Serum angiopoietin-like protein 2 as a potential biomarker for diagnosis, early recurrence and prognosis in gastric cancer patients

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Abstract

Chronic inflammation of gastric mucosa by Helicobacter pylori infection can initiate gastric carcinogenesis. As angiopoietin-like protein 2 (ANGPTL2) mediates inflammation and inflammation-associated carcinogenesis, we investigated the functional and clinical significance of ANGPTL2 in human gastric cancer (GC). SiRNA knockdown studies were performed for the functional assessment of ANGPTL2 in GC cell lines. ANGPTL2 expression was evaluated immunohistochemically in 192 tissue specimens from GC patients. In addition, we screened serum ANGPTL2 levels from 32 GC patients and 23 healthy controls; and validated these results in 194 serum samples from GC patients and 45 healthy controls by ELISA. ANGPTL2 knockdown caused anoikis and inhibited proliferation, invasion and migration in GC cells. ANGPTL2 expression was upregulated in GC tissues compared to normal gastric mucosa; and high ANGPTL2 expression was significantly associated with tumor progression, early recurrence ($p = 0.003$) and poor prognosis ($p = 0.007$). Serum ANGPTL2 levels in GC patients was significantly higher than for healthy controls ($p < 0.05$), and accurately distinguished GC patients from healthy controls ($AUC = 0.865$). The validation step confirmed significantly higher serum ANGPTL2 levels in GC patients than healthy controls ($p < 0.0001$). Receiver operating characteristic curves yielded robust AUC value (0.831) accompanied by high sensitivity (73.0%) and specificity (82.2%) in distinguishing GC patients from healthy controls. High serum ANGPTL2, rather than its expression in matched tissues, was significantly associated with tumor progression, and emerged as an independent marker for recurrence (HR: 5.05, $p = 0.0004$) and prognosis (HR: 3.6, $p = 0.01$). Serum ANGPTL2 expression is a potential noninvasive biomarker for diagnosis, early recurrence and prognosis of GC patients.

Introduction

Although the incidence of gastric cancer (GC) has significantly reduced in developed countries, it remains one of the most common malignancies and ranks second in global cancer-related mortality (1,2). Early detection is the most promising...
approach in improving long-term survival of GC patients, but ~70% of GC patients are firstly diagnosed at late stages with locally advanced or metastatic disease, which precludes the opportunity of surgical resection (3). Although several molecular biomarkers, including CEA (4), CA72-4 (5) and CA19-9 (6) have been used in clinical practice, none of these markers are highly specific for GC. In addition, tumor recurrence and metastasis often occurs even after surgery, and remains one of the main causes of death from GC. Therefore, early diagnosis, and managing postoperative recurrence and metastasis are the greatest challenges for improving survival rates in GC patients.

It is now a well-established paradigm that chronic inflammation plays a significant role in cancer development, including carcinogenesis and tumor progression such as invasion, migration and metastasis (7). Inflammation induced by environmental exposures is also known to increase cancer risk (8,9). Chronic and subclinical levels of inflammation; for example, obesity-induced inflammation (10), immune-mediated inflammatory diseases (11) and viral infections (12) reportedly may increase cancer risk as well. Epidemiological studies have suggested that Helicobacter pylori infection is an important risk factor for GC patients. Gastric carcinogenesis is regarded as a multistep process that manifests in an intestinal metaplasia–dysplasia–invasive carcinoma sequence—which can be initiated by H. pylori infection leading to chronic active inflammation with severe oxidative damage of the gastric mucosa (13,14).

Cellular stress in the endoplasmic reticulum (ER) is easily induced by various conditions such as hypoxia, oxidative stress, hypoglycemia and viral infections—all commonly observed in the primary tumor microenvironment (15), including H pylori-related gastric carcinogenesis (16). Angiopoietin-like protein 2 (ANGPTL2) is a causative mediator of chronic inflammation in obesity and related metabolic abnormalities (17). In obesity, adipose tissue-related ER stress increases ANGPTL2 secretion or expression in adipocytes (17). In addition, increased ANGPTL2 expression has also been detected in tumor cells in hypoxic regions and undernourishment, suggesting that the tumor microenvironment induces ANGPTL2 expression in GC. On the other hand, ANGPTL2 expression in isolated tumor cells is highly associated with metastasis to lymph nodes or distant organs due to increased angiogenesis in the tumor environment and increased tumor cell invasion and migration through epithelial-mesenchymal transition (18,19). Thus, cancer cell- and/or tumor microenvironment-derived ANGPTL2 is considered to be a critical factor in inflammation-induced carcinogenesis and cancer progression. Furthermore, ANGPTLs have an N-terminal signaling sequence for protein secretion (17), which has been detected in systemic circulation under obesity-induced inflammation (20,21), suggesting that serum ANGPTL2 levels may be a useful diagnostic and predictive biomarkers in cancer patients. We previously demonstrated that ANGPTL2 accelerates cell growth, proliferation, invasion and migration in esophageal cancer (EC) and colorectal cancer (CRC) (22,23). We also showed that serum ANGPTL2 is diagnostic marker for both EC and CRC. In addition, recently, Yoshida et al. (24) reported that serum ANGPTL2 was a potential diagnostic biomarker for GC. However, the biological mechanisms and clinical significance of ANGPTL2 expression in both serum and matched primary tumors in GC remain unclear.

Accordingly, we investigated the biological function of ANGPTL2 using RNA interference studies in cultured GC cells. We then evaluated ANGPTL2 expression in GC tissues by immunohistochemistry (IHC) to analyze the clinical significance of ANGPTL2. Finally, we quantified serum ANGPTL2 levels from patients with GC and normal controls to assess its capacity as a non-invasive biomarker. Thus, we, for the first time, demonstrate that high ANGPTL2 expression in tumor cells promotes proliferation, invasion and migration, and is significantly associated with GC tumor progression. From a clinical standpoint, our data provide novel evidence that serum ANGPTL2 may serve as a robust non-invasive biomarker for diagnosis, early recurrence and poor prognosis in GC patients.

Methods

Study design
This study analyzed 498 serum and tissue specimens that were obtained from healthy volunteers and consecutively enrolled patients with GC at the Mie University Medical Hospital, Japan, between 1 January, 2006 and 31 December, 2011. We interrogated the functional significance of ANGPTL2 through siRNA-induced knockdown in GC cell lines, followed by evaluation of the clinical significance of ANGPTL2 expression in GC surgical tissue specimens (n = 192) and adjacent normal gastric mucosa (n = 12) by IHC, and by correlating its expression levels with various clinicopathological factors and survival in GC patients. We also quantified serum levels of ANGPTL2 in order to determine its potential usefulness as a clinical biomarker. In the screening phase, a small subset of preoperative serum specimens were collected from 16 GC patients with stages I and IV disease, as well as 23 sex- and age-matched healthy subjects. To further assess the significance of serum ANGPTL2 in GC patients, we performed an independent validation step with a large, independent cohort of 194 GC patients and 45 healthy subjects from whom results from preoperatively obtained sera were directly compared with IHC expression in matched surgical tissue specimens (Supplementary Table 1, available at Carcinogenesis Online). A total of 194 patients (129 men, 65 women) with GC were enrolled in this study. The mean age was 66 years (range: 18–90 years). None of the patients received chemotherapy or radiotherapy before surgery and no perioperative mortalities were observed. All patients were classified according to the Japanese Classification of Gastric Carcinoma (25); 98 patients had stage I disease, 32 had stage II, 34 had stage III and 30 were stage IV. Median follow-up duration was 23 months (range: 1–79 months). All patients were followed up after their initial hospital discharge, with physical examination and tumor marker assays (CEA, CA19-9) performed every 1–3 months and computed tomography performed every 6 months. Endoscopic examination was performed when necessary. The entire study and patient sample collection was approved by the Institutional Review Board at the Mie University Hospital in Japan. All participants provided written informed consent and willingness to donate blood and tissue samples for research.

Cell lines
Human GC cell lines, MKN1, MKN7, MKN45, MKN74, KATO3, NUGC3 and NUGC4, were provided by Riken Bio-resource Center Cell Bank (Tsukuba, Ibaragi, Japan) and maintained in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics at 37°C in a 5% humidified CO2 atmosphere. The authenticity of various cell lines was routinely monitored by analyzing a series of genetic and epigenetic markers specific for each cell line.

ANGPTL2 RNA interference
ANGPTL2-specific siRNA (Silencer® Select Validated siRNA, standard purity) and negative control siRNA (Silencer™ Negative Control siRNA)
were purchased from Ambion (Austin, TX). Transfections were performed by mixing cell suspensions with siRNA oligonucleotides (20nM), OptimEM I (Invitrogen) and Lipofectamine RNAiMAX (Invitrogen) before cell plating. Cells were maintained in a humidified atmosphere and assays were performed after 48-h incubation.

MTT assay
Forty-eight hours after transfection, ANGPTL2 siRNA and control siRNA-transfected cells were seeded at 5 × 10^4 cells per well in 96-well flat-bottomed microtiter plates, in a final volume of 100 μl culture medium per well, and incubated in a humidified atmosphere. After 0–48 h culture, MTT assays were used to assess cell viability. Briefly, 200 μl sterile MTT dye (5 mg/ml Sigma, St Louis, MO) was added. After incubating for 4 h at 37°C in 5% CO₂, MTT medium mixture was removed and 200 μl of dimethyl sulfoxide was added to each well. Absorbance was measured using SoftMax Pro (Molecular Devices Corp., Sunnyvale, CA) at a wavelength of 450 nm. Each experiment was performed independently three times in triplicates.

BrDU assay
Forty-eight hours after transfection, ANGPTL2 siRNA and control siRNA-transfected cells were seeded at 5 × 10^4 cells per well in 96-well flat-bottomed microtiter plates, in a final volume of 100 μl culture medium per well. After 0–48 h culture, BrDU assays were used to assess cell viability according to the manufacturer's instructions. Absorbance was measured using SoftMax Pro (Molecular Devices Corp., Sunnyvale, CA) at a wavelength of 450 nm. Each experiment was performed independently three times in triplicates.

Cell cycle analysis
ANGPTL2 siRNA- and control siRNA-transfected cells (1 × 10^5/well) were pre- incubated for 24 h in medium with serum (10% fetal bovine serum) at 37°C in 5% CO₂. Cells were collected and fixed in 70% ethanol at −20°C. Then, DNA content was evaluated using a cell cycle assay kit (Millipore, Billerica, MA) according to the manufacturer's instructions when using the Muse Cell Analyzer (Millipore). Each independent experiment was performed three times.

Apoptosis assay
Annexin V & Dead Cell Assay was performed utilizing Muse Cell Analyzer from Millipore (Billerica, MA) according to manufacturer’s instructions. Briefly, after the transfection of siRNAs, the cells were incubated with Annexin V and Dead Cell Reagent (7-AAD) and the events for dead, late apoptotic, early apoptotic, and live cells were counted. Each independent experiment was performed three times.

Anoikis assays
Anoikis assays were performed in six-well Costar Ultra Low Attachment Microplates (Corning). Forty-eight hours after transfection, ANGPTL2 siRNA and control siRNA-transfected cells were seeded at a concentration of 5 × 10^4 cells/ml, and 2 ml cell suspension was added to each well and incubated in the microplates for 24 h in a humidified (37°C and 5% CO₂) incubator. Next, the MTT assay was performed. After induction of anoikis, cells were seeded at 5 × 10^4 cells/well in microtiter plates (96 wells, flat bottom) in a final volume of 100 μl culture medium per well. Spectrophotometric absorbance of the samples was measured as described earlier. Each independent experiment was performed six times.

Invasion assay
Transfected cells (2.5 × 10^5 cells/well) were seeded in serum-free media (in triplicate) in 24-well (8 μm pore size) Matrigel™ Invasion Chambers (BD Biosciences, Franklin Lakes, NJ). Inserts were placed into Falcon companion plates containing 10% fetal bovine serum and incubated for 48 h. The incubation media and cells were then removed from the top chamber using cotton swabs and phosphate-buffered saline and the number of cells invading the membrane underside was determined. Membranes were fixed and stained with Diff-Quik stain™ (Sysmex, Kobe, Japan) and mounted on glass slides. The numbers of migrating or invading cells in 10 microscopic fields were subsequently counted with a light microscope at 10× magnification.

Wound healing assay
Transfected GC cells were incubated until confluent in six-well plates and wounds were generated using a sterile 200 μl pipette tip. Cells were then grown for an additional 48 h. Wound closure was assessed using an Olympus IX71 microscope (Olympus, Center valley, PA) at 40× magnification. Cell migration distance was measured using Adobe Photoshop 9.0.2 software and compared with baseline measurements.

Total RNA extraction and cDNA synthesis
Total RNA from cell lines were isolated using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. cDNA was synthesized by random hexamers using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA).

Quantitative real-time reverse transcription PCR
ANGPTL2 and GAPDH were quantified in duplicate by quantitative real-time reverse transcription analysis (qRT-PCR), using TaqMan probes for ANGPTL2 ( assay ID, Hs0017191) and GAPDH ( assay ID, Hs02758991) (Applied Biosystems, Foster City, CA). The following cycling conditions were used: 95°C, 10 min; followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The average expression levels of ANGPTL2 were normalized against GAPDH using the 2−ΔΔCt method. qRT-PCR assays were performed in duplicate for each sample; mean values were used to calculate mRNA expression levels.

Immunohistochemistry
Formalin-fixed, paraffin-embedded (FFPE) sections (2-3 μm thickness) from specimens from 192 GC patients were used for IHC analysis of ANGPTL2 expression. After deparaffinization and dehydration, specimens were boiled in 10mM sodium citrate buffer to unmask antigens. Specimens were then blocked and incubated with primary antibody overnight at 4°C. Antibody binding was detected by Dako’s HRP Envision kit (Dako Cytomation, Denmark). All sections were counterstained with hematoxylin. Primary antibody against ANGPTL2 (Cell Signaling Technology, Boston, MA) was diluted 1:100. Positive and negative controls were also run simultaneously.

ANGPTL2 expression in stained FFPE sections from GCs was analyzed separately by two expert pathologists without prior knowledge of any subject's clinicopathological or survival data. Expression of ANGPTL2 was evaluated by scanning the entire tissue specimen under low-power magnification (40×), and then confirming the findings under high-power magnification (200× and 400×). An immunoreactivity scoring (IRS) system was applied using the following criteria: (1) fraction of positive-stained cells: 0, 50%; 0; 6–25%; 1; 26–50%; 2; 51–75%; 3; and >75%, 4 (Supplementary Figure 2A–D, available at Carcinogenesis Online), (ii) intensity of stain: negative, 0, weak, 1, medium, 2 and strong, 3 (Supplementary Figure 2A–D, available at Carcinogenesis Online). Scores obtained from A and B were multiplied together to make the staining score according to the proportion and intensity of positively stained cancer cells. Specimens were rescored if the difference between the scores by the two pathologists was more than 3.

Enzyme-linked immunosorbent assay
Serum concentrations of ANGPTL2 for screening assay were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (USCN Life ScienceWuhan) according to manufacturer’s protocol. We calculated optical density of each sample and duplicate measurements were obtained for all the samples. For the validation assay, serum ANGPTL2 concentration was measured using the human ANGPTL2 ELISA kit (IBL, Japan) according to manufacturer’s protocol. With regards to ANGPTL2 protein expression in GC cell lines, the cells were homogenized and cell lysates were obtained. The protein concentration of cell lysates was measured by the BCA protein assay (Pierce, Rockford, IL) and ANGPTL2 protein expression in human GC cell lines were quantified by ELISA (USCN Life Science, Wuhan).

Statistical methods
The significance of ANGPTL2 expression in both sera and matched corresponding tumors was determined by the Mann–Whitney test, Kruskal–Wallis test or the χ² test, as appropriate. Association between serum
ANGPTL2 and ANGPTL2 IHC scores from matched primary tumors was analyzed by Spearman’s correlation. Receiver operating characteristic (ROC) curves were used to obtain AUC values and to determine optimal cut-off values to predict diagnosis, early recurrence and prognosis by Youden’s index. Overall survival (OS) and disease-free survival (DFS) curves were analyzed by the Kaplan-Meier method and differences were examined by log-rank tests. Cox’s proportional hazard regression test was used to estimate univariate and multivariate hazard ratios for OS and DFS. All P values were two-sided; P < 0.05 was considered statistically significant. All statistical analyses used Medcalc 12.3 for Windows (Broekstraat 52, 9030, Mariakerke, Belgium).

Results

Functional analyses of ANGPTL2 in GC cells

ANGPTL2 expression in GC cells

We investigated ANGPTL2 expression by quantitative RT-PCR in a panel of established GC cell lines (Supplementary Figure 3A, available at Carcinogenesis Online). Among various cell lines, MKN1 and KATOIII showed highest ANGPTL2 expression. In all other cell lines, expression was distinctly low or undetectable. ELISA was used to confirm ANGPTL2 protein expression in GC cell lines and the results were consistent with the quantitative RT-PCR data (Supplementary Figure 3B, available at Carcinogenesis Online). Based on these results, we selected MKN1 and KATOIII for siRNA knockdown experiments and functional characterization. Transfection of GC cell lines with ANGPTL2-siRNA dramatically reduced ANGPTL2 mRNA expression compared to negative control-siRNA treated cells 48 h post-transfection (Supplementary Figure 3C, available at Carcinogenesis Online). ELISA results were consistent with the real-time PCR data (Supplementary Figure 3D, available at Carcinogenesis Online). Based on these results, we next performed additional in vitro experiments to assess function of ANGPTL2 in GC cells.

ANGPTL2 inhibits anoikis and promotes proliferation, invasion and migration ability in GC cells

We assessed proliferation, anoikis, migration and invasion in GC cell lines transfected with control-siRNA and ANGPTL2-siRNA. MTT assays revealed that ANGPTL2 downregulation significantly inhibited tumor cell growth at 24 and 48 h in MKN1 and KATOIII cells (Figure 1A and B). Further examination of cell proliferation and apoptosis by BrdU and apoptosis assay by flow cytometry analysis revealed that ANGPTL2 downregulation significantly inhibited BrdU incorporation in both MKN1 and KATOIII cells (Figure 1C). In contrast, no significant apoptosis was observed between negative control-siRNA and ANGPTL2-siRNA treated cells in both MKN1 and KATOIII cells at 48 h after transfection (Figure 1D). Furthermore, cell cycle analysis revealed that reductant ANGPTL2 expression reduced the S-phase population and increased the G0/G1 population in both GC cell lines (Figure 1E). We next measured anoikis by evaluating the number of viable MKN1 and KATOIII cells that were floating in low-attachment plates, because anoikis is a form of apoptosis characterized by loss of cell adhesion. Transfection of ANGPTL2-siRNA significantly decreased the number of viable MKN1 and KATOIII cells compared to controls (Figure 1F). Wound healing assays were performed to compare the migratory potential of GC cells transfected with ANGPTL2-siRNA versus control-siRNA. The number of migratory cells treated with ANGPTL2-siRNA was markedly decreased compared to control siRNA-treated cells (Figure 1G and H). In addition, we performed invasion assays to determine whether attenuated ANGPTL2 levels might affect cellular invasion. ANGPTL2-siRNA transfection of MKN1 and KATOIII revealed a weakened invasive capacity compared to cells transfected with control-siRNA (Figure 1I and 1J). Taken together, these results indicate that ANGPTL2 expression inhibits anoikis and promotes cell proliferation by inducing G0/G1 to S-phase progression, invasion and migration in GC cells.

ANGPTL2 expression is associated with tumor malignancy, early recurrence and poor prognosis in GC patients

We performed IHC analysis to investigate the expression pattern (location and intensity) of ANGPTL2 protein, and to evaluate associations between its protein expression and various clinicopathological variables. ANGPTL2 protein expression was primarily witnessed in the cytoplasm and nuclei of GC cells (Figure 2A). In contrast, normal gastric mucosal epithelia showed no ANGPTL2 staining, but weak expression in the stromal components (Figure 2B). ANGPTL2 staining scores were significantly elevated in GC compared to normal gastric mucosa and significantly increased in advanced stages (stages III and IV) compared to early stages (stages I and II) (P < 0.0001) (Figure 2C). Based upon the median value of 2, we defined a cut-off value of >2 as the high-staining group (n = 112) and <2 as the low-staining group (n = 80). The high-staining group was significantly associated with large tumor size (P < 0.001), serosal invasion (P = 0.0002), lymphatic invasion (P = 0.001), venous invasion (P = 0.0008), lymph node metastasis (P = 0.0001) and distant metastasis (P = 0.01) (Table 1). We next evaluated whether ANGPTL2 protein expression predicted prognosis in GC patients. The high staining group had significantly poorer OS (P = 0.007, Figure 2D) and poorer DFS (P = 0.003, Figure 2E) compared to patients in the low-staining group.

Serum ANGPTL2 in GC patients was significantly higher than that in healthy controls in the screening cohort

ANGPTL2 protein is a secreted glycoprotein, homologous to angiopoietins. Therefore, in order to determine the feasibility of conventional ELISA, we measured serum ANGPTL2 levels in a screening cohort of 16 patients with stage I GC, 16 with stage IV GC and 23 healthy volunteers. Compared to healthy controls, serum ANGPTL2 levels in patients with stage I and stage IV GC were significantly elevated (P < 0.05 for both stages; Figure 3A), and these levels in stage IV were significantly higher than in stage I (Figure 3A).

Next, we generated ROC curves to assess the potential of serum ANGPTL2 as a biomarker for GC diagnosis, and found that serum ANGPTL2 levels discriminated GC patients from healthy controls with a sensitivity of 84.37% and a specificity of 82.61% and an associated AUC value of 0.865 (95% CI: 0.746–0.942; Figure 3B). Serum ANGPTL2 levels also differentiated early stage GC (stage I) from control subjects with a sensitivity of 75.0% and a specificity of 82.61% and a corresponding AUC of 0.822 (95% CI: 0.666–0.926; Figure 3C).

Validation of serum ANGPTL2 expression as a biomarker

Serum levels of ANGPTL2 are robust for the identification of GC patients

To evaluate the diagnostic potential of ANGPTL2, we examined 239 serum samples that were obtained from 194 patients with GC and 45 healthy controls. Compared with healthy controls, serum ANGPTL2 levels were significantly higher in GC patients (P < 0.0001; Figure 3D). Furthermore, when GC patients were
segregated by TNM stage, a gradual increase in serum ANGPTL2 levels was clearly discernible, with significantly higher expression levels in stage IV patients compared to stages I, II or III patients \((P < 0.05\) for all; Figure 3E); all the findings that were consistent with the data from screening step. ROC analyses to validate the potential for using serum ANGPTL2 as a noninvasive biomarker for GC diagnosis revealed that serum ANGPTL2 levels robustly discriminated GC patients from control subjects, with an AUC values of 0.831 \((95\%\ CI: 0.786–0.903;\ Figure 3F)\), and a sensitivity of 73.0% and a specificity of 82.2% calculated by cut-off value of serum ANGPTL2 \((1031.6\ pg/ml)\). Most notably from a screening perspective, the ROC analyses revealed that serum ANGPTL2 levels could differentiate patients with stage I GC from healthy controls, with 82.83% sensitivity and 67.65% specificity \((cut-off \ value: 1031.6\ pg/ml)\), at an AUC value of 0.81 \((95\%\ CI: 0.722–0.865;\ Figure 3G)\).

Serum levels of ANGPTL2 significantly correlate with tumor progression in GC patients

Next, we asked whether ANGPTL2 expression in serum samples correlated with any other clinicopathological variables. Table 1 illustrates that serum ANGPTL2 levels were significantly higher in GC patients with lymphatic invasion \((P = 0.001)\), vessel invasion \((P = 0.02)\), liver metastasis \((P = 0.004)\), peritoneal metastasis \((P = 0.049)\) and distant metastases \((P = 0.0002)\). To further enhance the specificity of our assay and validate that circulating ANGPTL2 expression accurately reflects concentrations found in GC tissues, we determined the relationship between ANGPTL2...
intensity scores in primary GC tissues and matched serum ANGPTL2 levels from individual GC patients. Interestingly, we observed a significantly positive correlation between ANGPTL2 expression in GC lesions and matched serum samples ($\rho = 0.145$, $P = 0.0446$; Supplementary Figure 4B, available at Carcinogenesis Online).

Serum ANGPTL2 may be a biomarker for predicting early recurrence and poor prognosis in GC patients

To further evaluate whether serum ANGPTL2 levels in GC from patients may serve as a predictor of patient outcome, we performed Kaplan–Meier survival analysis. As anticipated, patients with higher levels of serum ANGPTL2 had significantly worse OS ($P = 0.0007$; Figure 3H). Moreover, increased serum ANGPTL2 concentrations were significantly associated with decreased DFS ($P = 0.03$; Figure 3I). Results of Cox’s proportional hazard regression analyses for prognostic indicators are shown in Table 3. Univariate analysis associated with poor prognosis in GC patients with high levels of ANGPTL2 in both tumor and serum ($P = 0.008$ and $P = 0.002$, respectively), large tumor size ($>35$ mm, $P = 0.0009$), higher grade pathological stage (T3/T4, $P < 0.0001$), lymph node metastasis ($P < 0.0001$) and distant metastasis ($P < 0.0001$). More importantly, multivariate analysis revealed that high serum-ANGPTL2, but not high tumor tissue-ANGPTL2, to be an independent prognostic marker for OS in GC patients ($HR: 3.618; 95\%CI: 1.31–9.95; P = 0.0133$).

In addition, to determine whether serum ANGPTL2 could predict tumor recurrence in curative patients (stages I–III), Cox’s proportional hazard regression model was utilized (Table 3). Univariate analysis showed that male patients ($P = 0.03$), large tumor ($P = 0.0001$), serosal invasion ($P < 0.0001$), lymphatic invasion ($P = 0.0058$), venous invasion ($P = 0.0001$), lymph node metastasis ($P < 0.0001$) and high-ANGPTL2 in both tumor and serum (tumor: $P = 0.005$, serum: $P = 0.036$) were significantly associated with poor DFS. In contrast, multivariate analysis showed high serum ANGPTL2 to be an independent predictor for tumor recurrence in stages I–III GC patients ($HR: 5.04, 95\% CI: 2.06–12.32, P = 0.0004$).

Discussion

In the present study, for the first time, we analyzed the function of ANGPTL2 in GC cells exhibiting high metastatic potential through acquisition of proliferative, invasive and high motility phenotype. We used large independent cohorts of clinical specimens to confirm that ANGPTL2 protein expression in GC tissues was significantly higher than in the adjacent normal mucosa. We observed that high ANGPTL2 expression in GC was significantly associated with disease progression, including larger tumors; invasion to serosa, lymphatic ducts and vessels; and metastases to lymph node and distant organs. We also found that elevated ANGPTL2 expression in GC tissues could serve as a novel indicator for worse DFS and OS.

Recently, ANGPTL2 expression was found to accelerate carcinogenesis through enhanced susceptibility to both pre-malignant changes and tumor progression. Aoi et al. (18) elegantly demonstrated that ANGPTL2 is highly correlated with carcinogenesis frequency, using a chemically-induced skin squamous cell carcinoma model in mice. Tumor cell derived ANGPTL2 accelerates tumor progression by induction of cell growth in
In vitro and in vivo, Sato et al. (26) demonstrated that downregulation of ANGPTL2 by siRNA inhibits cancer cell growth, while stably overexpressed ANGPTL2 promotes cancer cell growth in prostate cancer. In addition, overexpression of ANGPTL2 promoted tumor formation in xenograft model in hepatocellular carcinoma (27). These reports are consistent with our findings for cellular proliferation, which indicate that ANGPTL2 increases cell growth by induction of progression from G0/1 to S-phase.

Our study also found that ANGPTL2 expression promoted the metastatic potential in GC cells, which were further supported by in vitro invasion and migration assays. Recently, human leucocyte immunoglobulin-like receptor B2 (LILRB2) has been identified as the receptor for ANGPTL2, and the autocrine signaling of ANGPTL2 and its receptor LILRB2 has been shown to play a key role in sustaining EMT-related invasiveness and migration ability, promotion of angiogenesis and stemness, resulting in induction of the early metastatic behavior of cancer cells (28,29). On the other hand, ANGPTL2 secreted from the tumor microenvironment could result in tumor cell migration and metastasis. In transgenic mice expressing ANGPTL2, EMT as well as tumor lymph-angiogenesis in squamous cell carcinoma were significantly increased, resulting in increased tumor cell metastasis and shortened survival compared with wild-type mice. Conversely, carcinogenesis and metastasis were markedly attenuated in Angptl2 knockout mice (18). Collectively, ANGPTL2 secreted from cancer and/or stromal cells increases cancer progression and metastasis in an autocrine/paracrine manner. These evidences could explain the fact that not all cell lines express this protein.

Clinically, elevated ANGPTL2 expression in cells within primary tumors was associated with lymph node metastasis and shorter DFS after surgery in patients with lung cancer (19,30). In addition, we previously demonstrated that ANGPTL2 is overexpressed in cancer tissues and significantly associated with tumor progression including advanced T stage, lymph node metastasis and liver metastasis, consequently early recurrence and poor prognosis in patients with EC and CRC (22,23). These reports are in accordance with our data, and support our observations for previously unrecognized mechanistic role for ANGPTL2 overexpression in tumor aggressiveness and metastases, as well as in highlighting its potential clinical significance as an outcome-predictive biomarker for patients with GC.

Although the mechanisms of ANGPTL2 alterations in cancer cells is not fully investigated, recent evidences suggest that tumor cell autonomous responses to the microenvironment, such as activation of the ER stress, calcineurin, the nuclear factor of activated T cell pathway and/or activating transcription factor and cAMP-responsive element-binding protein family proteins induce ANGPTL2 expression in tumor cells (19). In addition, specific microRNAs (miRNAs) such as miR-25 and miR-221 also regulate ANGPTL2 expression post-transcriptionally (31,32), which might indicate the discrepancy between mRNA and protein levels of ANGPTL2 expression in our in vitro study.

This study also revealed a very intriguing and vital clinical implication for serum ANGPTL2 in GC patients. Data from initial screening phase of our study showed a statistically significant increase in serum ANGPTL2 in GC patients compared with healthy controls, and a significant higher ANGPTL2 expression in GC patients with stage IV than with stage I disease. Increased serum ANGPTL2 expression was thereafter successfully validated in a large, independent set of serum samples, which are consistent with recent published paper (24). In addition, our results are the first to demonstrate that high levels of ANGPTL2 in both primary GC tissues and matched serum samples are associated with large tumor size, distant metastasis and advanced TNM stage. Another interesting finding was a statistically significant correlation between ANGPTL2 expression in primary lesions and those in sera, suggesting that circulating

| Category                  | ANGPTL2 high (n = 112) | ANGPTL2 low (n = 80) | P value | Serum ANGPTL2 × 10² pg/ml (mean ± SD) | P value |
|---------------------------|------------------------|----------------------|---------|--------------------------------------|---------|
| Age                       | ≤67 years *            | 62 (55%)             | 0.2     | 1.22 ± 0.50                          | 0.0025  |
|                           | > 67 years             | 50 (45%)             |         | 1.47 ± 0.58                          |         |
| Sex                       | Male                   | 66 (59%)             | 0.01    | 1.33 ± 0.60                          | 0.4     |
|                           | Female                 | 46 (41%)             | 1.37 ± 0.45 |                                      |         |
| Histology                 | Well                   | 17 (15%)             | 0.19    | 1.30 ± 0.48                          | 0.66    |
|                           | Mod, poor or mucinous  | 95 (85%)             |         | 1.36 ± 0.57                          |         |
| Tumor size                | ≤35mm*                 | 68 (61%)             | 0.001   | 1.35 ± 0.53                          | 0.9827  |
|                           | >35mm                  | 44 (39%)             |         | 1.35 ± 0.58                          |         |
| Serosal invasion          | Present                | 76 (68%)             | 0.0002  | 1.30 ± 0.55                          | 0.16    |
|                           | Absent                 | 36 (32%)             | 1.40 ± 0.56 |                                      |         |
| Lymph node metastasis     | Present                | 32 (29%)             | 0.0001  | 1.29 ± 0.49                          | 0.09    |
|                           | Absent                 | 80 (71%)             | 1.43 ± 0.64 |                                      |         |
| Lymphatic invasion        | Present                | 47 (42%)             | 0.001   | 1.24 ± 0.54                          | 0.03    |
|                           | Absent                 | 65 (58%)             | 1.46 ± 0.57 |                                      |         |
| Venous invasion           | Present                | 81 (72%)             | 0.0008  | 1.28 ± 0.54                          | 0.02    |
|                           | Absent                 | 31 (28%)             | 1.33 ± 0.55 | 0.0046 |         |
| Liver metastasis          | Present                | 111 (99%)            | 0.09    | 1.97 ± 0.40                          |         |
|                           | Absent                 | 1 (1%)               |         | 3.33 ± 0.55                          | 0.049   |
| Peritoneal metastasis     | Present                | 105 (94%)            | 0.9     | 1.62 ± 0.63                          |         |
|                           | Absent                 | 7 (6%)               |         | 1.28 ± 0.52                          | 0.0002  |
| Distant metastasis        | Present                | 101 (90%)            | 0.01    | 1.26 ± 0.52                          |         |
|                           | Absent                 | 11 (10%)             | 1.71 ± 0.60 |                                      |         |

*Median age and tumor size 67 years and 35mm. SD, standard deviation.
ANGPTL2 in serum of GC patients is likely produced by the GC and adjacent normal H. pylori-infected mucosa.

In addition, our study demonstrates the potential role of serum ANGPTL2 in the early detection of GC. This was supported by markedly high AUC values derived from comparisons between GC patients and healthy control subjects (screening: AUC = 0.865; validation: AUC = 0.831). Yoshinaga et al. also demonstrated the diagnostic performance of ANGPTL2 in serum.
from small number of patients and healthy controls compared to our cohort. The AUC value in this report was 0.744 for discriminating patients with GC from healthy individuals (24). More importantly from a screening perspective, our current study demonstrated additional evidence that serum ANGPTL2 expression manifested with higher AUC values (screening AUC: 0.822; validation AUC: 0.81) than other serum biomarkers, including CEA (0.593) and CA19-9 (0.527) for discemining early (stage I) GC patients (33). As early-stage GC patients have a 5-year survival rate >95%, an accurate, noninvasive means of screening for early GC is very encouraging for the clinical management of these patients. Moreover, our data suggest that serum ANGPTL2 could serve as a predictive biomarker for patients with early recurrence and poor prognosis in GC. Our study has already shown the potential of tissue ANGPTL2 IHC scores as a prognostic and predictive marker for early relapse in GC. However, the finding that high levels of serum (rather than tissue) ANGPTL2 indicate poor DFS and OS in patients with GC is an important step in identifying a noninvasive biomarker for this disease. Furthermore, multivariable analysis revealed that high serum ANGPTL2 is an independent predictor of DFS and OS, whereas ANGPTL2 expression in tumors is significantly compromised by other clinicopathological factors. Therefore, serum ANGPTL2 levels may not only help diagnose gastric neoplasia, but may also help predict metastases or tumor recurrence with higher accuracy.

As promising as this screening tool is, we acknowledge a potential limitation of using ANGPTL2 as a singular biomarker for early detection of GC, since higher circulating ANGPTL2 levels have been described in several lifestyle-related diseases, including hypertension, diabetes, dyslipidemia, obesity, cardiovascular disease and several malignancies (20–24,34–37). Therefore, we need to further analyze whether circulating ANGPTL2 expression is specifically associated with GC itself or is a common phenomenon that manifests during progression of any cancer resulting from inflammation in cancer microenvironment and/or perturbations in the host immune response (38). In our study, the differences in ANGPTL2 expression between cancer and controls were not too high, questioning its clinical significance as a diagnostic biomarker. These data suggest that future studies are required to clarify its usefulness as a standalone biomarker, or its potential application by combining it with other serum tumor markers such as CEA and CA19-9 for improving diagnostic accuracy of GC.

Our study provides novel and previously unrecognized evidence for the biological and clinical significance of ANGPTL2

**Table 2.** Multivariable logistic analyses for serum ANGPTL2 levels and various diagnostic factors in patients with gastric cancer (GC) in validation step

| Variables                                      | OR (% CI) | P value |
|------------------------------------------------|-----------|---------|
| GC patients versus control subjects            |           |         |
| Age, > 66 versus ≤ 66 years\(^a\)              | 2.02 (0.91–4.47) | 0.08 |
| Sex, male versus female                        | 1.67 (0.78–3.58) | 0.18 |
| ANGPTL2 in serum, >1031.6 versus ≤1031.6 pg/ml\(^b\) | 11.2 (4.81–26.04) | <0.0001 |
| Early GC patients versus control subjects      |           |         |
| Age, ≤ 62 versus > 62 years\(^a\)              | 1.24 (0.55–2.79) | 0.60 |
| Sex, male versus female                        | 1.12 (0.50–2.50) | 0.78 |
| ANGPTL2 in serum, >1031.6 versus ≤1031.6 pg/ml\(^b\) | 9.27 (3.83–22.44) | <0.0001 |

CI, confidence interval; OR, odds ratio.

\(^a\)Median ages were 66 and 62 years, respectively.

\(^b\)The cutoff values of serum ANGPTL2 in GC patients versus control subjects and early GC patients versus control subjects were derived by receiver operating characteristic curves with Youden’s index.

**Table 3.** Univariate and multivariate analysis for overall survival and disease-free survival (Cox’s proportional hazards regression model)

| Factors                                      | Univariate analysis | Multivariate analysis |
|----------------------------------------------|---------------------|-----------------------|
|                                              | HR      | 95% CI               | P value | HR      | 95% CI       | P value |
| Overall survival                             |         |                       |         |         |              |         |
| Age (> 67/≤ 67 years)\(^a\)                  | 2.5111  | 1.1217–5.6216        | 0.0259  | 1.5586  | 0.6709–3.6210 | 0.3035  |
| Sex (male/female)                            | 1.8821  | 0.7950–4.6670        | 0.1745  | —       | —              | —       |
| Histology (moderate, poor or mucinous/well)  | 5.0745  | 0.6944–37.0826       | 0.1113  | —       | —              | —       |
| Tumor size (>35 mm/≤35 mm)\(^a\)             | 5.2437  | 1.9857–13.8475       | 0.0009  | 1.186   | 0.4006–3.5111 | 0.7593  |
| Serosal invasion (present/absent)            | 12.5461 | 3.7846–41.5910       | <0.0001 | 1.1424  | 0.3873–5.1515 | 0.6028  |
| Lymph node metastasis (present/absent)       | —       | —                    | <0.0001 | —       | —              | —       |
| Distant metastasis (present/absent)          | 11.7114 | 5.3384–25.6925       | <0.0001 | 2.9322  | 1.2518–6.8684 | 0.0137  |
| ANGPTL2 expression score (high/low)          | 2.8842  | 1.2903–6.4470        | 0.008   | 1.0386  | 1.3133–9.9689 | 0.9331  |
| Serum ANGPTL2 (high/low)                     | 4.6605  | 1.7630–12.3203       | 0.002   | 3.6183  | 1.3117–9.9502 | 0.0133  |
| Disease-free survival                        |         |                       |         |         |              |         |
| Age (>67/≤ 67 years)\(^a\)                  | 2.0742  | 0.9334–4.6093        | 0.0748  | —       | —              | —       |
| Sex (male/female)                            | 3.1801  | 1.0971–9.2174        | 0.034   | 4.0229  | 1.1999–11.7417 | 0.0204  |
| Histology (moderate, poor or mucinous/well)  | 2.6662  | 0.6330–11.2308       | 0.1836  | —       | —              | —       |
| Tumor size (>35 mm/≤35 mm)\(^a\)             | 11.286  | 3.3960–37.5079       | 0.0001  | 6.0301  | 1.7339–20.9718 | 0.0049  |
| Serosal invasion (present/absent)            | 17.413  | 5.2351–57.9153       | <0.0001 | 7.7249  | 2.1126–28.2459 | 0.0021  |
| Lymph node metastasis (present/absent)       | 21.338  | 6.4116–71.0134       | <0.0001 | 15.0608 | 2.7506–82.4634 | 0.0019  |
| Lymphatic invasion (present/absent)          | 7.6514  | 1.8170–32.2202       | 0.0058  | 0.438   | 0.0534–3.5916 | 0.4442  |
| Venous invasion (present/absent)             | 5.5049  | 2.3853–12.7044       | 0.0001  | 0.8485  | 0.2949–2.4410 | 0.7617  |
| ANGPTL2 expression score (high/low)          | 3.2194  | 1.4237–7.2566        | 0.005   | 0.7608  | 0.2918–1.9839 | 0.5781  |
| Serum ANGPTL2 (high/low)                     | 2.4581  | 1.0651–5.6731        | 0.036   | 5.0491  | 2.0679–12.3283 | 0.0004  |

CI, confidence interval; HR, hazard ratio.

\(^a\)Median age and tumor size are 67 years and 35 mm, respectively.
expression in GC. First, GC cells that expressed high ANGPTL2 levels were highly proliferative, invasive and migratory. ANGPTL2 protein expression in GC tumors was significantly associated with metastatic GC phenotypes. Second, our results provide compelling evidence that serum ANGPTL2 could not only be used as a noninvasive diagnostic, prognostic tool in patients with GC, but could be superior to CEA or pathological TNM staging. Finally, our data suggest that the source of ANGPTL2 in serum might be primary tumors and/or adjacent normal H. pylori-infected gastric mucosa. In conclusion, serum ANGPTL2 as a biomarker for GC could be incorporated into routine clinical practice in the near future, pending validation in large-scale prospective trials.

Supplementary material

Supplementary Table 1 and Figures 1–4 can be found at http://carcin.oxfordjournals.org/

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