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ORIGINAL ARTICLE

Placental growth factor is a predictive biomarker for ramucirumab treatment in advanced gastric cancer

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Abstract

Purpose Ramucirumab (RAM) has been used as the second-line standard chemotherapy for advanced gastric cancer (AGC) either alone or combination with paclitaxel (PTX). However, no predictive biomarkers have been identified for RAM treatment in AGC.

Methods We retrospectively identified 26 patients who received either RAM monotherapy or RAM + PTX therapy for AGC refractory to fluoropyrimidine and platinum agents from 2015 to 2018 at Nagoya City University Hospital. First, we extracted RNA using gastric cancer (GC) tissues from two responders and two non-responders, and then analyzed 24 VEGFR-related angiogenic genes. Subsequently, we examined the relationship between the expression of each angiogenic gene and RAM clinical activity in the entire cohort. Finally, we validated using in vitro angiogenesis assays using GC cells and microvascular endothelial cells.

Results We identified five angiogenic genes with aberrant expression between RAM responders and non-responders and *placental growth factor (PlGF)* was the most significant gene among them. Overall survival ($P = 0.046$) and progression-free survival ($P = 0.016$) were significantly shorter in the *PlGF*-high group than in the *PlGF*-low group. Overall response rates were 50% in the *PlGF*-low group and 0% in the *PlGF*-high group. In GC cells co-cultured with endothelial cells, *PlGF* gene silencing from GC cells significantly reinforced the inhibitory effect of RAM in the in vitro angiogenesis assay (tube formation assay and endothelial migration) through the inactivation of ERK, in comparison to the control GC cells.

Conclusions *PlGF* gene expression in gastric cancer tissues could be a predictive indicator of AGC treatment by RAM.

Keywords Angiogenesis · Biomarker · Gastric cancer · PlGF · Ramucirumab · VEGF

Introduction

Gastric cancer (GC) is the fifth most common malignancy and the second leading cause of cancer-related deaths worldwide [1]. Owing to recent advancements in chemotherapy, the current median overall survival of advanced GC (AGC) cases that cannot undergo curable surgical resection is beyond 1 year [2, 3].

Angiogenesis is a critical and indispensable step in tumorigenesis [4]. Proteins belonging to the vascular endothelial growth factor (VEGF) family, including VEGF-A, are secreted by tumor cells. VEGF binds to membrane-bound tyrosine kinase receptors VEGFR-1 and VEGFR-2 in endothelial cells, facilitating new vessel sprouting toward tumor cells through the activation of downstream proliferative signaling [5].

In particular, interaction between VEGF-A and VEGFR-2 is considered a key regulator of tumor angiogenesis, and the blockade of this axis has already been applied as a molecular target for treating numerous malignancies, including blockade by bevacizumab as an anti-VEGF-A antibody [6]. Ramucirumab (RAM) is a humanized immunoglobulin G1 monoclonal antibody, which shows high

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affinity toward the extracellular VEGF-binding domain of VEGF receptor 2 (VEGFR-2) [7]. Globally, RAM has been applied as the standard second-line chemotherapy for AGC, based on results of randomized controlled trials, and it is applied as either monotherapy [8] or combination therapy with paclitaxel (PTX) [9].

Since molecular agents target specific molecules associated with cancer development, simultaneous discovery of associated biomarkers is desirable. Predictive biomarkers have been clinically applied in some agents, such as human epidermal growth factor 2 (HER2) expression for trastuzumab [8] and RAS mutation for anti-EGFR antibody [9]. Such biomarkers facilitate appropriate patient selection, which minimizes risks and reduces unnecessary costs. However, no biomarkers have been established for anti-angiogenic agents, including RAM. The specific biomarker for RAM would particularly be beneficial for AGC treatment considering the relatively short life expectancy of patients for its indication, in which median survival is approximately 6 months after the failure of first-line chemotherapy. Therefore, the aim of this study was to identify biomarkers for RAM.

Materials and methods

Patients

Consecutive AGC patients who had received RAM therapy for over 28 days (combination therapy of PTX plus RAM or RAM monotherapy) at Nagoya City University Hospital Nagoya City University Hospital from August 2015 to August 2017 were enrolled in the present study. Patient data were retrieved from computerized databases and reviewed retrospectively.

The present study complied with the STROBE statement [10]. The study protocol was approved by the institutional review board of Nagoya City University Hospital, and the study was conducted according to the ethical guidelines in the 1975 Declaration of Helsinki (6th revision, 2008).

Chemotherapy schedule

In patients receiving PTX plus RAM, 80 mg/m^2 PTX was administered intravenously on days 1, 8, and 15 and 8 mg/kg RAM was administered intravenously on days 1 and 15 and repeated every 4 weeks [11]. In patients receiving RAM monotherapy, 8 mg/kg RAM was administered intravenously once every 2 weeks [12].

Definitions

Overall survival (OS) was measured from the first day of ramucirumab therapy until death or the last day of follow-up (whichever occurred first). Progression-free survival (PFS) was defined as duration from the first day of ramucirumab therapy to disease progression, death, or the last day of follow-up (whichever occurred first).

Treatment response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) guideline version 1.1 [13] using computed tomography (CT). In all patients, CT evaluations were routinely performed every 6–9 weeks, but emergent CT was performed at the time of aggravation, as appropriate. Performance status and toxicity were graded according to the Eastern Cooperative Oncology Group Performance Status.

Samples

GC tissue samples were obtained from primary stomach tumors at the time of surgical resection or at the time of biopsy in patients who did not undergo surgical resection. Immunohistochemical staining was performed as follows.

All urine samples were collected before chemotherapy for AGC, immediately frozen, and stored at − 80 °C until assayed, as previously reported [14–16]. Urinary protein concentrations of PlGF were measured using a Quantikine ELISA kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

RNA extraction and PCR

According to the manufacturers' instruction, total RNA from formalin-fixed paraffin-embedded (FFPE) GC tissue slides (10-μm thick) at the time surgical resection or at the time of biopsy in patients was extracted using The ReliaPrep™ FFPE Total RNA Miniprep System (Promega Corporation, Madison, WI). cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA) and it was performed using PCR Thermal Cycle Dice (Takara, Shiga, JAPAN). RT-PCR was performed using power up SYBR green Master mix (Thermo Fisher Scientific). Cycling conditions were one cycle at 50 °C for 2 min and one cycle at 95 °C for 2 min and 45 cycles at 95 °C for 15 sec and 45 cycles at 60 °C for 1 min.RT-PCR was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) according to the manufacture's recommendations. Breakdown of the specific primers is shown in Fig. 1a. Data are normalized to β-actin.

A

Gene	Forward primer	Reverse primer
VEGFA	GCAGAAGGAGGAGGGCAGAAT	GCACACAGGATGGCTTGAAGA
VEGFB	GATGGCCTGGAGTGTGTG	CACACTGGCTGTGTTCTTCC
VEGFC	GGCTGGCAACATAACAGAGA	GTGGCATGCATTGAGTCTTT
VEGFD	CTGCCTGATGTCAACTGCTTAG	TGAGATGATCGCTTCACTGGTC
PIGF	GTTCTCTCAGCACGTTCGCT	TGGCAGTCTGTGGGTCTCTG
VEGFR1	CGACGTGTGGTCTTACGGAGTA	CTTCCCTCAGGCGACTGC
VEGFR2	TGCCTCAGAAGAGCTGAAAACTT	CACAGACTCCCTGCTTTTGCT
VEGFR3	ATCCGAGGAGCTACTAGAGGG	AGCGCAGATGCTCGTACTTG
NRP1	AGGATCTACCCCGAGAGAGC	CAGTTGGCCTGGTCGTCATC
NRP2	GGTCGCCGGCGGGGATTGG	TCGGTGGGGTAGGGGGTGGTTGTC
SEMA3A	GGTTGCCCAGCTCCCTTTAC	TATCTTGTCGTCTTGTGCGTCTCT
SEMA3B	CCTCAACCTGGACAACATCAGC	CGAAGTTCATGCACTCAGTACCAAT
SEMA3C	TTTGCGTGTTGGTTGGAGTAT	TCCTGTAGTCTAAAGGATGGTGG
SEMA3D	TCAAGAAGGCAGTACCTCCG	TATCAGGCTGCGTTGTCCTC
SEMA3E	CGGTTACGCCTGTCACATAA	CCACGAAGAGCCTCTCTTGATA
SEMA3F	CGCGCCCAGGCCACACCAT	CATCGGGCGGAGGGCACCAT
SEMA3G	CGGGTGCTGGTGAACAAATG	CTCTAGCTGGTCAAAGTGGGT
PLXNA1	CGTGCTGTTCACTGTGTTCG	ACTGGATGCGCTCCTTAATCT
PLXND1	TGAGTCTGTTGTACGCTGTGA	GCCCCTTTAGTTGGAGGCT
FGF1	TGGGACAGCACTGAGCGAGTGT	AGCTGCTGCTTGTGGCGCTT
FGF2	CTGGCTATGAAGGAAGATGGA	TGCCCAGTTCGTTTCAGTG
ANGPT1	CAATGGGGGAGGTTGGACTGTA	GAGGGATTTCCAAAACCCATTTTAT
ANGPT2	CTCGAATACGATGACTCGGTG	TCATTAGCCACTGAGTGTTGTTT
Tie2	CTGTGAAGGGCGAGTTCGA	TGGTAGGAAGGAAGCTTGTTGAC

B

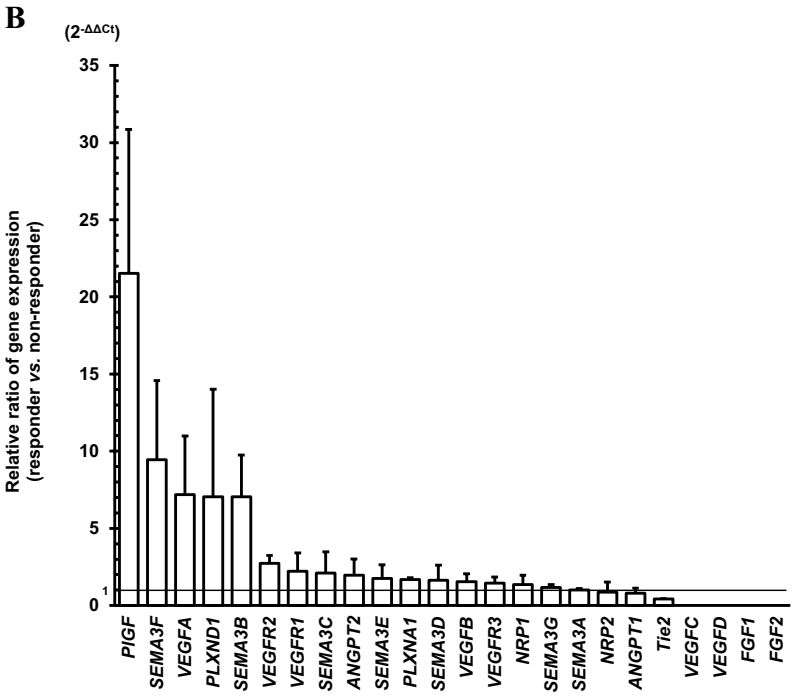

Fig. 1 Semi-comprehensive PCR analysis of VEGFR-related genes. VEGFR-related genes were comprehensively analyzed between two respondents and two non-respondents to ramucirumab. **a** Each primer used in the present study. **b** Relative ratios of VEGFR-related gene expressions in ramucirumab respondents and non-respondents. Relative ratios are expressed with $2^{-\Delta\Delta Ct}$, calculated based on differences: ΔΔCt = (ΔCt of respondents) − (ΔCt of non-respondents), where ΔCt indicates difference in Ct values between each gene and β-actin [ΔCt = Ct (gene) − Ct (β-actin)]

Cell culture

MKN45 and MKN74 (ATCC, Manassas, VA) were used as GC cell lines in the present study. Cultures were maintained in RPMI1640 medium (WAKO, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS). Human microvascular endothelial cells (HMVECs) were cultured in EGM-2 medium (Lonza, Basel, Switzerland).

RNA interference knockdown

siRNA (20 nM) transfection was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacture's instructions. MKN45 and MKN74 cells were transfected with desired siRNA using siGENOME Non-Targeting siRNA (siNT) control pool and

siGENOME human PlGF siRNA SMART pool (siPLGF) (Dharmacon, Lafayette, CO).

Tube formation assay

MKN45 and MKN74 cells were incubated in 0% EBM2 media (Lonza) for 24 h and then conditioned media were collected from each condition. Incubated HMVECs were washed with PBS and changed to serum-free medium. After 2 h of serum starvation, HMVECs were mixed with 1% FBS condition media from each condition of gastric cancer cells in the presence or absence of RAM, and 15,000 cells/well were placed in 96 wells coated with growth factor-reduced Matrigel (Corning, Corning, NY). The chambers were incubated with conditioned medium from GC cells for 6 h and photographed under a microscope.

Endothelial cell recruitment assay

Endothelial cell (EC) recruitment assay was performed using a transwell (Corning) which consisted of 24-well transwell tissue culture plates with membranes of 8.0-μm pores (top chamber). GC cells of each condition were seeded with a density of 100,000 cells on 24-well plate. After 24 h of serum starvation, HMVECs (50,000 cells/well) in 0.1% FBS EBM-2 with or without RAM (2 μg/ml or 10 μg/ml) were placed in the top chamber, and media of the bottom chamber were also switched to the same media, 0.1% FBS EBM-2. After 24 h of incubation, the cells migrated through the top chamber membrane were counted with microscopy.

Western blotting

Cultured HMVECs were washed with PBS and resuspended in serum-free media. After 1 h of serum starvation, HMVECs were placed in a condition media from GC cells with or without RAM (2 μg/ml). After 30 min, cells were rinsed with PBS and disrupted in cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with phenylmethanesulfonyl fluoride (Sigma-Aldrich, St Louis, MO) for 5 min. Each sample was normalized against an equal protein concentration using protein assay (BioRad, Hercules, CA) and then added to equal quantity of sample buffer. Samples were heated to 100 °C for 5 min and mounted samples were separated by 10% polyacrylamide gel using TGX™ FastCast™ Acrylamide kit (BioRad) transferred on membrane. The membrane was blocked with 5% skim milk in phosphate-buffered saline–Tween 20 (PBST) for 1 h at room temperature, and the membrane was incubated with the primary antibodies against phospho-p44/42MAPK (ERK1/2) antibody (#9106) (Cell Signaling Technology) 1:1000, p44/42MAPK (ERK1/2) antibody (#4696) (Cell Signaling Technology) 1:1000 and β-actin monoclonal antibody (Wako) 1:5000 overnight at 4 °C The membranes were washed three times in PBST for 5 min and incubated with secondary antibody for anti-rabbit IgG, HRP-linked antibody 1:5000 (Cell signaling Technology) or anti-mouse IgG, HRP-linked antibody 1:4000 (Cell signaling Technology) for 1 h at room. Chemiluminescence was measured using an analyzer, GE ImageQuant LAS-4000mini (GE Healthcare, Chicago, IL).

Statistical analysis

Data were analyzed using the Mann–Whitney *U* test, Chi-square test, or Fisher's exact probability test, as appropriate. Nonparametric Spearman's rank correlation coefficient (*r*) was used as a measure of correlation. Kaplan–Meier curves were constructed to analyze OS and PFS, and differences between two groups were compared with the log-rank test. $P < 0.05$ was considered statistically significant. Data analyses were performed in IBM SPSS statistics, version 25 (IBM Corp., Tokyo, Japan).

Results

Semi-comprehensive analysis of angiogenic genes related to ramucirumab resistance

RNA was extracted from GC tissue samples of two respondents and two non-respondents to RAM, and 24 VEGFR-related angiogenic genes were semi-comprehensively compared between respondents and non-respondents. Relative ratios of each VEGFR-related gene expression in non-responders to responders are shown in Fig. 1b. Among these, placental growth factor (*PlGF*), *SEMA3F, VEGFA, PLXND1*, and *SEMA3B* were the five angiogenic genes with the highest expression levels in the non-respondents compared with those in the respondents. Subsequently, OS and PFS were analyzed for the entire cohort ($n = 26$) based on expression levels of these five genes.

Patient characteristics

Patient characteristics are summarized in Table 1. A total of 26 patients who received RAM for GC were included in this study.

Among the five candidate genes, tumoral *PlGF* expression level was a poor prognostic factor for RAM therapy in the entire cohort, but other genes were not significant (Supplementary Fig. 1). Patients were categorized into two groups based on tumoral *PlGF* gene expression: *PlGF*-high group with $\Delta Ct < 12$ ($n = 11$); the *PlGF*-low group with $\Delta Ct \geq 12$ ($n = 15$). Baseline characteristics were well

Table 1 Characteristics

	Total ($n=26$)	*PlGF*-high ($n=11$)	*PlGF*-low ($n=15$)	*P*
Age [median(range)]	67 (40–81)	65 (54–81)	68 (40–80)	0.198
Gender (male/female)	8/18	3/8	5/10	1.000
Histology (poorly/well-differentiated adenocarcinoma)	17/9	8/3	9/6	0.683
Regimen (RAM alone/PTX + RAM)	4/22	1/10	3/12	0.614
Regimen line (2nd/3rd/4th)	24/1/1	11/0/0	13/1/1	0.452
Previously used drug				0.606
Fluoropyrimidine	26	12	14	
CDDP	16	7	9	
Oxaliplatin	7	5	2	
Docetaxel	4	2	2	
Trastuzumab	7	2	5	
Location (cardia/non-cardia)	5/21	1/10	4/11	0.356

PlGF placental growth factor, *RAM* ramucirumab, *PTX* paclitaxel, *CDDP* cisplatin

balanced and there were no statistical differences between two groups.

Effectiveness of ramucirumab based on PlGF gene expression

Median OS was 243 days [95% CI, 111–375] in the *PlGF*-high group and 422 days [95% CI, 169–675] in the *PlGF*-low group, and there was a significant difference in median OS between two groups ($P=0.046$) (Fig. 2a). Median PFS was 56 days [95% CI, 34–78] in the *PlGF*-high group and 169 days [95% CI, 71–267], with statistically significant difference ($P=0.016$) (Fig. 2b).

Overall response rate (ORR) is presented in Table 2. Among the 26 patients, 14 patients had target lesions that could be evaluated according to the RECIST criteria. ORR was 50% (3/6) in the *PlGF*-low group, and 0% (0/8) in the *PlGF*-high group. Despite borderline significance for ORR due to a small sample size ($P=0.055$), obvious differences were observed in responses to RAM.

These results suggested that high *PlGF* expression in GC tissues could be a negative predictive biomarker for RAM therapy. Among the 26 patients, urine samples were collected from 7 patients at similar time points. Although it was not significant due to small sample size, urinary level of PlGF reveals a good correlation with *PLGF* gene expression in GC tissues (Fig. 2c).

In vitro angiogenesis assay with ramucirumab treatment

To validate clinical data suggesting that RAM is effective at low *PlGF* expression, the association between *PlGF* expression and anti-angiogenic effects of RAM was analyzed in vitro using GC cell lines and HMVECs. *PlGF* expression was knocked down well in both GC cell lines (MKN 45 and MKN 74) with siPlGF compared with that in controls and cell lines with siNT (Fig. 3a). EC recruitment assay revealed that when HMVECs were co-cultured with GC cells, HMVEC migration under RAM treatment significantly decreased under co-culture with siPlGF GC cell lines compared with that under co-culture with siNT GC cell lines (Fig. 3b, c). This inhibitory effect in siPlGF GC cells was investigated under different RAM concentrations (Fig. 3d). Similarly, conditioned media from two types of siPlGF GC cells significantly inhibited tube formation in vascular endothelial cells under RAM treatment when compared with conditioned media from siNT GC cells (Fig. 3e, f). Regardless of RAM concentrations, this inhibitory effect was maintained (Fig. 3g).

Conditioned media from two GC cell lines induced phosphorylation of ERK in HMVECs, but RAM treatment inhibited ERK phosphorylation in HMVECs. In addition, compared with siNT, siPLGF significantly enhanced the inhibition of ERK phosphorylation by RAM treatment (Fig. 3h), these results suggested that anti-angiogenic effect by RAM is inhibited by secreted PlGF from GC cells, through the activation of downstream signaling, ERK.

Discussion

To the best of our knowledge, using multiple approaches, the present study presents the first evidence that tumoral *PlGF* gene expression is a negative predictive biomarker for RAM in AGC. RAM is one of the anti-angiogenic agents that act

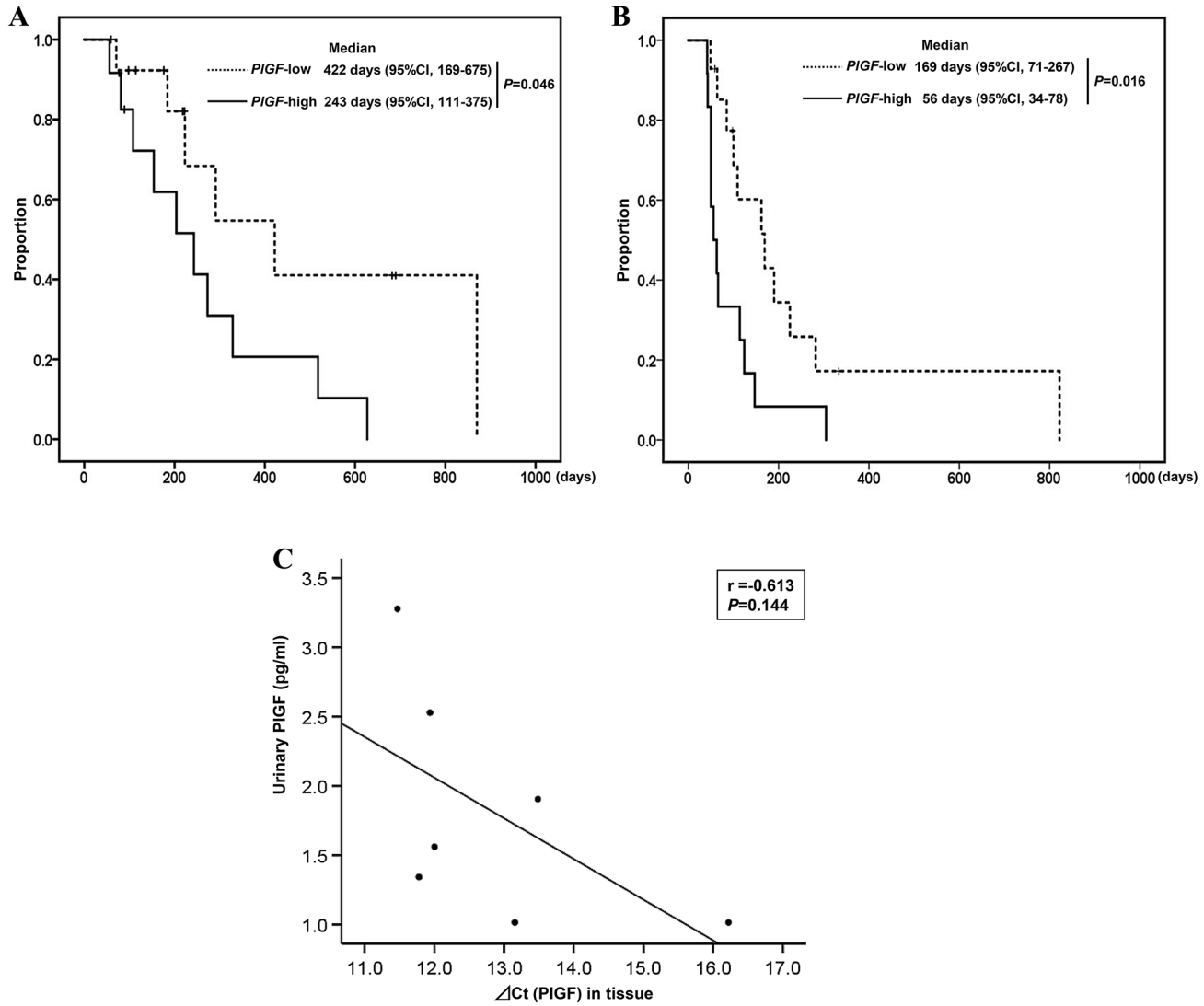

Fig. 2 Survival curves according to PlGF gene expression levels. **a** Overall survival. **b** Progression-free survival. *PlGF*-low was defined as $\Delta Ct \geq 12$ and *PlGF*-high was defined as $\Delta Ct < 12$. 95% CI, 95% confidence interval. **c** Correlation between tumoral *PlGF* gene expression and urinary PlGF levels. Data are analyzed using Spearman rank correlation

Table 2 Overall response rate according to *PlGF* gene expression

For measurable lesion ($n=14$)	CR	PR	SD	PD	ORR (%)	*P*
PlGF-low ($n=6$)	0	3	0	3	50	0.055
PlGF-high ($n=8$)	0	0	4	4	0	

PlGF placental growth factor, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *ORR* overall response rate

via the blocking of VEGFR-2. Anti-angiogenic agents are currently the representative molecular target agents for malignancies including gastrointestinal cancers. Despite the principle goal of translational research, no biomarkers have been revealed and validated for anti-angiogenic agents, which could be due to the complexity of the mechanisms of action of anti-angiogenic agents. Anti-angiogenic agents do not directly target cancer cells. They target the more complex vascular microenvironments surrounding tumors.

To reveal key biomarkers, we analyzed tumoral VEGFR-associated genes and then determined that *PlGF* gene expression could be a predictive biomarker for RAM in

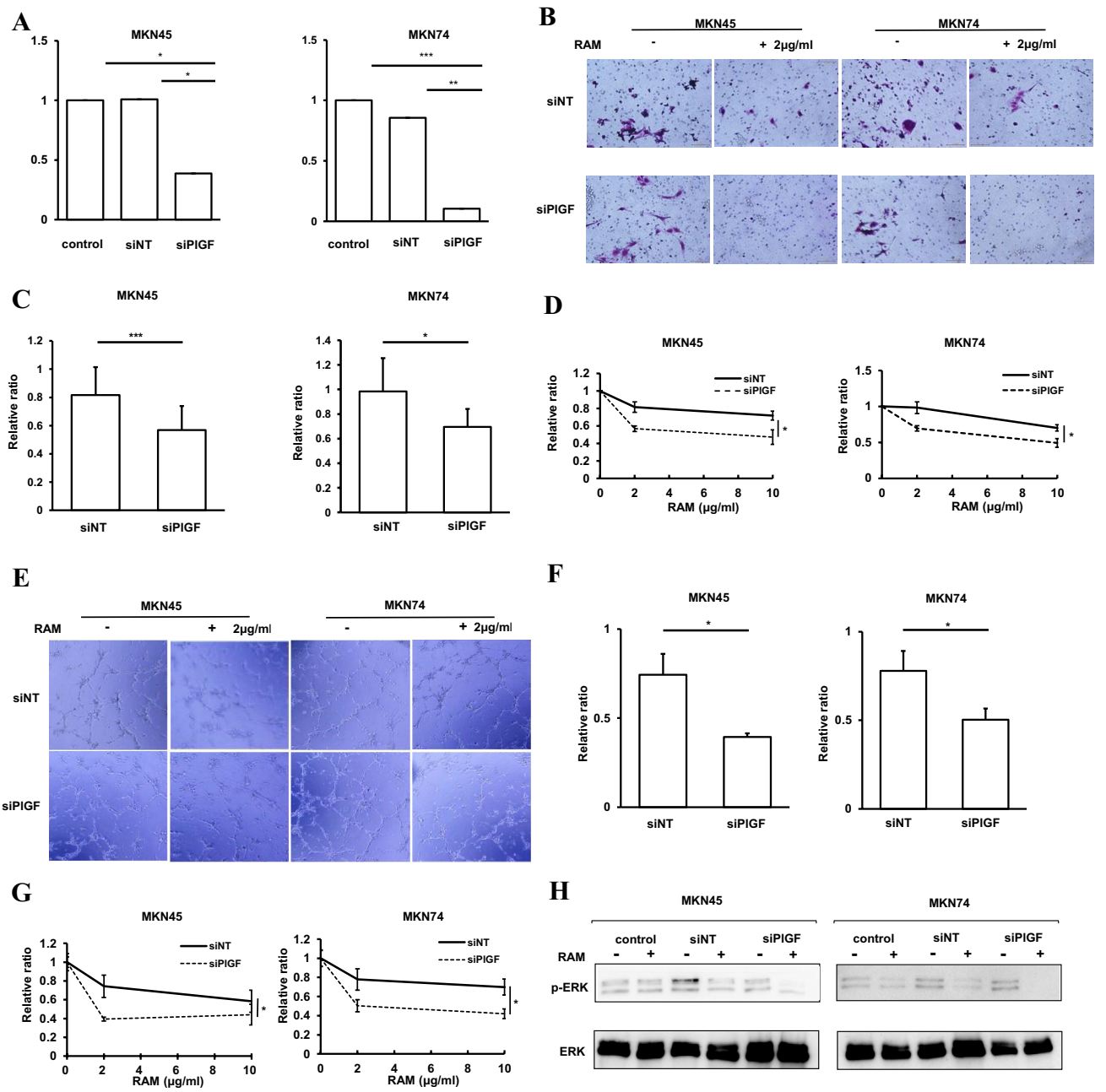

Fig. 3 In vitro validation. siNT, siRNA of non-targeting genes; siPlGF, siRNA of *PlGF*; RAM, ramucirumab; HMVECs, human microvascular endothelial cells. *$P<0.05$; **$P<0.01$; ***$P<0.001$. **a** *PlGF* gene expression in siRNA-mediated gastric cancer cells. Each graph represents mean ± SD from three independent experiments. **b–d** Endothelial cell recruitment assay. HMVECs were used for endothelial cell (EC) recruitment assay. HMVECs were seeded in the upper chamber and co-cultured with siRNA-mediated GC cells (siNT, siPlGF) in the lower chamber. RAM was added into the lower chamber of GC cells. HMVECs that migrated toward GC cells were counted after 24 h. Migrated HMVECs were counted from averages at four microscopic fields, and each result was presented as a mean of at least three independent experiments. **b** Representative images of EC recruitment assay. GC cells were treated with or without 2 ug/ml of RAM (×200). **c** Quantification of EC recruitment assay. Each value represents a mean relative ratio of migrated HMVECs under RAM treatment against control (non-RAM). **d** Quantification of EC recruitment assay with or without RAM treatment. Each value represents a mean relative ratio of migrated HMVECs under RAM treatment against control (non-RAM). **e–g** Tube formation assay. HMVECs were incubated with conditioned media from siRNA-mediated GC cells (siNT, siPlGF), in the condition with or without 2 ug/ml of RAM. **e** Representative images of tube formation assay (×100). **f** Quantification of tube formation assay. Cells with more than three branches were considered as tube formation. Each value represents a mean relative ratio of tube formation under RAM treatment against control (non-RAM). **g** Quantification of tube formation assay under RAM treatment. Each value represents a mean relative ratio of tube formation under RAM treatment against control (non-RAM). **h** Western blotting. HMVECs were incubated with conditioned media from siRNA-mediated GC cells (siNT, siPlGF), in the condition with or without 2 ug/ml of RAM. ERK phosphorylation of HMVECs were analyzed with western blotting analysis

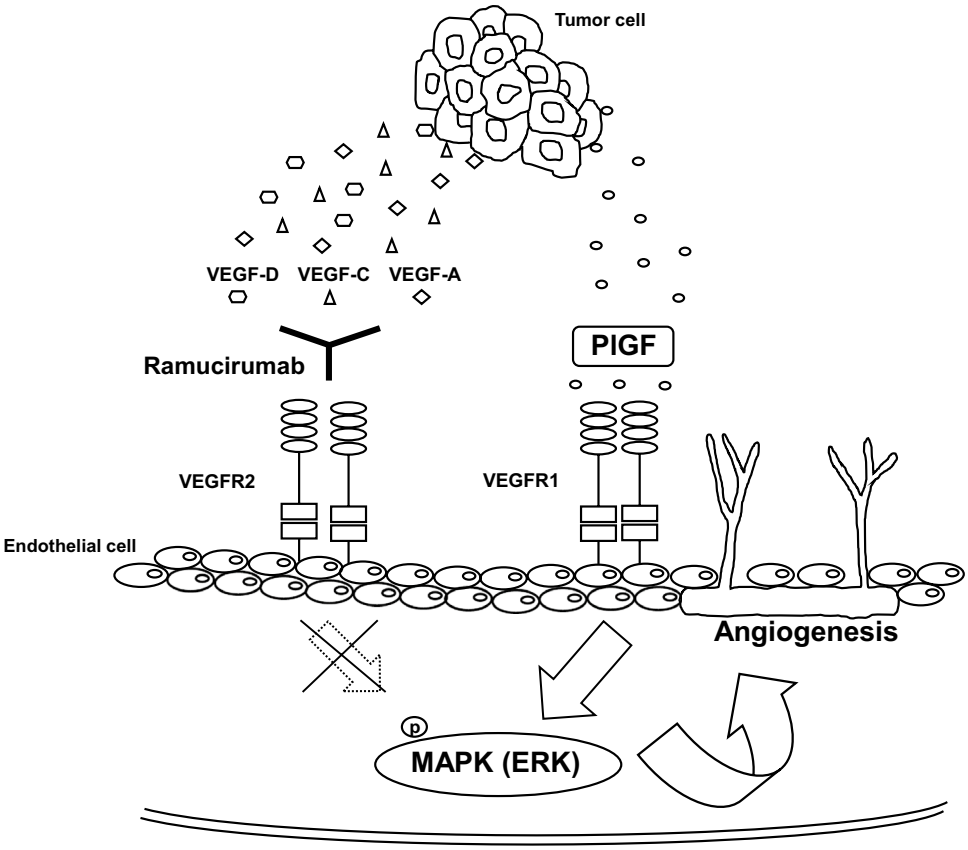

Fig. 4 Efficacy of ramucirumab and tumoral *PlGF* expression. When *PLGF* expression was low in gastric cancer cells, ramucirumab (RAM) could inhibit angiogenesis by blocking ERK phosphorylation through VEGFR-2 downstream signaling. In contrast, anti-angiogenic effects of RAM were evaded due to the incomplete blocking of ERK phosphorylation when tumoral *PLGF* expression was high

AGC, with significantly shorter OS and PFS in AGC when *PlGF* was highly expressed. In addition, in an in vitro study using GC and endothelial cells, the anti-angiogenic effects of RAM were influenced by *PlGF* expression levels in GC cells.

Two studies have attempted to identify predictive RAM biomarkers. The first study analyzed VEGFR2 and HER2 protein expression in GC tissues and serum levels of VEGF-C, VEGF-D, sVEGFR1, and VEGFR2 in biomarker analyses of REGARD participants, which was a phase III study that compared RAM monotherapy with the best supportive care for AGC patients. However, no apparent relationship was observed between protein expression levels and clinical results [17]. The second study measured VEGF-C, VEGF-D, sVEGFR-1, sVEGFR-2, sVEGFR-3 in plasma and VEGFR-2 expression level in colorectal cancer (CRC) tissues, and analyzed the correlation between these expression levels and clinical outcomes in a RAISE study, which was a phase III study that assessed the effect of integrating RAM in standard chemotherapy care for CRC patients. In this biomarker study, VEGF-D levels in plasma were a predictive biomarker of RAM in metastatic CRC [18]. In terms of VEGF-D, *VEGF-D* gene expression levels were very low and were not significant in our discovery phase in four cases. The inconsistency could be due to a small sample size or differences between GC and CRC. On the other hand, PlGF expression had not been analyzed in both previous studies. Nevertheless, other studies have demonstrated that PlGF levels in serum increased following treatment with RAM in hepatocellular cancer [19] and ovarian cancer [20], suggesting that PlGF expression could be modulated by RAM administration.

Although VEGF-A binds to VEGFR-1 and VEGFR-2, the VEGF-A–VEGFR-2 axis is generally considered a major signaling pathway for angiogenesis, and VEGF-A stimulation leads to weaker autophosphorylation of VEGFR-1 than VEGFR-2 phosphorylation [21]. PlGF is a member of the VEGF family, which binds to VEGFR-1 and induces angiogenesis. Notably, PlGF stimulation activates VEGFR-1 more effectively than VEGF-A stimulation by the activation of downstream MAPK signaling [22]. Indeed, our data are consistent with the above findings, wherein the inhibitory effects of ERK/MAPK phosphorylation and angiogenesis by RAM are weaker in GC cells with high *PIGF* expression levels than in GC cells with low *PGIF* expression levels. Therefore, the present finding that *PlGF* gene expression levels are a predictive biomarker for RAM treatment in AGC is plausible.

As summarized in Fig. 4, RAM inhibits angiogenesis through the blocking of VEGFR-2 signaling and ERK

phosphorylation in GC cells with low PlGF expression levels. However, when GC tissues express high levels of PlGF, only VEGFR-2 blocking is not adequate to downregulate ERK phosphorylation by escaping to PlGF–VEGFR-1 signaling, which induces RAM resistance.

A limitation of the present study is the use of tumor tissue samples for biomarker detection. Although tumoral gene analysis using biopsy sample remains invasive for patients, additional invasive examination is not required because *PlGF* expression can be analyzed using a sample stock. In addition, urinary level of PlGF may also be useful biomarkers in the future since tumoral PlGF expression levels were positively correlated with urinary PlGF levels in the present study.

In conclusion, *PlGF* gene expression could be a predictive biomarker for AGC response to RAM.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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