Higher autocrine motility factor/glucose-6-phosphate isomerase expression is associated with tumorigenesis and poorer prognosis in gastric cancer

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Introduction
Gastric cancer (GC) is the fifth common cancer and a major cause of cancer-related deaths in worldwide.1 Adenocarcinoma is the major type of GC. According to Lauren’s classification, it can be further subdivided into intestinal and diffuse types.2 GC is often either asymptomatic or it may cause only nonspecific symptoms in its early stages. By the time of diagnosis, the cancer often reached an advanced stage with poor prognosis. When the tumor is invading the serosa or distant metastases, 5-year overall survival (OS) rate is only <5%.3 Therefore, there is a critical challenge to identify new diagnostic and treatment targets to improve GC patients’ outcomes.

Glucose-6-phosphate isomerase (GPI) is encoded by a GPI gene and is a member of the glucose phosphate isomerase protein family. In the cytoplasm, GPI functions as a glycolytic enzyme that interconverts glucose-6-phosphate and fructose-6-phosphate in the cytoplasm. This protein is also secreted into the extracellular matrix by cancer cells and is, therefore, also called autocrine motility factor (AMF).

Background: Glucose-6-phosphate isomerase (GPI) is a glycolytic-related enzyme that interconverts glucose-6-phosphate and fructose-6-phosphate in the cytoplasm. This protein is also secreted into the extracellular matrix by cancer cells and is, therefore, also called autocrine motility factor (AMF).

Methods: To clarify the roles of AMF/GPI in gastric cancer (GC), we collected 335 GC tissues and the corresponding adjacent noncancerous tissues, performed immunohistochemical studies, and analyzed the relationship between AMF/GPI expression and the patients’ clinicopathologic features.

Results: AMF/GPI expression was found to be significantly higher in the GC group than in the corresponding noncancerous tissue group (P<0.001). Additionally, AMF/GPI expression positively associated with a higher TNM stage and poorer prognosis in patients. Through Kaplan–Meier analysis and according to the Oncomine database, we found that AMF/GPI was overexpressed in GC tissues compared to normal mucosa, and the patients with higher AMF/GPI expression had poorer outcomes. We used AMF/GPI-silenced GC cell lines to observe how changes in AMP/GPI affect cellular phenotypes. AMF/GPI knockdown suppressed proliferation, migration, invasion, and glycolysis, and induced apoptosis in GC cells.

Conclusion: These findings suggest that AMF/GPI overexpression is involved in carcinogenesis and promotes the aggressive phenotypes of GC cells.

Keywords: gastric cancer, glucose-6-phosphate isomerase, autocrine motility factor, tumorigenesis, prognosis, metabolism
in gluconeogenesis and the pentose phosphate pathway.\(^5\) Additionally, the same protein is secreted into the extracellular matrix by cancer cells, acts as a tumor-secreted cytokine and angiogenic factor, and is, therefore, called autocrine motility factor (AMF).\(^6\) Finally, GPI is also studied as a neurotrophic factor that promotes survival of skeletal motor neurons and sensory neurons and induces immunoglobulin secretion.\(^7\)

AMF/GPI was widely studied in rheumatoid arthritis as a prognosis and therapy marker.\(^8\)–\(^10\) In cancer research, AMF/GPI is a known biomarker of cancer progression that is found in the serum and urine of patients\(^11\)–\(^12\) and explored for early diagnosis purposes.\(^13\) AMF/GPI is also known as a tumor-secreted cytokine that plays a role in induction of cell migration,\(^14\) tumor angiogenesis,\(^15\) tumor metastasis,\(^16\) cell proliferation,\(^17\) and resistance to apoptosis.\(^18\) Remarkably, higher AMF/GPI expression was found to correlate with poor prognosis of human GC.\(^19\) Additionally, as the second glycolytic enzyme, AMF/GPI is possibly involved in the "Warburg effect", which proposes that for energy production, tumor cells rely more on glycolysis than on mitochondrial respiration.\(^20\) AMF/GPI targeting might offer therapeutic benefits for drug-resistant cancers and allow effective treatment of GC. Therefore, we aimed to clarify the clinicopathologic and prognostic significance of AMF/GPI expression in GC and its role in the regulation of the phenotype of GC cells.

**Materials and methods**

**Study population and immunohistochemistry**

Tissue microarrays (TMA) included 335 cases of GC tissues and paired adjacent nonneoplastic mucosa from GC patients who underwent radical gastrectomy at Peking University Beijing Cancer Hospital from January 2004 to December 2012. All patients signed a general informed consent to agree to the use of GC tissues for clinical research. The study was approved by Ethics Committee of Beijing Cancer Hospital. The original data of patients were reviewed in the context of clinicopathologic and follow-up information. Stage of GC was classified according to 2010 TNM classification recommended by the American Joint Committee on Cancer (AJCC, seventh edition). T and N classification were assessed based on the final pathologic result and M classification was determined by surgical findings. The OS was calculated starting from the date of the initial surgery to the time of death, counting death from tumor cause as the end point or the last date of follow-up as the end point, if no event was documented. All patients were followed up until 2012. None of the patients received chemotherapy or radiation therapy prior to surgery.

Formalin-fixed and paraffin-embedded tissue blocks were cut into 4-μm-thick sections, which were deparaffinized in xylene and rehydrated. Antigen retrieval was done upon incubation in an EDTA solution (pH 8.0; Santa Cruz Biochemistry, Dallas, TX, USA) for 10 minutes in a pressure cooker. Endogenous peroxidase activity was blocked with a 3% H\(_2\)O\(_2\) solution at 25°C for 15 minutes. After blocking with 5% BSA, the sections were incubated with anti-AMF/GPI antibody (1:800, Bethyl Laboratories, Inc.) at 4°C overnight, followed by washing with PBS and incubation with goat anti-rabbit IgG (1:1000, Santa Cruz Biotechnology, Inc.) at 25°C for 1 hour. All TMA slide sections were quantified by an automated image analysis software. The percentage of cells with positive AMF/GPI immunostaining was estimated from 0 to 100. The staining intensity was scored as “1” (no staining or weakly stained), “2” (moderate staining), or “3” (strong staining). The value indicating the percentage of stained cells was multiplied by the corresponding intensity value (intensity × proportion) to obtain a score ranging from 0 to 300: a final value <50 indicated negative expression and ≥50 positive expression.

**Cell culture**

SGC7901 and BGC823 cells were obtained from the Cell Research Institute (Shanghai, China) and routinely cultured as previously reported.\(^21\) All the cells were cultured in DMEM (Sigma-Aldrich Co., St Louis, MO, USA) with 10% FBS (Sigma-Aldrich) and 1% antibiotics at 37°C and 5% CO\(_2\) incubator.

**shRNA-mediated RNA interference**

shRNAs against AMP/GPI were designed with MIT’s siRNA designer (http://sirna.wi.mit.edu/home.php). At least four quadruplexes were designed, and the most effective shRNAs were used for subsequent studies. The sequences of the effective shRNA were provided as follows: 5′-gtgcccgttgtcactaca-3′. shRNAs against AMP/GPI and control hairpins were cloned into pSICO R vector. Production of lentiviral particles and transduction of GC cell lines were performed according to protocols from the RNAi consortium (http://www.broadinstitute.org/mal/tre). Cells were infected with lentiviral constructs expressing shAMP/GPI or shControl as described earlier for 24 hours. Stable cells were selected with puromycin. Then, cells were collected for protein and RNA analysis.

**Proliferation assays**

Cell proliferation was measured with the MTT assay. Cells were seeded into 96-well plates and incubated for 0, 24, 48, and 72 hours. At each time point, the supernatant was...
removed, and 10 μL of MTT solution was added to each well for a 4-hour incubation. Then, the supernatant was discarded, and 100 μL dimethyl-sulfoxide was added to each well. Absorbance was measured with a reference wavelength of 450 nm in a microplate reader (Bio-Rad, Hercules, CA, USA). Cell viability was calculated as (absorbance of test sample/absorbance of control)×100%.

Transwell assay
Cell migration/invasion was assayed using transwell chambers (Cell Biolabs, San Diego, CA, USA). Cells (5×10⁴) were harvested and added to the upper chambers with serum-free medium; culture medium containing 10% FBS was added into the lower chambers. For cell invasion assays, the transwell chambers were coated with 100 μL Matrigel before the cells were added. Finally, the cells on the upper surface of the filters were removed. The membranes were fixed with methanol for 10 minutes and stained with 0.5% crystal violet for 10 minutes. The cells on the underside of the filter were photographed and counted in five randomly selected microscopic views.

Flow cytometry
Cells were synchronized in the G0/G1 phase by incubating them in a serum-free medium overnight and then with medium containing 10% FBS for 24 hours. The cells were then trypsinized, washed three times with PBS, and fixed with 70% ethanol for 16 hours at −20°C. The samples were then trypsinized, washed three times with PBS, and stained with staining buffer for 15 minutes before flow cytometry (BD Biosciences, San Jose, CA, USA). For apoptosis analysis, the cells were stained using an Annexin V/propidium iodide (PI) double staining kit (DOJINDO, Kumamoto, Japan).

Seahorse XF24 analysis
Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured with the Seahorse XF24 analyzer (Seahorse Bioscience, North Billerica, MA, USA). Cells (2×10⁴ cells/well) were cultured in DMEM in Seahorse XF24 plates at 37°C. Each well was sequentially injected with three mitochondrial inhibitors, oligomycin, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone, and rotenone. OCR and ECAR were calculated with the Seahorse XF-24 software.

Western blotting
Cells were lysed in RIP A lysis buffer (Pierce Biotechnology, Rockford, IL, USA) containing a protease inhibitor cocktail (Roche, Basel, Switzerland), and centrifuged at 15,000×g for 20 minutes. Proteins (100 μg) were separated by 10% SDS-PAGE and transferred onto a 0.45 μm poly-vinylidene difluoride membrane (Whatman, Germany). The membrane was blocked with blocking buffer (5% skim milk in 0.1% tween tris-buffered saline solution) for 1 hour at 25°C, and then incubated with diluted primary antibodies (1:1000, Bethyl Laboratories, Inc., Montgomery, TX, USA) in the blocking buffer at 4°C overnight. The membrane was then washed with PBS and incubated in horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1000, Santa Cruz) for 1 hour. Finally, the membrane was developed using a chemiluminescence detection system (Pierce Biotechnology).

Animal studies
Animal studies were performed with the approval of the Ethics Committee of Peking University Beijing Cancer Hospital and conducted according to the institutional and national guidelines. The shControl and shAMF/GPI transfectants of SGC7901 and BGC823 cells (~2×10⁶ cells in 200 μL volume) were injected into both forelegs of BALB/c-nude mice (20 mice total, five mice per group). Tumors were monitored every 3 days and measured using a caliper. The tumor volume was calculated with the formula, \( V = 0.5 \times L \times W^2 \) (with L, length and W, width).

Statistical analysis
The demographic and clinical information of the patients and samples were summarized by descriptive analyses. The chi-squared test was used to evaluate the correlation between AMF/GPI expression and the clinicopathologic characteristics of the patients with GC. Survival curves were obtained with the Kaplan–Meier (KM) method and compared with the log-rank test. The Cox proportional hazard regression model was used to estimate the effect of AMF/GPI expression on mortality risk, ultimately controlling for confounders. The 95% CI for the median time to event was computed. Differences were considered significant at \( P < 0.05 \). All the statistical analyses were performed using STATA 15.0.

Results
AMF/GPI expression in GC tissues
Using the Oncomine database, we found that AMF/GPI expression was significantly higher in GC tissues than in normal tissues (Figure S1). To confirm this observation, we collected four pairs of fresh GC and adjacent noncancerous tissues and, by Western blot, found a higher AMF/GPI expression in GC tissues than in the paired mucosa (Figure 1A,B). As shown in Table 1, AMF/GPI expression in the GC group...
was significantly higher than that in adjacent nonneoplastic mucosa (53.73% vs 36.72%, \( P < 0.001 \)).

### Association between AMF/GPI expression and clinicopathologic features of GC

As shown in Table 2, higher AMF/GPI expression was positively associated with lymph node metastasis (\( P = 0.021 \)) and pathologic TNM staging (\( P = 0.022 \)). Additionally, the diffuse-type GC displayed a lower AMF/GPI expression than intestinal-type and mixed-type ones (\( P = 0.033 \)), in agreement with the results from the Oncomine database.

### Higher AMF/GPI expression predicts worse outcomes in patients with GC

KM analysis showed that among all 335 cases, patients with higher AMF/GPI expression have a lower 5-year OS rate than those with lower AMF/GPI expression (\( P = 0.007 \) with the log-rank test; Figure 1B). Also, patients with lower AMF/GPI expression showed a better OS rate in 231 cases with intestinal type (\( P = 0.003 \) with the log-rank test; Figure S2).

Additionally, by analyzing data from KM plotter data set, we found that the OS rate and progression-free (PF) survival rate of patients with GC in the AMF/GPI-positive group are poorer than those of patients in the AMF/GPI-negative group (Figure S3, \( P < 0.05 \)).\(^{22}\) Multivariate Cox regression revealed that there was some evidence to suggest the expression of AMF/GPI to be an independent prognostic factor in patients with GC (HR = 1.30, 95% CI = 0.96–1.76; Table 3).

### Knockdown of AMF/GPI restrains proliferation and tumorigenesis of GC cells

AMF/GPI knockdown with a lentivirus expressing anti-AMF/GPI shRNA was confirmed by Western blotting (Figure 2A). As shown in Figure 2B, shRNA knockdown groups had a...
Table 2  Relationship between AMF/GPI expression and clinicopathologic features of gastric cancer patients

<table>
<thead>
<tr>
<th>Clinicopathologic characteristics</th>
<th>N</th>
<th>AMF/GPI expression</th>
<th>χ²</th>
<th>P</th>
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<tr>
<td></td>
<td></td>
<td>Negative (%)</td>
<td>Positive (%)</td>
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<td>77</td>
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<td>90 (54.22)</td>
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<td>&gt;60</td>
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<td>159</td>
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<td>12</td>
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<td>Diffuse</td>
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<td>Mixed</td>
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<td>13 (32.50)</td>
<td>27 (67.50)</td>
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<td>Depth of invasion</td>
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<td></td>
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<tr>
<td>T1</td>
<td>9</td>
<td>6 (66.67)</td>
<td>3 (33.33)</td>
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<td>T2</td>
<td>30</td>
<td>16 (53.33)</td>
<td>14 (46.67)</td>
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<td>T3</td>
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<tr>
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<td>150 (58.37)</td>
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<td>IV</td>
<td>32</td>
<td>10 (31.25)</td>
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Note: *P<0.05.
Abbreviations: AMF, autocrine motility factor; GPI, glucose-6-phosphate isomerase.

Knockdown of AMF/GPI induced G2/M arrest and apoptosis in GC cells

To clear whether AMF/GPI commands cell growth by influencing DNA synthesis or apoptosis, we monitored cell cycle progression using fluorescence-activated cell sorting. A higher proportion of shAMF/GPI-infected cells both in SGC7901 and BGC 823 were arrested in the G2/M phase of the cell cycle compared to the shControl-infected ones (Figure 2A). In addition, apoptosis was assessed by annexin V and PI staining in SGC7901 and BGC 823 cells. shAMF/GPI-infected
cell groups showed increased apoptosis rate compared to their corresponding control ones (Figure 3B).

**Knockdown of AMF/GPI attenuates GC cell migration and invasion**

We had found that AMF/GPI expression was associated with the TNM stage in patients with primary GC. We hypothesized that AMF/GPI augmented the migration and invasion capabilities of GC cells. Therefore, we performed Transwell assays to investigate whether changes in AMF/GPI levels affected the migration and invasion of the cells. We found that migration was inhibited and found a significant reduction of invasion in AMF/GPI-knockdown cells (Figure 4).

**Knockdown of AMF/GPI disrupts the metabolism in GC cells**

To determine the metabolic effect of AMF/GPI on GC cells, we performed Seahorse XF24 assays and analyzed OCR and ECAR in each group. We found blockage of these two metabolic indicators in AMF/GPI-silenced cells (Figure 5). The results confirmed that AMF/GPI knockdown causes a metabolic disturbance in GC cells.

**Discussion**

AMF/GPI is a housekeeping enzyme that catalyzes the interconversion between glucose-6-phosphate and fructose-6-phosphate in glycolysis and gluconeogenesis and also acts as a multifunctional cytokine associated with invasion and metastasis in cancer cells. According to the theory of the “Warburg effect”, cancer cells favor glycolysis for ATP production even in an aerobic environment. In previous studies, de Padua et al found that GPI knockdown can suppress glucose consumption and lactic acid secretion in human colon cancer and mouse melanoma. Niizeki et al proved that hypoxia enhances the expression of AMF/GPI and the motility of human pancreatic cancer cells, and inhibitors of
The association between AMF/GPI expression, tumorigenesis and prognosis of GC

**Figure 2** Effects of AMF/GPI knockdown in GC cell growth and proliferation in vitro and in vivo.

**Notes:**
(A) AMF/GPI expression confirmed by Western blotting. Lane 1, wild type; lane 2, scrambled shControl; lane 3, shAMF/GPI. (B) Cell proliferation measured by MTT assay. (C) Tumor growth curve of shControl and shAMF/GPI cells. (D) Pictures show tumor formation in nude mice injected with shControl and shAMF/GPI cells. (E) H&E and Ki-67 stainings of xenograft tumor tissues. Magnification: ×100. The data are shown as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

**Abbreviations:** AMF, autocrine motility factor; GC, gastric cancer; GPI, glucose-6-phosphate isomerase.
AMF/GPI could counteract these effects. A different study has shown that overexpression of hypoxia-inducible factor 1-alpha (HIF-1α) or a dominant-negative HIF-1α enhances or suppresses the expression of AMF/GPI and cell motility, respectively. These findings suggest that AMF/GPI overexpression is involved in the molecular mechanism of carcinogenesis caused by the adaption of cancer cells to hypoxia. In this study, we found that AMF/GPI overexpression was related to the metabolism disruption in GC cells.

A previous study has shown that AMF/GPI binds to HER2, and triggers HER2 phosphorylation and metalloprotease-mediated ectodomain shedding, and activation of the
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Phosphoinositide 3-kinase and mitogen-activated protein kinase (MAPK) signaling, finally interfering with the ability of trastuzumab to inhibit breast carcinoma cell growth.26 Another study has found that an anti-AMF/GPI monoclonal antibody (9A-4H) significantly suppresses the growth of tumors in Balb/c nude mice transplanted with the human GC cell line NCI-N87.27 Therefore, AMF/GPI overexpression may cause drug resistance in GC and its suppression may increase the sensitivity to chemotherapy.

AMF/GPI expression was positively linked to lymph node metastasis and TNM staging of GC, indicating that AMF overexpression may play a positive role in the progression and differentiation of cancer. Gong et al19 have found AMF/GPI overexpression in GC with lymph node metastasis, an observation that supports our data. Our functional experiments showed the inhibitory effect of AMF/GPI knockdown on proliferation, metabolism, and metastasis of GC cells, in accordance with the previous findings. In breast cancer, AMF/GPI overexpression increases the DNA-binding activity of NF-κB to upregulate the expression of ZEB1/ZEB2, which induces epithelial–mesenchymal transition (EMT).28 In hepatocellular carcinoma, AMF/GPI enhances cell invasion by stimulating the adhesion, motility, and matrix metalloproteinase 2 secretion.29 In melanoma, migration induced by AMF/GPI is mediated by autocrine production of IL-8.30 AMF/GPI knockdown inhibits cell invasion, migration, and proliferation, and tumor metastasis in endometrial carcinoma via the suppression of the MAPK-ERK1/2 pathway.31 Moreover, Tsutsumi et al32 indicated that AMF/GPI overexpression stimulates invasion in vitro, tumor growth in vivo, and promotes liver metastasis of pancreatic cancer cells through downregulation of E-cadherin and upregulation of SNAIL. These data support the hypothesis that AMF/GPI may be positively linked to the progression of GC by inducing proliferation and EMT.

Previously, AMF/GPI overexpression has been found to be an independent prognostic factor of poor survival.11,19 Furthermore, high levels of AMF/GPI and its receptor have been reported in patients with breast cancer and high AMF expression has been related to local recurrence.33 In our study, the results of immunohistochemistry and bioinformatics analysis showed that AMF/GPI overexpression is positively correlated with lower OS and PF survival rates in patients with GC. This result might be explained by the positive correlation between AMF/GPI expression and aggressive cell behavior. Hence, we propose that AMF/GPI might be a novel marker for adverse prognosis in patients with GC. Future studies are necessary to confirm the biologic effects of AMF/GPI on drug resistance and/or angiogenesis and to explore the relationship between tumor metabolism and other aggressive features.

Figure 5  Effects of AMF/GPI knockdown on metabolism in GC cells.
Notes: (A) OCR of shControl and shAMF/GPI cells. (B) ECAR of shControl and shAMF/GPI cells.
Abbreviations: AMF, autocrine motility factor; ECAR, extracellular acidification rate; GC, gastric cancer; GPI, glucose-6-phosphate isomerase; OCR, oxygen consumption rate; WT, wild type.
Collectively, we found that AMF/GPI overexpression was associated with carcinogenesis and tumor progression and with a poorer prognosis in patients with GC. AMF/GPI knockdown reverses the aggressive phenotypes of GC and might, therefore, be a novel target for the treatment of GC.

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Disclosure
The authors report no conflicts of interest in this work.

References
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Supplementary materials

Figure S1 AMF/GPI mRNA in normal gastric tissues and primary gastric cancer tissues (Oncomine).
Abbreviations: AMF, autocrine motility factor; GPI, glucose-6-phosphate isomerase.

Figure S2 Kaplan–Meier survival curves of overall survival in 56 patients of diffuse type and 231 patients of intestinal type (AMF/GPI negative vs AMF/GPI positive).
Abbreviations: AMF, autocrine motility factor; GPI, glucose-6-phosphate isomerase.
Figure S3 Kaplan–Meier survival curves of overall and progression-free survival for patients with AMF/GPI-negative and AMF/GPI-positive, from Kaplan–Meier Plotter (Szasz, Lanczky et al. 2016).

Abbreviations: AMF, autocrine motility factor; GPI, glucose-6-phosphate isomerase.

Reference