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ORIGINAL PAPER

Expression of the anaphylatoxin C5a receptor in gastric cancer: implications for vascular invasion and patient outcomes

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Abstract The C5a receptor (C5aR) expressed in various types of cancers is involved in C5a-induced cancer cell invasion. However, its role in gastric cancer has not yet been fully elucidated. Therefore, we studied the clinical significance of C5aR expression in gastric cancer. The association of C5aR expression in gastric cancer, determined by immunostaining using the anti-C5aR antibody, with clinicopathological parameters and outcomes was evaluated in 148 patients. Further, the association of C5aR expression in liver metastatic sites with clinicopathological parameters was investigated in a separate cohort of 58 patients who underwent hepatectomy. High tumoral C5aR expression ($n = 45$, 30.4 %) was significantly related to tumor location, cancer invasion depth, vascular and lymphatic invasion, and tumor stage. The 5-year recurrence-free and overall survival rates of patients with high tumoral C5aR expression were significantly lower than those of patients with low tumoral C5aR expression (50.9 vs. 84.2 %, $P = 0.002$ and 58.8 vs. 86.1 %, $P = 0.007$, respectively). The incidence of liver metastasis was significantly higher in patients with high tumoral C5aR expression (13.3 %) than in those with low tumoral C5aR expression (3.9 %; $P = 0.04$). C5aR expression at liver metastatic sites was associated with the C5aR expression status at the primary site ($P = 0.0004$), vascular invasion at the primary site ($P = 0.04$), and tumor size at the metastatic site ($P = 0.01$). C5aR expression in gastric cancer was associated with cancer progression, liver metastasis, and poor prognosis. Therefore, C5aR may represent a prognostic factor and therapeutic target in gastric cancer.

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Keywords C5a · C5a receptor · Gastric cancer · Invasion · Liver metastasis

Introduction

Gastric cancer is a common gastrointestinal malignant disease with a markedly high mortality rate in Asian countries, including Japan [1]. The clinical stage and aggressiveness of tumors are critical factors for predicting the outcomes of gastric cancer; thus, the early detection of gastric cancer and subsequent endoscopic or surgical treatments can provide favorable patient outcomes [2–4]. Despite the availability of several anticancer drugs and molecular targeted agents, the prognosis of gastric cancer is unsatisfactory in patients with late-stage disease and in those who receive multidisciplinary therapies [5]. The aberrant expression of adhesion molecules has been shown to be associated with accelerated tumor growth and metastatic potential [6–8].

The complement system is a biochemical cascade involved in the immune response [9]. The anaphylatoxin C5a exerts potent leukocyte chemoattractant effects by binding to its corresponding cell surface receptor [C5a receptor (C5aR)], first identified in leukocyte cell lines [10]. C5a functions in the initiation of inflammation by stimulating the migration of leukocytes, production of radical oxygen, and release of histamines [10–12]. We previously demonstrated that C5aR is aberrantly expressed in various human cancer cells isolated from surgically resected tissues, including gastric cancer tissues, and that the C5a–C5aR axis promotes bile duct cancer cell invasion via the enhancement of cell motility and metalloproteinase secretion [13]. Previous reports have shown that the complement system is activated in cancer tissues in both experimental animal [14] and human studies [15], suggesting that a byproduct of C5a is an important component of the cancer microenvironment. Thus, the C5a–C5aR axis may be associated with the poor prognosis of gastric cancer in humans. However, the biological and clinical significance of the C5a–C5aR axis in gastric cancer has not yet been elucidated.

Therefore, in the present study, we evaluated gastric cancer C5aR expression in primary and liver metastatic sites and the association of C5aR expression with clinicopathological parameters and patient outcomes.

Materials and methods

Patients and tissues

Surgically resected gastric cancer specimens were obtained from 168 patients who underwent gastrectomy and lymph node dissection at Kumamoto University Hospital between March 2001 and December 2009. Among these patients, 20 at stage IV (peritoneal washing cytology positive, $n = 11$; peritoneal dissemination, $n = 6$; and liver metastasis, $n = 3$) were excluded, and the remaining 148 patients who underwent curative gastrectomy (stage I–III) were analyzed. Informed consent was obtained from all patients, and the study was approved by the Human Ethics Review Committee of Kumamoto University Graduate School of Medicine.

Gastric cancer tissues from primary and liver metastatic sites of 103 patients with liver-limited metastasis of gastric cancer treated with surgery, microwave coagulation therapy, or radiofrequency ablation were collected from 28 institutions of the Kyushu Study Group of Clinical Cancer (KSCC) in Japan (KSCC1302 study) [16]. Among these patients, the primary site and metastasized liver-paired tissues of 58 patients who synchronously or metachronously underwent gastrectomy for primary gastric cancer and hepatectomy for liver metastasis from January 1, 2000, to December 31, 2010, were collected from 23 institutions. In addition, patient clinicopathological parameters were obtained. The ethical, medical, and scientific aspects of the present study were reviewed and approved by the institutional review board of each participating institution.

Differentiation, cancer invasion depth and pattern, lymph node metastasis classification, vascular and lymphatic invasion, tumor size, and intestinal connective tissue volume were evaluated by histopathological examination using the Japanese Classification of Gastric Carcinoma (14th edition) [17].

Immunohistochemistry

C5aR expression of gastric adenocarcinomas from primary and liver metastatic sites was immunohistochemically evaluated. Deparaffinized 2-μm-thick sections were pretreated with 0.3 % H_2O_2 in methanol for 20 min, followed by treatment with a serum-free protein block (Dako Cytomation, Glostrup, Denmark) for 20 min. The sections were incubated with a primary monoclonal antibody against human C5aR (2 μg/mL; Hycult Biotechnology, Uden, the Netherlands) at 4 °C overnight. After washing, the sections were stained using EnVision + kits (Dako Cytomation) and 3,3′-diaminobenzidine tetrahydrochloride solution containing 0.006 % H_2O_2, according to the manufacturer's instructions. Nuclei were counterstained with hematoxylin. We confirmed the immunoreactivity to proximal renal tubular cells as positive control as previously reported [18]. Normal mouse IgG was used instead of the primary antibody as the negative control, and it did not react with the tissue sections. The level of C5aR expression was quantified using scores for staining intensity (0, no staining; 1, light yellow staining; 2, brownish yellow staining; and 3, dark brown

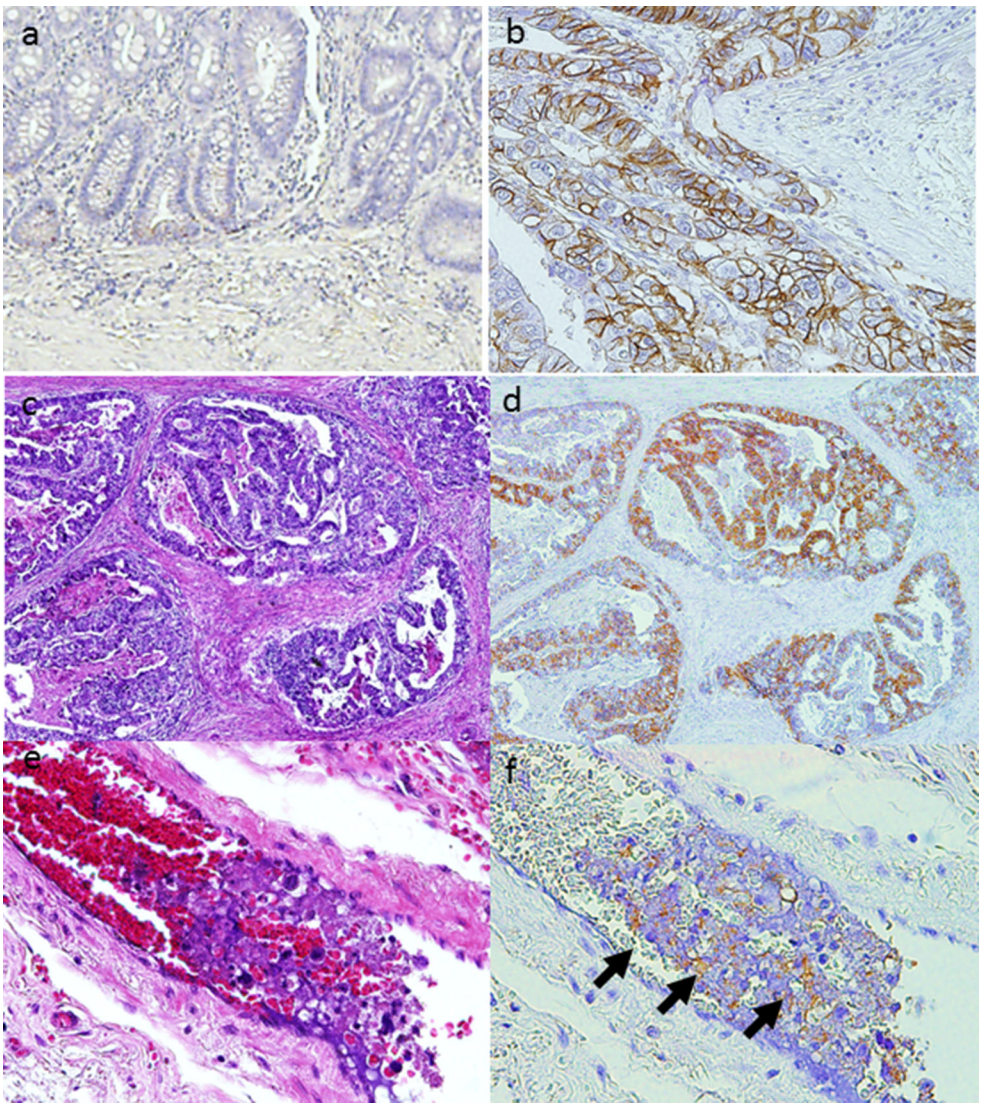

Fig. 1 Immunohistochemical staining of gastric cancer tissues using mouse monoclonal antibody against human C5aR. **a** Non-cancerous gastric tissue (C5aR, ×100), **b** cancer tissue (C5aR, ×200), **c** cancer tissue [hematoxylin and eosin (HE) staining, ×40], **d** cancer tissue (C5aR, ×40), **e**, **f** cancer cells invading an adjacent blood vessel (**e** HE, ×200; **f** C5aR, ×200)

staining) and the C5aR-positive cancer cell occupying ratio (0, no positive cells; 1, 1–30 %; 2, 30–60 %; and 3, >60 %) according to the evaluation methods of prior reports [19, 20]. For each tissue section, five high-power fields (100×) were randomly selected, and the average intensity and area scores were added. High C5a expression was defined as a total C5aR expression score of ≥3.

Statistical analyses

C5aR expression was analyzed as binomial data (high or low). The χ^2 test was used to analyze associations of C5aR expression with clinical and histological parameters. Recurrence-free survival (RFS) was calculated from the date of surgery to the date of the first relapse, to the date of death without relapse, or to the date of data censoring. Overall survival (OS) was calculated from the date of surgery to the date of death or to the date of data censoring. RFS and OS were calculated using the Kaplan–Meier method and were compared using the log-rank test. A two-sided P value of <0.05 was considered statistically significant. All statistical analyses were performed with SAS version 9.3 and JMP® 10 (SAS Institute Inc., Cary, NC, USA).

Result

C5aR expression in gastric cancer tissues at primary sites

Negligible C5aR staining was observed on non-cancerous gastric epithelial cells (Fig. 1a). However, significant

Table 1 Baseline clinicopathological data of 148 gastric cancer patients divided according to whether they had high or low tumoral C5aR expression

Clinicopathological factors	C5aR expression		*P* value‡
	High ($n = 45$)	Low ($n = 103$)	
Gender			
Male	32	76	0.74
Female	13	27	
Age (years)[a]			
<72	18	50	0.34
≥72	27	53	
Tumor size (mm)[a]			
<35	22	62	0.20
≥35	23	41	
Location			
Upper	22	20	0.001
Middle	13	39	
Lower	10	44	
Differentiation			
Intestinal	32	64	0.29
Diffuse	13	39	
Invasion depth			
pT1 (M, SM)	13	67	0.0006
pT2 (MP)	7	11	
pT3 (SS)	20	19	
pT4 (SE, SI)	5	6	
N classification			
N0	27	72	0.62
N1	8	16	
N2	6	8	
N3	4	7	
pStage			
I	15	73	<0.0001
II	20	18	
III	10	12	
Lymphatic invasion			
−	26	82	0.004
+	19	20	
Unknown	0	1	
Vascular invasion			
−	14	64	0.0005
+	31	39	
Amount of interstitial connective tissue			
Medullary	7	26	0.19
Intermediate	34	55	
Scirrhous	3	8	
Unknown	1	14	
Infiltrative pattern			
α	4	19	0.08
β	38	62	
γ	3	13	
Unknown	0	9	

C5aR C5a receptor

‡ χ^2 test

[a] Median value

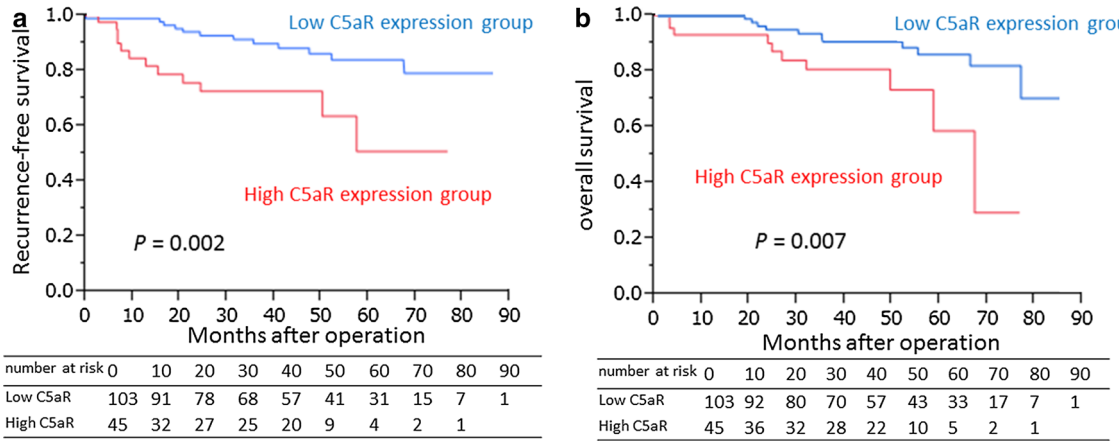

Fig. 2 Kaplan–Meier survival plots following gastric cancer resection at the primary site. **a** Recurrence-free survival (RFS) of patients with stage I–III disease ($n = 148$), **b** overall survival (OS). High and low tumoral C5aR expression groups are represented by *red and blue lines*, respectively

C5aR expression was observed on the cell membranes of cancer cells (Fig. 1b–d). Cancer cells invading the adjacent blood vessel showed strong positivity to the anti-C5aR antibody (Fig. 1e, f). Patients were classified into the following groups according to the C5aR tumoral expression intensity and C5aR-positive cancer cell occupying ratio: the high tumoral C5aR expression group ($n = 45$, 30.4 %) and the low tumoral C5aR expression group ($n = 103$, 69.6 %). The number of patients classified according to the staining intensity score was as follows: 0, $n = 29$; 1, $n = 82$; 2, $n = 18$; and 3, $n = 19$. The occupying ratio of C5aR-positive cells ranged from 0 to 90 % (average, 13.1 %; score 0, $n = 61$; 1, $n = 53$; 2, $n = 17$; 3, $n = 17$). C5aR expression scores ranged from 0 to 6 (0, $n = 29$; 1, $n = 32$; 2, $n = 43$; 3, $n = 13$; 4, $n = 5$; 5, $n = 20$; and 6, $n = 6$).

Association of tumoral C5aR expression with clinicopathological parameters in gastric cancer patients

We compared the clinicopathological parameters between patients with high and low tumoral C5aR expression (Table 1). High C5aR expression was significantly associated with upper tumor location ($P = 0.001$), cancer invasion depth ($P = 0.0006$), lymphatic invasion ($P = 0.004$), vascular invasion ($P = 0.0005$), and clinical stage ($P < 0.0001$). No associations with other parameters were observed.

Association of tumoral C5aR expression with clinical outcomes in gastric cancer patients

Next, we analyzed the association of tumoral C5aR expression with the prognosis of gastric cancer patients. The median follow-up duration was 36.6 months. The 5-year RFS rates of patients in the high ($n = 45$) and low ($n = 103$) tumoral C5aR expression groups were 50.9 and 84.2 %, respectively ($P = 0.002$; Fig. 2a). The 5-year OS rates in the high and low tumoral C5aR expression groups were 58.8 and 86.1 %, respectively ($P = 0.007$; Fig. 2b). Gastric cancer recurrence patterns are shown in Table 2. Twenty-four patients had disease recurrence during the follow-up period. A higher liver metastasis rate ($P = 0.04$) was observed in the high tumoral C5aR expression group than that in the low tumoral C5aR expression group; however, high tumoral C5aR expression was not associated with peritoneal dissemination or lymph node metastasis.

C5aR expression profile of gastric cancer liver metastases

C5aR expression in gastric cancer liver metastases was evaluated in a separate cohort of 58 gastric cancer patients with liver metastasis. C5aR expression was specifically detected in the membrane of cancer cells at liver metastatic sites (Fig. 3). High tumoral C5aR expression was observed at primary and liver metastatic sites in 23 (39.7 %) and 24 (41.4 %) patients, respectively. The associations of tumoral C5aR expression in liver metastases with clinicopathological parameters are shown in Table 3. Although no significant difference was observed between the primary site C5aR status and clinicopathological parameters in patients with liver metastases, patients with liver metastasis having high C5aR expression were more likely to be younger (<71 years), have a liver metastatic size of ≥31 mm, and have venous invasion at the primary site than those with liver metastasis with low C5aR expression ($P = 0.004$, $P = 0.01$, and $P = 0.04$, respectively). A higher proportion of patients with liver metastasis having high C5aR

Table 2 Association of tumoral C5aR expression level with recurrence pattern in 148 gastric patients

	C5a receptor expression		*P* value‡
	High ($n = 45$)	Low ($n = 103$)	
Liver			0.04
–	39	99	
+	6	4	
Lung			0.17
–	43	102	
+	2	1	
Bone			0.51
–	45	102	
+	0	1	
Distant metastasis			0.05
–	38	97	
+	7	6	
Peritoneal dissemination			0.34
–	41	98	
+	4	5	
Lymph node			0.22
–	41	99	
+	4	4	
Local recurrence			0.17
–	43	102	
+	2	1	
Total recurrence			0.02
–	33	91	
+	12	12	

C5aR C5a receptor

‡ χ^2 test

expression had ≥2 liver metastases than those with liver metastasis having low C5aR expression; however, this difference did not reach statistical significance ($P = 0.07$).

Discussion

Complex networks of chemokines and their corresponding receptors have been shown to influence the development of primary cancers and metastasis, suggesting that these factors have a chemoattractant effect on cancer cells [21]. Originally identified on leukocytes, C5aRs have also been found on vascular endothelial cells [22, 23], mesangial cells [24], and alveolar and bronchial epithelial cells [25, 26]. We previously demonstrated that C5aR is not expressed on non-cancerous epithelial cells, with the exception being renal tubular cells. However, cancer cells were found to aberrantly express C5aR, with tumoral C5aR expression observed in 35 % of gastric cancer patients ($n = 20$) [13]. A similar proportion of patients with high tumoral C5aR expression (30.4 %; Table 1) was observed among the 148 gastric cancer patients in the present study. The observed association of high tumoral C5aR expression with cancer invasion depth, tumor stage, and vascular invasion (Table 1) suggests that C5aR is involved in cancer progression. This concept is supported by the observation of C5aR expression on cancer cells invading adjacent vascular structures (Fig. 1f) and the high incidence of liver metastasis in gastric cancer patients with high tumoral C5aR expression (Table 2), which was positively associated with liver metastatic tumor size (Table 3). This is in accordance with the poor prognosis observed in gastric cancer patients with high tumoral C5aR expression (Fig. 2). To our knowledge, the present study is the first to demonstrate an association of tumoral C5aR expression with disease progression in gastric cancer patients.

The enhancing effect of C5a on cancer cell invasion and growth through C5aR has been shown in cholangiocarcinoma [13], nasopharyngeal carcinoma [27], renal cell carcinoma [28], non-small cell lung cancer [29], ovarian cancer [30], and breast cancer [31]. The C5a–C5aR axis promotes cancer cell invasion through various

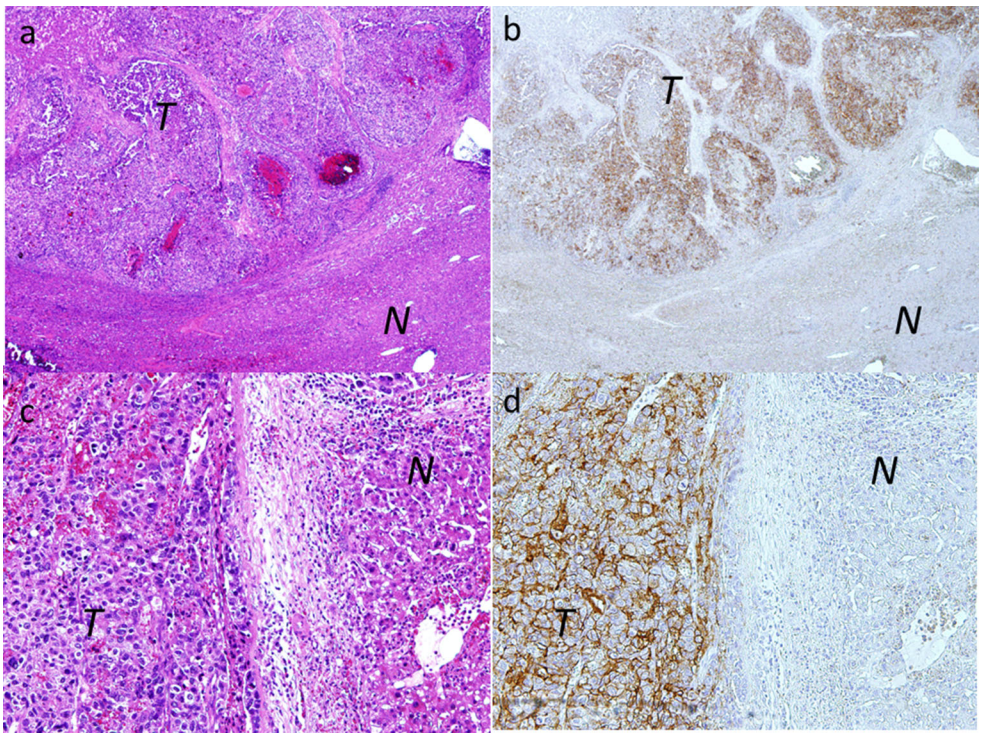

Fig. 3 Immunohistochemical staining of gastric cancer liver metastatic lesions using a mouse monoclonal antibody against human C5aR. **a**, **c** HE staining (**a** ×20; **c** ×100). **b**, **d** C5aR immunostaining (**b** ×20; **d** ×100). *T* tumor, *N* non-cancerous liver tissue

mechanisms, such as the release of matrix metalloproteases, activation of the ERK–PI3 K pathway, and down-regulation of E-cadherin [13, 28, 29]. The results of the present study demonstrating significant associations of high tumoral C5aR expression with cancer invasion depth, tumor stage, and vascular invasion (Table 1) corroborate the results of these previous studies. A higher incidence of metastasis in patients with C5aR-positive cancer than in those with C5aR-negative cancer has been reported for renal carcinoma [28], urothelial carcinoma [32], and breast cancer [31]. The observed association of high tumoral C5aR expression with liver metastasis (Table 2), together with vascular invasion (Table 1), suggests an involvement of C5aR in the blood-borne liver metastasis of gastric cancer. This is supported by the finding of significantly higher rates of vascular invasion in primary sites with high tumoral C5aR expression at liver metastatic sites (Table 3). Thus, the C5a–C5aR axis likely contributes to cancer progression, which is consistent with the observed unfavorable outcomes of gastric cancer patients with high tumoral C5aR expression (Fig. 2).

The strong association of the C5aR expression status in the primary site and liver metastatic site ($P = 0.0004$) suggested that almost all gastric cancer cells maintained their status of C5aR expression during and after metastasis. However, seven patients who had high C5aR expression in the primary site lost their C5aR expression in the liver metastatic site. On the other hand, eight patients with low C5aR expression in the primary site had high C5aR expression in the metastatic site. This difference in the status of C5aR expression might be explained by the effect of several cytokines and proteases; IFN-γ, IL-6, and urokinase-type plasminogen activator upregulated C5aR expression in various organs [24, 33–35]. Because these inflammation-related factors are produced from gastric cancer or microenvironment [36–38], the status of C5aR expression in cancer cells may be affected by these factors and upregulation of C5aR expression may promote cancer progression at the site.

The production of the C5a is essential for the stimulatory effects of C5aR on cancer cell invasion. C5a has been posited as a tumor microenvironment factor [14, 39]. Our previous study demonstrated that a cell membrane serine protease of cancer cells cleaves the precursor C5 to release C5a independent of complement activation [40]. Cho et al. [30] demonstrated C5a secretion by cervical cancer cells in an autocrine manner. C5a generated in the tumor microenvironment recruits myeloid-derived suppressor cells, leading to the suppression of antitumor $CD8^+$ T-cell-mediated responses, thereby enhancing tumor growth [14]. Moreover, C5a induces vascular endothelial growth factor expression, promoting angiogenesis [39, 41]. In addition to enhancing the direct invasion of cancer cells, C5a secretion into the tumor microenvironment may promote cancer cell

Table 3 Association of tumoral C5aR expression level at liver metastatic sites with clinicopathological parameters in patients who underwent gastrectomy and hepatectomy

Clinicopathological factors	C5aR expression in liver metastatic lesions		*P* value‡
	High ($n = 24$)	Low ($n = 34$)	
Gender			
Male	20	28	0.92
Female	4	6	
Age (years)[a]			
<71	17	11	0.004
≥71	7	23	
Location			
Upper	5	8	0.67
Middle	5	10	
Lower	14	16	
Differentiation			
Intestinal	17	22	0.66
Diffuse	7	11	
Other	0	1	
Metachronous/synchronous			
Metachronous	14	17	0.53
Synchronous	10	17	
Lymphatic invasion			
−	5	11	0.33
+	19	23	
Venous invasion			
−	1	8	0.04
+	23	26	
Number of lymph node metastasis			
<3	10	22	0.06
≥3	14	11	
Unknown	0	1	
Size of liver metastasis[a]			
<31 mm	7	22	0.01
≥31 mm	17	12	
Number of liver metastasis			
1	12	25	0.07
≥2	12	9	
CEA (ng/ml)[a]			
<5	11	11	0.58
≥5	9	16	
Unknown	4	7	
CA19-9 (U/ml)[a]			
<11.25	8	15	0.67
≥11.25	11	12	
Unknown	5	7	
C5aR expression status at the primary site			
High	16	7	0.0004
Low	8	27	

C5aR C5a receptor

‡ χ^2 test

[a] Median value

growth and invasion by indirect effects. Therefore, therapies targeting the C5a–C5aR axis using the anti-C5a antibody, C5aR antagonists, or inhibitors specific to the C5a-producing protease may be used in improving the prognosis of gastric cancer patients, including those with liver metastases.

In conclusion, we demonstrated tumoral C5aR expression in approximately 30 % of gastric cancer patients. C5aR expression was found to be associated with tumor invasiveness and poor prognosis. Further in vitro and in vivo studies are required to clarify the precise molecular functions of C5aR in gastric cancer for developing targeted therapies against the C5a–C5aR axis.

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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 substitute for it was obtained from all patients for being included in the study.

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