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ORIGINAL ARTICLE

Signature of cytokines and angiogenic factors (CAFs) defines a clinically distinct subgroup of gastric cancer

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Abstract

Background Little is known about cytokine and angiogenic factors (CAFs) in gastric cancer (GC) in terms of tumor classification and prognostic value. Here, we aimed to correlate CAF signature with overall survival (OS) in GC.

Methods We measured pretreatment serum levels of 52 kinds of CAFs in 68 GC patients who were treated with fluoropyrimidine and platinum combination chemotherapy using multiplex bead immunoassays and enzyme-linked immunosorbent assay. We evaluated correlations between CAF levels and pathological features and OS.

Results Three distinct patient groups were identified: one with high levels of proangiogenic factors, another with high levels of proinflammatory factors, and the other with high levels of both factors. Eleven CAFs [interleukin (IL)-2 receptor-alpha, growth-regulated alpha protein, hepatocyte growth factor, macrophage colony-stimulating factor, stromal cell-derived factor, IL-6, IL-8, IL-10, interferon-gamma, vascular endothelial growth factor, and osteopontin] were independently correlated with poor OS. Clustering analysis of these 11 CAFs revealed distinct high and low 11-CAF signature groups. High 11-CAF signature was associated with shorter OS (10.1 vs. 17.9 months, $p = 0.026$) along with poor performance status, and the presence of signet ring cell components in multivariate analysis of OS (HR 1.76, $p = 0.029$). The patients' traditional clinicopathological characteristics were not significantly different between the high and low 11-CAF signature groups.

Conclusion Serum CAF profiling differentiated GC patient groups. A high 11-CAF signature could identify GC patients with a poor prognosis when treated with standard chemotherapy who need urgent new treatment strategies.

Keywords CAF · Cytokine · Angiogenesis · Gastric cancer · Overall survival

Introduction

Gastric cancer (GC) is the third leading cause of cancer death worldwide [1]. Metastatic or recurrent GC has a median overall survival (OS) of approximately 1 year, if treated with cytotoxic chemotherapy [2]. Recent advances in genomic approaches have revealed that GC is not a single disease, but can be classified as four subtypes: EBV-positive, microsatellite instability, genomically stable, and chromosomal instability [3]. Also, recurrent amplification of genes associated with the receptor tyrosine kinase pathway, such as *EGFR*, *ERBB2*, *ERBB3*, *JAK2*, *FGFR2*, and *MET*, and the angiogenesis pathway, such as *VEGFA*, have been reported in GC [4, 5]. With the exception of a subset of patients with *ERBB2* amplification who experienced benefits from trastuzumab [6], targeted agents for aberrant gene amplification have not yet been successful in therapeutic applications [7–9].

Growing evidence shows that not only the tumor itself but also its niche and inflammatory cytokines are important

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considerations when defining a tumor [10, 11]. Moreover, recent successes in clinical trials that studied immune checkpoint inhibitors provided support for the new anti-tumor strategy of immune modulation [12, 13]. Comprehensive genomic research in GC has already shown that a subset of EBV-positive GC exhibited immune evasion mechanisms by amplification of programmed death ligand 1 (*PD-L1* or *CD274*) [3], and recent data have shown promising results of PD-1 inhibition in GC [14]. The development of the optimal anti-tumor immunological strategy should begin with an exploration of immunological factors, such as cytokines and angiogenic factors (CAFs), which are associated with the tumor. To understand the immunological landscape of a tumor, subgroups have been defined on the basis of circulating CAFs and their clinical implications have been explored in solid tumors [15–19]. In renal cell carcinoma, 6 CAFs that were predefined as the "angiogenic group" were also identified as significant predictive factors for adding interferon-alpha treatment to sorafenib [16]. Moreover, levels of CAFs that were known as a "hypoxia signature" were higher in patients who experienced progression after induction chemotherapy in head and neck cancer [18]. In these studies, the used CAFs were various in terms of item and number. The CAFs work in the context of complex network. An understanding of CAF networks has been significantly correlated with clinical outcomes in several cancers, but little has been studied in GC. In different tumor types, the involved CAF networks might be different.

In this study, serum levels of all available CAFs were analyzed with clinicopathological features and clinical outcomes of GC patients. We hypothesized that patients with GC could be identified by distinct groups based on the serum CAF profile. Moreover, we aimed to find the key CAF signature that was significantly correlated with OS of GC patients treated with conventional chemotherapy.

Patients and methods

Patients

This study was a retrospective analysis of de-identified patient-level data collected from medical charts. Patients diagnosed with GC at Seoul National University Hospital, Republic of Korea, from April 2005 to December 2011 were included in the analysis if they were older than 18 years of age and had histologically confirmed recurrent or metastatic GC, an Eastern Cooperative Oncology Group performance status of 0–2, adequate organ function, and received chemotherapy with fluoropyrimidine and platinum combination.

Sample preparation and CAF analysis

Patients provided written informed consent for the collection of blood samples for biomarker analysis. Specimens were obtained before initiation of palliative chemotherapy. A total of 52 CAFs present in serum were analyzed according to the manufacturers' instructions with multiplex bead suspension array kits using the Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA, USA), including human group I and II cytokine panels as described in previous reports [15, 16]. Serum concentrations of soluble carbonic anhydrase IX (sCA9), soluble vascular endothelial growth factor receptor-2, placental growth factor, and osteopontin (OPN) were determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). Each serum sample was analyzed in duplicate and mean CAF concentrations were reported in pg/ml. Analytes for which more than 50 % of patients had nondetectable levels or coefficients of variation greater than 20 % were not included in the subsequent analyses, based on the previous reports of CAF analysis [16, 18]. Analytes with nondetectable levels were recorded as one-half of the lower threshold value.

Statistical analysis

The primary objective of this study was to determine whether pretreatment serum CAF levels correlated with clinicopathological characteristics of GC and clinical outcomes such as OS of patients with GC who were treated with conventional chemotherapy. The CAF concentrations analyzed in the study were log transformed because concentrations were highly skewed in all samples. For unsupervised hierarchical clustering, the log-transformed concentration of each baseline CAF was standardized by subtracting the sample mean and dividing by the standard deviation. Hierarchical clustering and data presentation were performed with Cluster 3.0 and TreeView v. 1 software (downloaded from http://www.eisenlab.org/). Wilcoxon rank-sum tests were applied to compare the differences of continuous variables between the groups.

OS was measured from the first day of palliative chemotherapy until death or the last follow-up date, if censored. Progression-free survival (PFS) was measured from the first day of palliative chemotherapy until disease progression or final follow-up visit and to date of disease progression confirmed by imaging modality. To identify the optimal cutoff of CAF levels to predict survival outcomes, differences in OS of binary groups according to each CAF level were compared and optimal cutoffs were determined by the lowest p value of the log-rank test [20]. All p values were two sided, and $p < 0.05$ was considered statistically significant. Analyses were completed with

STATA version 12 software (StataCorp LP, College Station, TX, USA).

Ethics

The study protocol was reviewed and approved by the Institutional Review Board of Seoul National University Hospital (H-1411-022-623). The study was conducted according to guidelines for biomedical research outlined in the Declaration of Helsinki.

Results

Patient characteristics

Of the 68 GC patients enrolled in the study, 40 patients received combination chemotherapy with 5-fluorouracil and oxaliplatin (FOLFOX) and 28 patients received capecitabine and cisplatin (XP) as first-line palliative chemotherapy. Table 1 shows the general clinicopathological characteristics of the 68 GC patients who were included in the final study analysis. Seven patients (10.3 %) were HER2 positive and 16 patients had diffuse-type disease, according to the Lauren classification system [21]. Of 22 patients (32.4 %) who had signet ring cell components, 13 had pure poorly cohesive carcinoma. The median follow-up duration was 81.6 months (range, 32.6–113 months), and the median OS and progression-free survival (PFS) of first-line palliative chemotherapy were 12.1 and 6 months, respectively. Clinicopathological characteristics were not significantly different between the two chemotherapy regimens (FOLFOX and XP; data not shown).

Unsupervised hierarchical clustering by CAF concentration

A total of 52 CAFs were initially measured and analyzed, but 10 CAFs were excluded from the final analysis because more than half the samples were outside the detection range. The mean, standard error, median, and range of the 52 CAFs are listed in Table 2.

Unsupervised hierarchical clustering identified three groups of patients (Fig. 1). Half the patients were characterized by relatively high concentrations of proangiogenic and hypoxia-regulated factors (angiogenic group, $N = 34$), including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), macrophage colony-stimulating factor (M-CSF), growth-regulated alpha-protein (GRO-α), and stromal-derived factor 1-alpha (SDF-1α). The second group of patients (inflammatory group, $N = 17$) included patients with high levels of interleukins (ILs) and other proinflammatory factors. The third group of patients (dual group, $N = 17$) had elevated levels of both angiogenic and inflammatory factors. Clinicopathological features were not significantly different among the three patient groups (Supplementary Table 1). Survival analysis showed that patients who had elevated levels of angiogenic CAFs (angiogenic group and dual group) had unfavorable clinical outcomes compared to patients in the inflammatory group, but the difference was not statistically significant (median OS 11.2 vs. 16.7 months, $p = 0.301$; median PFS 6.0 vs. 5.1 months, $p = 0.697$; Supplementary Fig. 1).

CAF profile according to pathological characteristics of GC

CAF analytes were compared according to HER2 status, Lauren classification, and signet ring cell components. GRO-α was significantly lower in patients with HER2-positive status than in patients with HER2-negative status ($p = 0.037$; Fig. 2a), and GRO-α was higher in patients with diffuse-type disease compared to patients with intestinal-type disease ($p = 0.047$; Fig. 2b). Also, sCA9 was lower in patients with diffuse-type disease than in patients with intestinal-type disease ($p = 0.037$; Fig. 2c). Stem cell factor (SCF) and macrophage inflammatory protein 1-alpha (MIP-1α) were lower in patients with signet ring cell components than in patients without signet ring cell components ($p = 0.037$ and $p = 0.038$, respectively; Fig. 2d, e). All CAF analytes are summarized in Supplementary Fig. 2 according to HER2 status, Lauren classification, and signet ring cell components.

CAF groups predict OS in GC

For each CAF, repeat analyses were performed to compare the OSs of patient groups divided by each level of CAF and identify the optimal cutoff points for predicting OS. A total of 42 CAFs were analyzed, and 11 CAFs significantly predicted OS: IL-2 receptor-alpha, GRO-α, HGF, M-CSF, SDF-1α, IL-6, IL-8, IL-10, interferon-gamma (IFN-γ), VEGF, and OPN (Table 3). We then classified patients into two groups by clustering analysis using these significant 11 CAFs. Clustering analysis of these 11 CAFs revealed two distinct groups: patients with high levels of these CAFs (high 11-CAF signature, $N = 42$) and patients with low levels of these CAFs (low 11-CAF signature, $N = 26$; Fig. 3a). The median OS of the high 11-CAF signature group was 10.1 months and the median OS of the low 11-CAF signature group was 17.9 months (HR 1.77, $p = 0.026$; Fig. 3b). The PFS of high 11-CAF signature group was significantly prolonged compared to that of low 11-CAF signature group (HR 2.04, $p = 0.012$; Fig. 3c). Interestingly, clinicopathological characteristics were not significantly different between the

Table 1 Patient characteristics

	Total $N = 68$
Age, median years (range)	56 (26–77)
Sex, *N* (%)	
Male	42 (61.8)
Female	26 (38.2)
Palliative setting, *N* (%)	
Metastatic	55 (80.9)
Recurrent	13 (19.1)
ECOG, *N* (%)	
0	6 (8.8)
1	56 (82.4)
2	6 (8.8)
HER2, *N* (%)	
Positive	7 (10.3)
Negative	61 (89.7)
Tumor location, *N* (%)	
Stomach	63 (92.7)
GEJ	5 (7.4)
Pathology, *N* (%)	
Adenocarcinoma	53 (79.4)
(Pure) PCC	13 (19.1)
Adenosquamous	1 (1.5)
SRC component, *N* (%)	
No	46 (67.7)
Yes	22 (32.4)
Lauren, *N* (%)	
Intestinal	8 (11.8)
Diffuse	16 (23.5)
Mixed	1 (1.5)
Unknown[a]	43 (63.2)
Chemotherapy regimen, *N* (%)	
FOLFOX	40 (58.8)
XP	28 (41.2)
Metastatic site, *N* (%)[b]	
Lymph node	29 (52.7)
Liver	13 (23.6)
Peritoneum	36 (65.5)
Bone	4 (7.3)
Recurrent site, *N* (%)[b]	
Lymph node	6 (46.2)
Liver	2 (15.4)
Peritoneum	8 (61.5)
Bone	1 (7.7)
Remnant stomach	3 (23.1)
Overall survival, median months (95 % CI)	12.1 (9.8–17)
Progression-free survival, median months (95 % CI)	6 (4.3–6.9)
Follow-up duration, median months (range)	81.6 (32.6–113)

ECOG Eastern Cooperative Group performance status, *GEJ* esophagogastric junction, *HER2* human epidermal growth factor receptor 2, *PCC* poorly cohesive carcinoma, *SRC* signet ring cell, *FOLFOX* 5-fluorouracil plus oxaliplatin, *XP* capecitabine plus cisplatin

[a] Lauren classification: not evaluable in 43 of 68 because of small amount of biopsy tissue

[b] Numbers and proportions of metastatic and recurrent site were calculated respectively (metastatic for $N = 55$ and recurrent for $N = 13$)

high and low 11-CAF signature groups (Supplementary Table 2). Moreover, multivariate Cox analysis showed that the high 11-CAF signature was independently correlated with poor OS along with poor performance status and the presence of signet ring cell components (HR 1.76, $p = 0.029$; Supplementary Table 3).

OS prediction modeling with IL-8 and OPN

Eleven CAFs that significantly predicted OS were analyzed with other clinicopathological characteristics (Supplementary Table 4). Univariate Cox analysis of OS showed that performance status and signet ring cell components, along with 11 CAFs, were significantly correlated with poor OS. Multivariate Cox analysis showed that IL-8 (HR 2.31, $p = 0.009$) and OPN (HR 2.70, $p = 0.001$) were significantly correlated with poor OS. Using these 2 CAFs, which showed the strongest relationships with OS, we developed a model for predicting OS. The median OS of patients with elevated levels of both IL-8 and OPN was 8.2 months; the median OSs of patients with elevated levels of either one of these factors or neither factor were 15.8 and 19.9 months, respectively ($p = 0.002$ and $p < 0.001$, respectively; Supplementary Fig. 3a). The median PFS of patients with elevated levels of both IL-8 and OPN was worse than other groups ($p = 0.068$ and 0.007; Supplementary Fig. 3b).

Discussion

In this study, we analyzed pretreatment serum levels of 52 CAFs in patients with advanced GC. Clustering analysis of the CAFs revealed three distinct patient groups: patients with high levels of angiogenic factors, patients with high levels of inflammatory mediators, and patients with high levels of both angiogenic factors and inflammatory mediators. We found the 11 CAF-signature could identify patients who obtained poor prognosis when treated with standard conventional chemotherapy.

Although correlations between inflammation and cancer are well established [10, 22] and angiogenic sprouting of tumors is known as an important feature of carcinogenesis and cancer progression [23], few studies have been reported that define solid cancers according to circulating factors, such as CAFs. Clustering analyses of pretreatment serum CAFs to classify patients into subgroups have been reported in renal cell carcinoma and non-small cell lung cancer [16, 19], and several studies showed that a distinct CAF profile could predict clinical outcomes [15–19]. However, although associations between inflammatory cascades or angiogenic factors, such as VEGF, and clinical outcomes have been reported in GC [24], comprehensive

Table 2 Cytokines and angiogenic factors profile

CAF	N^a	Mean (pg/ml)	Standardized error[a]	Median (pg/ml)	Min (pg/ml)	Max (pg/ml)
CAFs included in the final analysis ($N = 42$)						
IL-2Rα	68	156.9	13.5	126.7	12.7	679.0
IL-3	54	145.6	24.4	90.2	1.0	1095.3
IL-16	68	694.4	150.3	342.1	26.2	7298.3
IL-18	68	123.4	14.1	82.4	23.2	564.0
CTACK	68	891.8	40.5	809.1	413.7	2175.5
GRO-α	67	262.9	44.3	181.4	5.7	2386.1
HGF	68	623.9	49.4	506.9	161.0	2428.2
IFN-α2	57	25.3	2.9	20.8	0.2	104.4
LIF	36	18.4	2.5	13.9	1.4	88.9
M-CSF	45	26.4	3.8	16.4	1.1	156.9
MIF	68	4603.3	1385.0	787.7	104.4	77622.3
MIG	68	2329.4	510.1	1449.0	421.4	34713.6
SCF	68	135.9	7.1	132.5	46.5	297.6
SCGF-β	68	33556.3	4596.5	26173.7	4610.6	310063.7
SDF-1α	67	249.6	21.0	209.5	31.9	1335.6
TRAIL	46	45.9	6.1	32.6	1.5	275.7
IL-1Rα	68	306.2	133.9	106.4	30.1	9082.5
IL-4	67	9.4	0.9	4.3	0.2	24.3
IL-6	62	34.2	10.6	11.3	0.9	643.5
IL-7	61	26.2	15.5	10.0	0.6	1060.0
IL-8	64	90.9	53.4	19.7	1.3	3578.4
IL-9	64	78.1	37.5	16.2	0.9	2171.1
IL-10	48	110.9	82.8	14.5	1.3	5583.3
IL-12p70	65	383.3	213.7	58.9	1.4	11784.3
IL-13	68	23.2	11.5	8.6	1.7	788.3
IL-17	59	59.9	5.8	49.5	0.9	191.3
Eotaxin	67	119.7	9.6	106.0	1.2	565.7
FGF-basic	67	40.3	4.1	32.1	1.2	185.8
G-CSF	68	582.9	495.4	76.3	17.6	33773.9
IFN-γ	68	116.8	39.7	61.8	14.4	2734.7
IP-10	67	2479.4	433.8	1686.1	299.2	27797.0
MCP-1	68	106.3	13.6	74.3	8.6	717.8
MIP-1α	68	28.0	22.1	4.9	1.0	1511.7
PDGF-bb	68	7844.4	648.8	6650.9	152.3	24895.8
MIP-1b	68	280.4	110.5	154.4	50.4	7648.1
RANTES	60	36042.6	3145.0	30257.6	2735.5	179879.4
TNF-α	45	35.2	11.9	22.0	2.6	806.2
VEGF	66	283.5	28.9	207.0	1.2	1146.8
PIGF	68	39.3	6.4	22.4	8.0	244.6
VEGFR2	68	1309.9	34.8	1315.3	742.8	2172.8
sCA9	67	161.6	27.8	97.1	18.7	1374.5
OPN	68	5.7	0.5	4.4	1.3	22.8
CAF excluded in the final analysis ($N = 10$)						
IL-1α	2	0.2	0.1	0.1	0.1	2.4
IL-12p40	22	1.7	0.3	0.1	0.1	7.7
MCP-3	24	0.2	0.2	0.1	0.1	4.2
BNGF	15	0.2	0.	0.1	0.1	3.9
TNF-β	15	0.7	0.2	0.1	0.1	3.5
IL-1β	19	0.1	0.2	0.1	0.1	4.4

Table 2 continued

CAF	N^a	Mean (pg/ml)	Standardized error[a]	Median (pg/ml)	Min (pg/ml)	Max (pg/ml)
IL-2	15	0.3	0.2	0.1	0.1	5.6
IL-5	5	0.1	0.1	0.1	0.1	3.6
IL-15	8	0.2	0.1	0.1	0.1	3.3
GM-CSF	29	1.5	0.3	0.1	0.1	5.8

CAF cytokines and angiogenic factors, *IL* interferon, *IL-2Rα* IL-2 receptor-alpha, *CTACK* cutaneous T-cell-attracting chemokine, *GROα* growth-regulated alpha protein, *HGF* hepatocyte growth factor, *IFN-α2* interferon-alpha 2, *LIF* leukemia inhibitory factor, *M-CSF* macrophage colony-stimulating factor, *MIF* macrophage migration inhibitory factor, *MIG* monokine induced by interferon-gamma, *SCF* stem cell factor, *SCGF-β* stem cell growth factor-beta, *SDF-1α* stromal cell-derived factor 1-alpha, *IL-1Rα* IL-1 receptor-alpha, *FGF-basic* fibroblast growth factor 2 basic, *G-CSF* granulocyte colony-stimulating factor, *IFN-γ* interferon-gamma, *IP-10* interferon gamma-induced protein 10, *MCP-1* monocyte chemotactic protein 1, *MIP-1α* macrophage inflammatory protein 1-alpha, *PDGF-bb* platelet-derived growth factor-beta polypeptide b, *MIP-1β* macrophage inflammatory protein 1-beta, *RANTES* regulated on activation, normal T cell expressed and secreted, *TNF-α* tumor necrosis factor-alpha, *VEGF* vascular endothelial growth factor-α, *PIGF* placenta growth factor, *VEGFR2* vascular endothelial growth factor receptor 2, *sCA9* soluble carbonic anhydrase 9, *OPN* osteopontin, *MCP-3* monocyte chemotactic protein 3, *BNGF* beta-nerve growth factor, *TNF-β* tumor necrosis factor-beta, *IL-1β* interferon 1-beta, *GM-CSF* granulocyte macrophage colony-stimulating factor

[a] Number of analytes within detection range

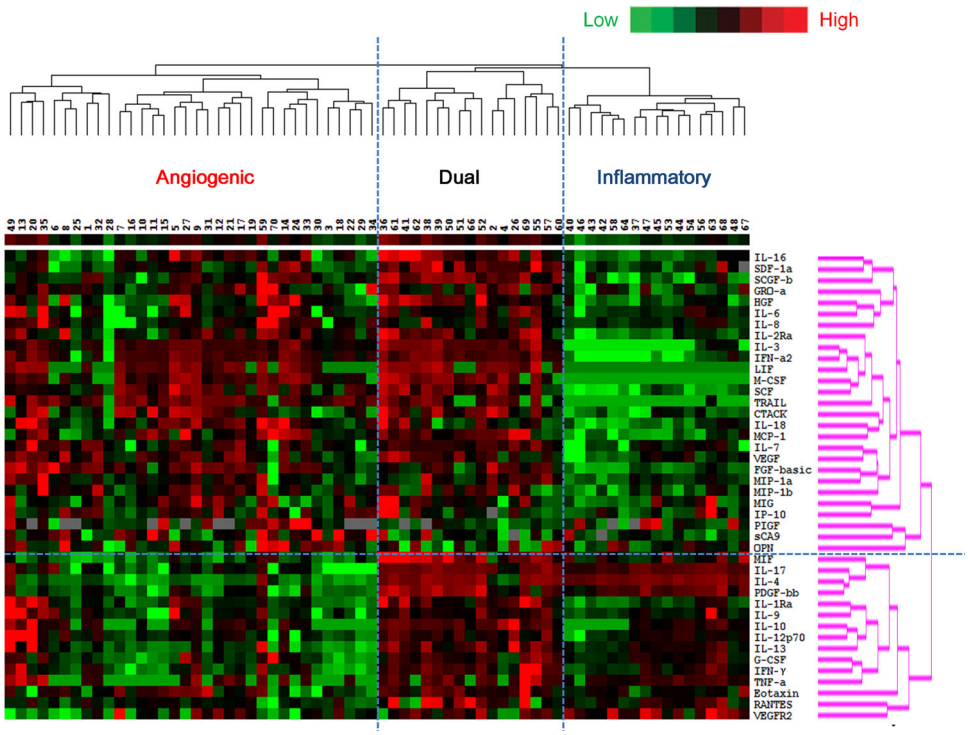

Fig. 1 Cluster analysis of cytokines and angiogenic factors (CAF). Unsupervised cluster analysis of CAFs in patients with gastric cancer. Dendrograms show 68 patient samples (*columns*) according to 42 soluble CAFs (*rows*). The CAF concentration ratios are depicted by a log-transformed pseudo-color intensity scale. Three patient groups were identified: angiogenic, inflammatory, and dual. Please see Table 2 for a complete list of CAFs

explorations of circulating factors and the relationships with clinical outcomes have not been published for GC.

We investigated pretreatment serum levels of 52 CAFs in GC patients. To the best of our knowledge, this is the most extensive investigation of CAFs in GC. Clustering analysis revealed three distinct groups of CAFs. One group, which was classified as the angiogenic group in a previous report of renal cell carcinoma, included VEGF, HGF, GRO-α, M-CSF, and OPN. The other group, which was also used in the previous report [16], included various IL series, as well as tumor necrosis factor-alpha and IFN-γ, which are well-known mediators of inflammatory cascades [10]. The third group included both factors. Although OSs were not significantly different among patients in the three CAF groups, patients who had elevated levels of angiogenic CAFs tended to have unfavorable OS (Supplementary Fig. 1). Moreover,

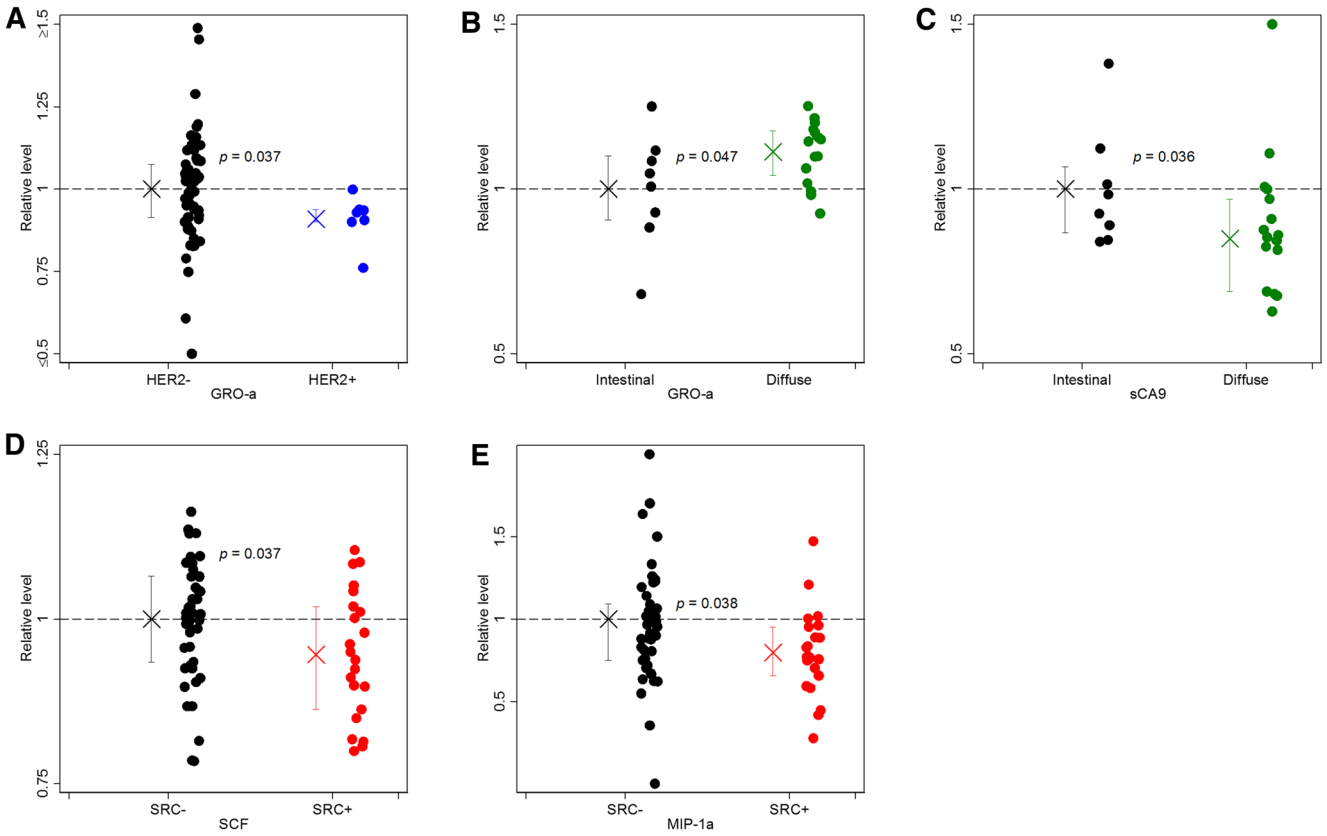

Fig. 2 Differences in CAF profiles according to HER2 status, Lauren type classification, and signet ring cell components. Mean levels (X on *left side*) plus 2 standard errors (*bar*) and all levels (*dots on right side*) of significantly different CAFs according to HER2 status (**a**), Lauren classification (**b**, **c**), and signet ring cell components (SRC, **d**, **e**). Relative levels of CAFs were calculated by dividing each value by the mean level of HER2-negative status (**a**), intestinal-type Lauren classification (**b**, **c**), and signet ring cell components (**d**, **e**), respectively. *GRO-a* growth-regulated alpha protein, *MIP-1a* macrophage inflammatory protein 1-alpha, *sCA9* soluble carbonic anhydrase 9, *SCF* stem cell factor

Table 3 Lists of 11 cytokines and angiogenic factors that predict overall survival

CAF	Cutoff (pg/ml)	Cox analysis			CAF < cutoff			CAF ≥ cutoff		
		HR	95 % CI	*p*	*N*	mOS	95 % CI	*N*	mOS	95 % CI
IL-2Rα	125.27	1.79	(1.08–2.96)	0.023	33	16.8	(11.5–21.2)	35	10.2	(7.5–12.1)
GRO-α	184.43	1.64	(1–2.67)	0.048	34	17.1	(9.8–22)	34	10.5	(7.1–13.2)
HGF	495.94	1.81	(1.11–2.95)	0.018	31	17.1	(13.6–22)	37	9.8	(7.1–11.3)
M-CSF	15.33	1.69	(1.04–2.76)	0.036	33	17.1	(11.5–22)	35	10.1	(7.5–12.1)
SDF-1α	209.49	1.7	(1.04–2.79)	0.035	33	17.6	(11.5–22)	34	9.5	(7.5–12.1)
IL-6	13.24	1.82	(1.11–2.98)	0.018	37	17	(12.5–22)	31	10.1	(5.8–11.3)
IL-8	18.33	1.88	(1.14–3.08)	0.013	31	17.1	(12.5–26.3)	37	10.1	(7.5–12.1)
IL-10	15.03	1.72	(1.04–2.85)	0.034	37	16.8	(9.5–21)	31	10.9	(7.5–13.2)
IFN-γ	65.58	1.79	(1.07–3)	0.028	37	15.8	(9.4–22)	31	10.9	(9–16.7)
VEGF	215.47	1.66	(1–2.75)	0.05	36	16.7	(9.2–22)	32	10.9	(7.8–15.8)
OPN	4.4	1.91	(1.15–3.16)	0.012	34	17.9	(10.1–26.3)	34	10.2	(7.5–13.2)

CAF cytokines and angiogenic factors, *IL-2Rα* IL-2 receptor-alpha, *GRO-α* growth-regulated alpha protein, *HGF* hepatocyte growth factor, *M-CSF* macrophage colony-stimulating factor, *SDF-1α* stromal cell-derived factor 1-alpha, *IFN-γ* interferon-gamma, *VEGF* vascular endothelial growth factor, *OPN* osteopontin

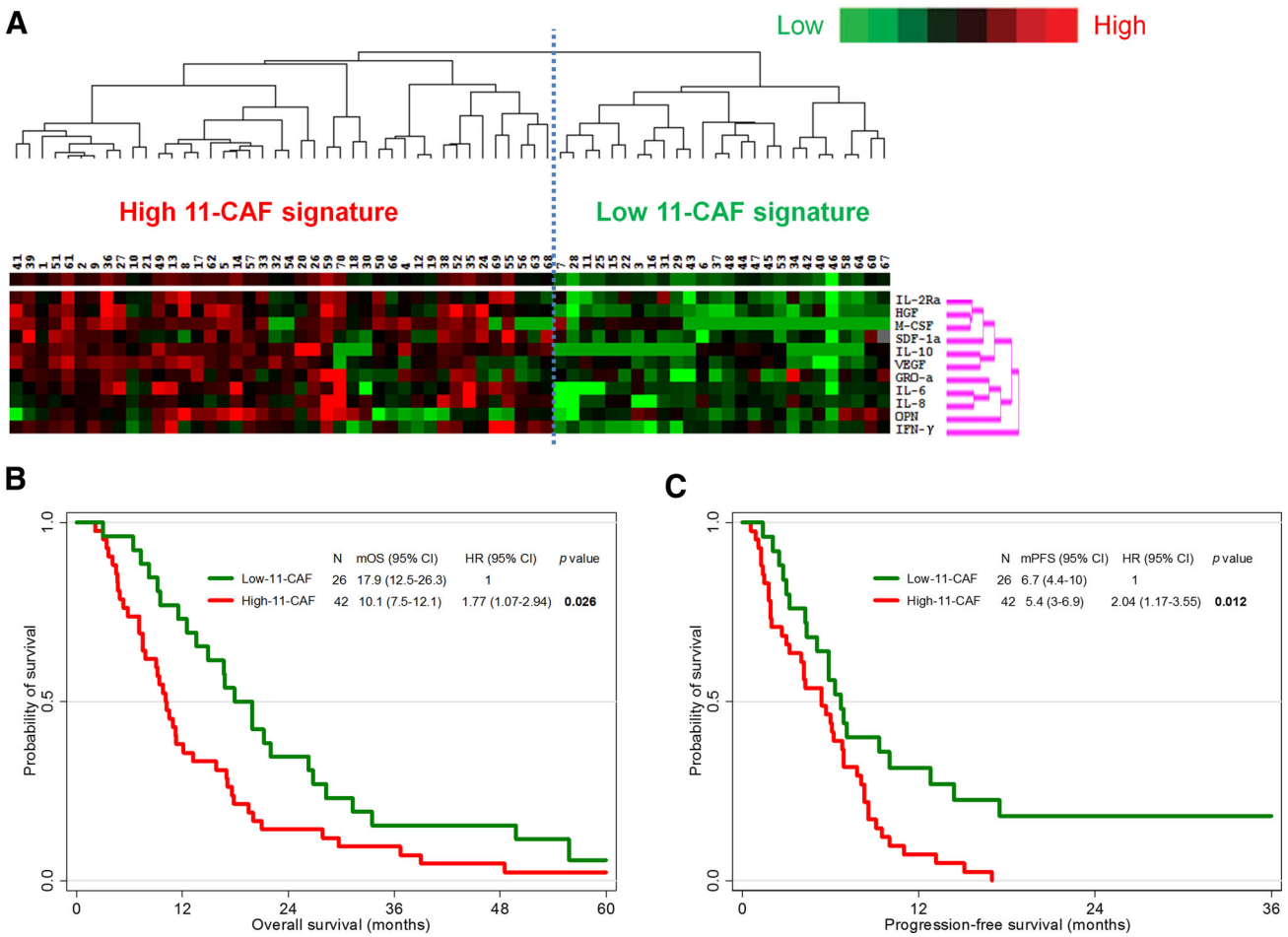

Fig. 3 Cluster analysis of 11-CAF signature. Unsupervised cluster analysis of 11 CAFs that significantly predicted poor overall survival. Dendrograms show 68 patient samples (*columns*) with baseline levels of 11 significant CAFs (*rows*). The CAF concentration ratios are depicted by a log-transformed pseudo-color intensity scale. Two patient groups were identified: high 11-CAF signature and low 11-CAF signature (**a**). Kaplan–Meier curves for overall survival (**b**) and progression-free survival (**c**) according to the 2 groups identified by cluster analysis of the 11-CAF signature. *CI* confidential interval, *HR* hazard ratio, *mOS* median overall survival, *IL-2Ra* interleukin-2 receptor alpha, *HGF* hepatocyte growth factor, *M-CSF* macrophage colony-stimulating factor, *SDF-1a* stromal cell-derived factor 1 alpha, *VEGF* vascular endothelial growth factor, *GRO-a* growth-regulated alpha protein, *SDF-1a* stromal cell-derived factor 1-alpha, *OPN* osteopontin, *IFN-γ* interferon-gamma

9 of the 11 CAFs that predicted poor OS according to the Cox analysis were classified as angiogenic CAFs. To the best of our knowledge, this is the first report to assess the interactions between circulating CAFs via clustering analysis in GC patients and identify patient subgroups on the basis of CAFs.

CAF levels varied according to pathological classifications of GC, such as HER2 status, Lauren classification [21], and signet ring cell components. GRO-α was significantly higher in HER2-negative GC and diffuse-type GC compared with HER2-positive GC and intestinal-type GC, respectively. GRO-α attracts immune cells via the chemokine receptor CXCR2 [25, 26]. Previous reports showed that GRO-α expression in tumors is higher in diffuse-type disease than intestinal-type disease [27]. Previous reports showed sCA9, SCF, and MIP-1a are associated with a hypoxic signature [18, 28–30]. As intestinal-type GC is partially caused by *Helicobacter pylori*-associated metaplasia and mediated by hypoxia-induced angiogenesis [31, 32], as such, increased levels of sCA9 in intestinal-type disease compared to the diffuse-type disease would be reasonable. Moreover, as signet ring cell pathology is associated with diffuse-type disease [21, 33], it is expected that serum levels of SCF and MIP-1a would be lower in patients with signet ring cell components. However, these findings and the statistical comparison of the groups according to HER2 status and Lauren classification should be interpreted with caution, because only a small number of patients ($N < 10$) were included in our study.

Previous studies of CAFs have indicated that an understanding of the delicate networking of CAFs is necessary to correlate CAF levels to clinical outcomes, because the biological activity of circulating factors might be the result of the interactions of CAFs rather than the action of single CAFs [15–19]. A previous study in renal cell carcinoma identified 6 CAFs as significant predictive factors for the selection of a combination regimen of sorafenib and interferon-alpha compared to sorafenib alone [16]. In head and neck squamous cell carcinoma, CAFs that were defined as a hypoxia signature were closely clustered and their levels were higher in patients who experienced progression after induction chemotherapy than in those who did not experience disease progression [18]. In our study, we identified 11 CAFs that independently predicted poor OS among 52 CAFs. A cluster analysis that included these 11 CAFs divided patients by prognosis, even after adjustment for other traditional significant clinicopathological features. Interestingly, the characteristics of patients were not different according to the 11-CAF signature, even though the OS was significantly different (Supplementary Table 2, Supplementary Table 3). According to The Cancer Genome Atlas of stomach adenocarcinoma [3], genomically stable tumor, which would be correlated with signet ring cell component or diffuse type, would have a high inflammatory signature; however, some portions of other molecular subtypes such as Epstein–Barr virus-positive, chromosomal instability, and microsatellite-high might also contain high CAF signature as a result of *CD274* (*PD-L1*) amplification and *VEGF-α* amplification, and abundant neo-antigen-promoting immunogenicity [34, 35], respectively. Therefore, tumors with high inflammatory markers or an 11-CAF signature would not be simply associated with the conventional clinicopathological factors. This finding indicates that using traditional clinicopathological prognostic factors limits the ability to identify patients who are likely to experience a poor prognosis after standard chemotherapy in GC. Using 11-CAF signature information, we can more accurately predict the prognosis of patients who receive standard chemotherapy, which fills a substantial gap in medical knowledge.

A multivariate Cox analysis of each of the 11 CAFs revealed that high levels of IL-8 and OPN independently predicted poor survival (Supplementary Table 4). IL-8 is a proinflammatory cytokine that attracts and activates neutrophilic granulocytes and it is associated with poor prognosis in many cancers [36]. Moreover, the close association of IL-8 with tumor angiogenesis has been reported [37, 38] and the dual inhibition of VEGF and IL-8 has been suggested as a potentially efficient strategy for treating cancer [39]. Previous studies have shown that serum levels of IL-8 are higher in patients with GC than in healthy control patients [40, 41], and serum IL-8 has been reported as a poor prognostic factor in a variety of malignant diseases, including melanoma, renal cell carcinoma, and hepatocellular carcinoma [42]. Our study adds the evidence of correlation between serum IL-8 and poor prognosis in GC for the first time.

The role of OPN in tumor progression has been reported to be extracellular remodeling to promote epithelial-mesenchymal transition and angiogenesis [43, 44]. Studies have shown that OPN could bind CD44, then activate the phosphoinositide 3-kinase/protein kinase B pathway, and upregulate VEGF [45–47]. Several studies showed that tumor expression of OPN was a poor prognostic factor in resectable cases of GC [48], and serum levels of OPN have also been represented as significant prognostic factors in many cancers, including GC [49–51]. However, our study gives the information on the prognostic significance of OPN in advanced GC patients who are treated with conventional standard chemotherapy for the first time.

The current study presents a new scope of GC classification on the basis of serum CAF clustering and survival analysis, but there are several limitations that should be considered. Although all patients who enrolled in the study were successfully followed for the survival analysis, the retrospective design of the study and relatively small number of patients limits the interpretation of the results. However, 52 different serum CAFs in our study that were concurrently analyzed with the survival of GC patients contributes to discriminating the types of circulating factors that influence clinical outcomes and recognizing the delicate networking of CAFs. The most valuable finding of the current study is the identification of the 11-CAF signature, which may be able to discern GC patients with a poor prognosis when treated with standard chemotherapy, which otherwise could not be identified. Although previous reports have shown that simple biomarkers of cancer-associated inflammation, such as high C-reactive protein or low albumin levels, would relate to poor prognosis of GC [52, 53], the involved mechanisms have not been known thoroughly. Our study suggests that this biological impact would be mediated by the 11-CAF signature. However, this gross shot of the CAF signature should be further clarified in terms of its exact networking and actions that result in the final clinical outcomes of patients. For patients with a high 11-CAF signature, treatment strategies targeting the factors included in this signature might be worthy to be investigated. For example, for the patient selection for ramucirumab, an anti-VEGFR2 antibody which has recently proved survival benefits in the second-line setting of GC [54], a single factor such as VEGF-α is not enough to identify the optimal patients. In that case, an 11-CAF signature might be a candidate to be evaluated. Another aspect is that patients with high CAF signature might benefit from the immunological agents because the high

inflammatory signature such as the IFN-γ signature has been suggested to be related to a favorable clinical outcome of immune checkpoint inhibitors [55].

In conclusion, serum CAF profiling differentiated three subgroups of GC patients: angiogenic, inflammatory, and dual groups. A high 11-CAF signature could identify GC patients with a poor prognosis when treated with standard chemotherapy, who need urgent new treatment strategies.

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Compliance with ethical standards

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

Ethical standards All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent or a substitute for it was obtained from all patients for being included in the study.

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