing 5% skimmed milk at 37°C for 2 h and incubated with primary antibody at 4°C overnight: anti-AMFR (1:500 dilution), anti-E-cadhein (1:500 dilution), anti-N-cadherin (1:500 dilution), or anti-β-actin (1:2,000 dilution, 4A Biotech, Beijing, China). After being washed with TBST and incubation with either anti-rabbit or anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (1:4,000 dilution, MultiSciences, USA) at 37°C for 2 h, immunocomplexes were finally visualized using the enhanced chemiluminesence (ECL) reagent (Santa Cruz, Dallas, Texas, USA).

Statistical analysis

Statistical analysis was performed using SPSS software (version 17.0, Chicago, IL, USA). Continuous data presented as mean ± SD (standard deviation) were measured by Student's t-test. For categorical data, chi-square or Fsher's exact test was used. Kaplan-Meier and Cox regression analyses were applied for overall survival and recurrence-free survival. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Aberrant expression of AMFR, E-cadherin and N-cadherin in GCs by IHC

We analyzed the expression of AMFR, E-cadherin and N-cadherin protein in paraffin-embedded cancerous and matched adjacent normal tissues from 122 cases of gastric adenocarcinomas. By IHC, 73 of 122 GC cancerous tissues (59.8%) were defined as AMFR positive staining. Among positive samples, 59 (80.8%) were strong positive and 14

Table 1. Expression of AMFR, E-cadherin and N-cadherin in 122 Cancerous and Matched Adjacent Normal Tissues

Group	AMFR			E-cadherin			N-cadherin		
	+	-	*P*-value[a]	+	-	*P*-value[a]	+	-	*P*-value[a]
Cancer	73	49	0.007	34	88	0.000	55	67	0.001
Normal	52	70		122	0		21	101	

[a]*P*<0.05, statistical significance by chi-square test

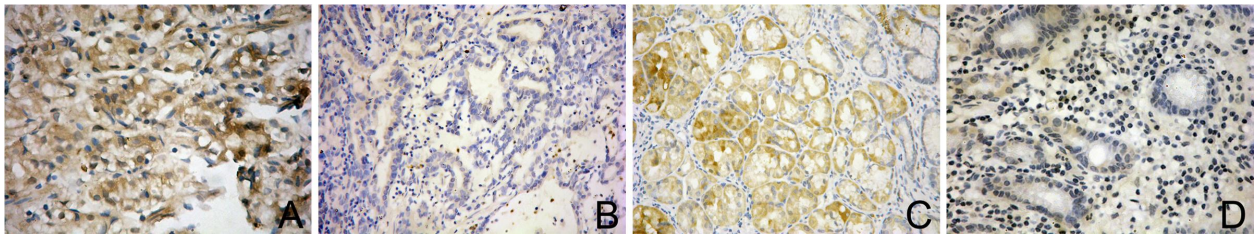

Figure 3. Expression of N-cadherin in Human Gastric Adenocarcinomas and Matched Adjacent Normal Tissues by Immunohistochemistry. (A) Positive staining (+) of N-cadherin in gastric adenocarcinoma. (B) Negative staining (-) of N-cadherin in gastric adenocarcinoma. (C) Positive staining (+) of N-cadherin in matched adjacent normal tissue. (D) Negative staining (-) of N-cadherin in matched adjacent normal tissue. Original magnification, ×200

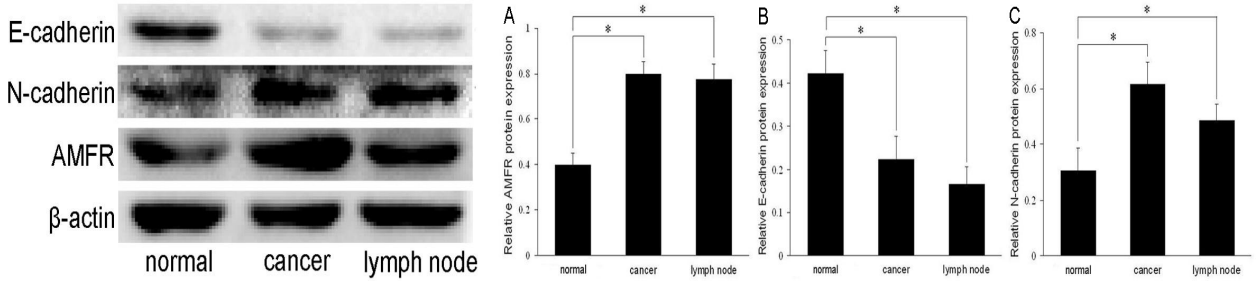

Figure 4. Expression of AMFR, E-cadherin and N-cadherin in Gastric Carcinoma, Adjacent Normal Tissues And Lymph Node By Western Blot Analysis. (A) Relative expression of AMFR. (B) Relative expression of E-cadherin. (C) Relative expression of N-cadherin. **P*<0.01 statistical significance by Student's t-test

(19.2%) were weak positive (Figure 1A and 1B). Whereas, all 122 matched adjacent normal samples showed weakly positive (52/122, 42.6%) or negative (70/122, 57.4%) AMFR staining on the mucosal tissue (Figure 1C and 1D). Moreover, AMFR was located in the cytoplasm and membrane. In addition, E-cadherin and N-cadherin were also located in the cytoplasm and membrane. Positive staining of E-cadherin protein was found in 34 gastric cancer samples (34/122, 27.9%, Figure 2A), whereas all 122 (100%) of the normal mucosae expressed the E-cadherin protein (Figure 2C). By contrast, 55 (45.1%) gastric cancer tissues were positive for N-cadherin (Figure 3A), whereas 21 (17.2%) of the normal mucosae expressed the N-cadherin protein (Figure 3C). Compared with the normal mucosae, AMFR expression was significantly increased in gastric cancer tissues (*P*=0.007; Table 1). The decreased E-cadherin expression and increased N-cadherin in gastric cancer tissues were also statistically significant (*P*=0.000 and *P*=0.001, respectively).

Expression of AMFR, E-cadherin and N-cadherin in gastric carcinoma, adjacent normal tissues and lymph node by western blot analysis

Seventeen cases selected randomly from these 122 clinic specimens, including different degrees of cellular differentiation or different stages of gastric cancer, were analysed by western blot. We found that these antibodies were specific against the proteins. Then, the western blot analysis revealed that AMFR was highly expressed in the tumor tissues and lymph nodes, whereas all the normal tissues showed a decreased AMFR expression. By contrast, the expression of E-cadherin protein was significantly decreased in tumor tissues and lymph nodes compared with the normal mucosae. Moreover, as compared with the normal mucosae, N-cadherin was highly expressed in the tumor tissues and lymph nodes. The alteration of the expression of these proteins was statistically significant (Figure 4, *P*<0.01).

Expression of AMFR correlated with invasion depth and lymph node metastasis in GCs

Clinicopathological features of the 122 GCs were stratified by AMFR expression (Table 2). The AMFR positive group was made by combining weakly and strong positive cases. Expression of AMFR was significantly associated with invasion depth (*P*=0.016) and lymph node metastasis (*P*=0.012), but not with age, gender, clinical staging, histological grade, and distant metastasis. To further analyze this finding, the odds ratio (OR) was used for estimating the relative risk (RR). The OR of AMFR positive staining for lymph node metastasis was 2.684 (95% confidence interval (CI) =1.228–5.867, *P*=0.017). Moreover, as compared with no serosal invasion, the OR of AMFR positive staining for serosa or djacent structures

Table 2. Clinicopathological Features and AMFR Expression in 122 Gastric Carcinoma (GC) Patients

Features	Number of cases	AMFR		P-value[c]
		Positive (n, %)	Negative (n, %)	
Age (Years)				
≤60	77	47, 61.0	30, 39.0	0.723
>60	45	26, 57.8	19, 42.2	
Gender				
Male	85	54, 63.5	31, 36.5	0.207
Female	37	19, 51.4	18, 48.6	
Clinical Staging[a]				
I+II	58	32, 55.2	26, 44.8	0.317
III+IV	64	41, 64.1	23, 35.9	
Histological grade				
Well differentiated	38	25, 65.8	13, 34.2	0.367
Poorly differentiated	84	48, 57.1	36, 42.9	
Invasion depth				
Mucosa or Submucosa	17	7, 41.2	10, 58.8	0.016[d]
Muscularis propria	20	8, 40.0	12, 60.0	
Serosa or adjacent structures	85	58, 68.2	27, 31.8	
Lymph node metastasis				
Yes	83	56, 67.5	27, 32.5	0.012
No	39	17, 43.6	22, 56.4	
Distant metastasis[b]				
Yes	21	14, 66.7	7, 33.3	0.483
No	101	59, 58.4	42, 41.6	
Survival				
≤2 years	88	58, 65.9	30, 34.1	0.028
>2 years	34	15, 44.1	19, 55.9	

[a]According to the 7th UICC-TNM classification; [b]Metastasizing to liver (5/21), pancreas (6/21), transverse colon (3/21), small intestine (3/21), parietal peritoneum (1/21) and greater omentum (3/21); [c]*P*-Value is for chi-square or Fisher's exact test; $P<0.05$, statistical significance; [d]Pairwise comparisonno significant difference between the two former groups ($P=0.942$); the third group was statistically different from the two former groups, respectively ($P=0.034$ and $P= 0.019$, respectively)

invasion was 3.151 (95% CI=1.416–7.008, $P=0.004$). As the high rate of lymph node metastasis (83/122, 68%) in GCs, and 56 cases happened lymph node metastasis in 73 AMFR positive staining cases, we then analyzed AMFR expression in cancerous tissues of GC patients with distant metastasis. Whereas, only 14 cases happened distant metastasis in those positive staining cases, possibly due to the small distant metastatic sample number. Together, the data suggested that expression of AMFR was significantly associated with an enhanced metastatic behavior in GCs.

Correlation between AMFR and EMT markers

To investigate the hypothesis that AMFR might play a critical role in EMT, we analyzed the expression of E-cadherin and N-cadherin protein in 122 cancerous samples and correlation between AMFR and these markers. Thirty-four cancerous samples (27.9%) showed E-cadherin positive staining; whereas, only 12 samples showed both AMFR and E-cadherin positive staining. In addition, expression of AMFR negatively correlated with E-cadherin expression in 122 GCs (rs=–0.311, $P=0.001$; Table 3). Conversely, the expression of N-cadherin in cancerous samples was higher than E-cadherin expression, which showed a significant positive correlation with AMFR expression (rs=0.381, $P=0.000$). These results suggested that AMFR might involve in the regulation of EMT.

Table 3. Correlation between AMFR and E-cadherin, N-cadherin Expression in 122 GCs

AMFR	E-cadherin				N-cadherin			
	+	-	rs	*P*-value[a]	+	-	rs	*P*-value[a]
+	12	61	-0.311	0.001	43	30	0.381	0.000
-	22	27			10	39		

[a]$P<0.05$, statistical significance by Spearman's correlation test

Expression of AMFR was associated with poor prognosis in GC patients following surgical resection

Fifteen (15/73, 20.5%) GC patients with AMFR positive staining survived more than 2 years, which was significantly lower in AMFR negative patients (19/49, 38.8%; $P=0.028$) during our observation period, suggesting that the expression of AMFR was associated with the poor prognosis in GCs. But this result confounded with censored data. Some patients were lost to follow-up during observation period, or survived at the end of our follow-up period, whose observation time was less than 2 years. To further validate this finding, Kaplan-Meier and Cox regression analyses were performed in 122 GC patients following surgical resection.

Kaplan-Meier analysis showed that the expression of AMFR significantly correlated with a notable reduction of overall survival (log-rank $P=0.001$, Figure 5A) and an increased risk of recurrence (log-rank $P=0.001$, Figure 5B) in 122 GCs. Multifactorial Cox regression analysis was used to avoid confounder effects (Table 4). To adjust the risk ratio (RR), factors including histological grade,

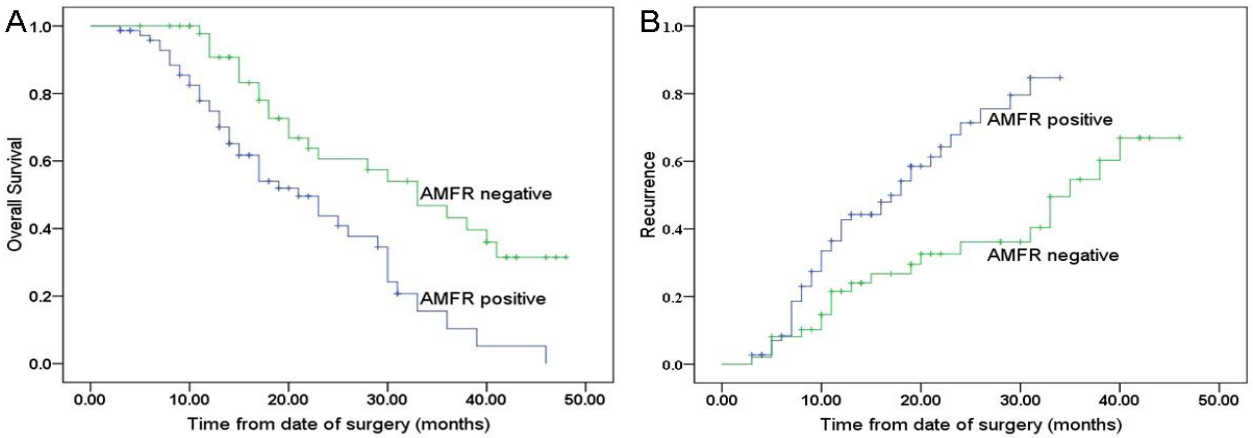

Figure 5. Expression of AMFR Impacted Overall Survival (A) and Recurrence (B) in 122 GCs Following Surgical Resection by Kaplan-Meier Analysis

Table 4. Cox Regression Analysis for Overall Survival and Recurrence-free Survival of GCs Following Surgical Resection (n=122)

Factors	Overall survival		Recurrence-free survival	
	RR (95% CI)	*P*-value[b]	RR (95% CI)	*P*-value[b]
Expression of AMFR (positive/negative)	0.401 (0.220–0.732)	0.003	0.525 (0.288–0.955)	0.035
Histological grade (well/poorly)	0.973 (0.540–1.752)	0.927	0.867 (0.484–1.555)	0.633
TNM stage[a] (I+II/III+IV)	4.732 (1.560–14.356)	0.006	3.641 (1.116–11.879)	0.032
Invasion depth (no serosa/serosa or adjacent structures)	0.433 (0.157–1.192)	0.105	1.471 (0.393–5.503)	0.566
Lymph node metastasis (yes/no)	0.559 (0.198–1.575)	0.271	0.204 (0.049–0.848)	0.029
Distant metastasis (yes/no)	0.397 (0.220–0.715)	0.002	0.443 (0.247–0.792)	0.006

[a]According to the 7th UICC-TNM classification; [b]$P<0.05$, statistical significance

TNM stage (7th), invasion depth, lymph node metastasis and distant metastasis were applied. The expression of AMFR was an independent predictor for poor overall survival and recurrence-free survival (RR=0.401, 95% CI=0.220–0.732, *P*=0.003; RR=0.525, 95% CI=0.288–0.955, *P*=0.035). Besides, advanced TNM stage (III and IV) was another independent predictor of overall survival and recurrence-free survival (RR=4.732, 95% CI=1.560–14.356, *P*=0.006; RR=3.641, 95% CI=1.116–11.879, *P*=0.032), which was consistent with the other studies (Peng et al., 2013; Li et al., 2013; Graziosi et al., 2013), supporting the accuracy of our study. Together, these data revealed the expression of AMFR was a risk factor indicating poor prognosis in GC patients following surgical resection.

Discussion

Cancer is the most important health and safety issues all over the world. In recent years, due to the tremendous advances in the treatments, including surgery, chemotherapy and radiotherapy, the survival rate of gastric cancer has significantly improved, and the death rate of gastric cancer in American continues to decline 3.1% annually (Siegel et al., 2013). However, approximately 1 million new stomach cancer cases and 740,000 deaths have occurred per year, accounting for 8% of the total cases and 10% of total deaths. Clinically, gastric cancer is characterised by high metastasis and poor prognosis. Surgical resection remains the curative option for GC patients. Several clinicopathologic parameters, such as TNM stage, histopathologic classification, invasion depth and metastasis, are useful prognostic predictors for GC after surgery. However, they have limitations. Patients with similar pathologic stages often display considerable variability in recurrence and survival. Therefore, it is necessary to screen more new biomarkers. Our recent studies have reported several molecules, such as KLF4 (Krüppel-like factor 4) (Zhang et al., 2012) and HMGA2 (high mobility group protein A2) (Zha et al., 2013), correlate with GC progression and prognosis. In present study, our data suggested that AMFR was a new candidate prognostic marker for GCs.

AMFR, which has a dual role in vivo, is initially isolated as the receptor for AMF (Fairbank et al., 2009). AMF is originally identified by its ability to induce the migration of AMF-producing human A2058 melanoma cells (Liotta et al., 1986). Elevated serum AMF is found in patients with malignant tumors such as colorectal, lung, kidney, breast and gastrointestinal carcinomas and is well correlated with the development of metastasis (Iiizumi et al., 2008). Moreover, overexpression of AMF in normal fibroblasts lead to a gain of tumorigenicity (Funasaka et al., 2007). During tumor progression, an additional role of AMF is revealed, namely, it is demonstrated that AMF not only stimulates AMF-producing tumor cell motility in an autocrine manner by binding to its receptor, but also acts as a paracrine factor for vein endothelial cells; when functioning as a paracrine factor, AMF induces angiogenesis by stimulating cell motility and up-regulating its VEGFR expression, and it may facilitate metastasis by becoming more active at the metastasis phase (Funasaka et al., 2001). Some other studies show that AMF also contributes to malignant progression by stimulating the migration and proliferation of endothelial cells via its receptor AMFR, followed by activation of small Rho-like GTPase (Tsutsumi et al., 2002). Since we didn't collect enough serum samples, and AMF is a secreted protein detected accurately from serum; we did not check the concentration of this ligand in present study.

Generally, solid tumor growth is largely dependent on angiogenesis. Quite separately from cardiovascular health, vasculature plays a fundamental role in cancer biology; furthermore tumor growth is rate-dependent on angiogenesis (Nussenbaum and Herman, 2010). AMFR is overexpressed in a variety of human cancers. Besides inducing cell migration, it is closely correlated with tumor progression, involved in angiogenesis, endothelial motility and increased permeability; and its secretion by tumor cells is reported to up-regulate the vascular endothelial growth receptor Flk-1 (Funasaka et al., 2002). It also shows increased expression in prostate cancers in African Americans (Wallace et al., 2008), which can contribute to more exuberant angiogenesis and is critical to tumor growth. In addition, angiogenesis is one of the important factors on tumor metastasis. In this study, we found that positive rate and level of AMFR expression were significantly increased in gastric cancer tissues by IHC and western blot analysis, whereas all the adjacent normal tissues showed a decreased AMFR expression (Figure 1, Figure 4 and Table 1). AMFR positive staining cases showed a deeper invasion and higher rate of lymph node metastasis; expression of AMFR was significantly associated with invasion depth (*P*=0.016) and lymph node metastasis (*P*=0.012). Whereas, only 14 cases occurred distant metastasis in AMFR positive staining cases,

showed no significant difference from those negative staining cases, possibly due to the small distant metastatic sample number (Table 2). It suggested that expression of AMFR was significantly associated with an enhanced metastatic behavior in GCs. And we speculated that this phenomenon might be related to angiogenesis, increased permeability and degradation of extracellular matrix (ECM).

Furthermore, AMFR also has a dual role as an E3 ubiquitin ligase. Expressions of various proteins demonstrate spatial and temporal heterogeneity in vitro and in vivo. Ubiquitylation and proteasomal degradation perform critical functions in degradation of misfolded, unassembled, and highly regulated proteins from the endoplasmic reticulum (ER) (Nakatsukasa and Brodsky, 2008). In mammals, there are more than 500 E3s; AMFR is one of them, having the ability to facilitate transfer of ubiquitin from E2 ubiquitin-conjugating enzymes to substrates or to growing chains of ubiquitin (polyubiquitin or multiubiquitin) (Das et al., 2009). KAI1 has been characterized as a metastasis suppressor. Reduced or abrogated expression of KAI1 is linked to elevated tumor cell migration, invasion, and proliferation in aggressive tumor (Miranti, 2009). Recent research indicates KAI1 is regulated post-translationally by proteasomal degradation after being targeted by the ubiquitin ligase AMFR (Tsai et al., 2007). Analyses of the transgenic AMFR mouse model overexpressing AMFR in the mammary gland show that AMFR induces a hyperplastic phenotype, increased ductal branching, and dense alveolar lobule formation as well as down-regulation of the KAI1 (Joshi et al., 2010). Then, reduced expression of KAI1 caused by AMFR may lead to preneoplastic hyperplasia as well as predispose these cells to metastasis upon subsequent transformation and tumor formation. RNAi-mediated knockdown of AMFR inhibits sarcoma metastasis but not primary tumor growth; furthermore, levels of KAI1 correlate inversely with AMFR (Tsai et al., 2007). Consistent with this idea, the metastatic activity of AMFR requires its ubiquitin ligase activity (Tsai et al., 2007) but not its putative function as the AMF receptor, which is proposed (Watanabe et al., 1991; Shimizu et al., 1999) prior to its characterization as a ubiquitin ligase involved in ERAD (Fang et al., 2001).

Metastasis remains by far the major cause of cancer-related mortality; more than 90% of cancer patients ultimately die from sequel of metastatic disease. A complete metastasis process is often portrayed as a succession of six distinct steps: localized invasion, intravasation, translocation, extravasation, micrometastasis and colonization. Successful metastasis is dependent on the balance and complex interplay of both the metastasis promoters and suppressors in each step (Hanahan and Weinberg, 2011). Our results showed that aberrant expression of AMFR was significantly associated with an enhanced metastatic behavior in GCs. In addition, the expression of AMFR significantly correlated with a notable reduction of overall survival and an increased risk of recurrence in 122 GCs, which was an independent predictor for poor overall survival and recurrence-free survival (RR=0.401, 95% CI=0.220–0.732, P=0.003; RR=0.525, 95% CI=0.288–0.955, P=0.035; Figure 5, Table 4). Increased metastasis induced by AMFR is not only related to its dual role as the AMF receptor and E3 ubiquitin ligase, but also involved in EMT process. Interestingly, we found that compared with the normal mucosae, E-cadherin expression was decreased in gastric cancer tissues; conversely, N-cadherin was increased. Expression of AMFR negatively correlated with E-cadherin expression in 122 GCs (rs=–0.311, Table 3), whereas N-cadherin expression showed a significant positive correlation with AMFR expression (rs=0.381). When carcinomas progress from epithelial tissues to higher pathological grades of malignancy, it reflects in local invasion and distant metastasis, the associated cancer cells typically develop alterations in their shape as well as in their attachment to other cells and to the ECM (Hanahan and Weinberg, 2011). Loss of cell adhesion plays an important role in EMT and metastasis. The best characterized alteration is the loss by carcinoma cells of E-cadherin, a key cell-to-cell adhesion molecule. By forming adherens junctions with adjacent epithelial cells, E-cadherin helps cells to maintain quiescence. Increased expression of E-cadherin is well established as an antagonist of invasion and metastasis, whereas reduction of its expression is known to potentiate aggressive phenotypes (Berx and van Roy, 2009). Conversely, adhesion molecules normally associated with cell migration are often upregulated. N-cadherin, which is normally expressed in migrating neurons and mesenchymal cells during organogenesis, is upregulated in many invasive carcinoma cells (Cavallaro and Christofori, 2004). Besides the abnormal expression of these EMT-related molecules in cancer, cancer cells at the invasive margin are repoted to undergo an EMT process, suggesting that these cells are subject to microenvironmental stimuli distinct from those received by cancer cells located in the cores of these lesions (Hlubek et al., 2007). In many cases, cancer cells may enter into an EMT program only partially, thereby acquiring new mesenchymal traits while continuing to express residual epithelial traits. In this study, these results suggested that AMFR might directly repress EMT-related molecules expression by post-translational regulation, or indirectly regulated these molecules by mediation of transcription factors. In short, AMFR might involve in the regulation of EMT.

Taken collectively, the aberrant expression of AMFR was associated with certain clinicopathological parameters, invasion depth and lymph node metastasis, correlated with poor prognosis in GCs. The enhanced expression of AMFR regulated tumor aggressive progression by involving in the regulation in EMT process. Although novel, these data should be further verified with a larger sample size and a longer follow-up period. In addition, future studies are required to investigate the molecular mechanism of AMFR in stomach carcinogenesis and progression and further evaluate it as a potential biomarker for gastric cancer.

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