C5b-9 Staining Correlates With Clinical and Tumor Stage in Gastric Adenocarcinoma

Jian Chen, PhD,* Wei-jun Yang, MD,† Hai-jian Sun, BSc,* Xia Yang, PhD,* and Yu-zhang Wu, PhD*

Abstract: The complement system is a critical part of the immune response, acting in defense against viral infections, clearance of immune complexes, and maintenance of tissue homeostasis. Upregulated expression of the terminal complement complex, C5b-9, has been observed on various tumor cells, such as stomach carcinoma cells, and on cells in the necrotic regions of these tumors as well; however, whether and how C5b-9 is related to gastric cancer progression and severity remains unknown. In this study, human gastric adenocarcinoma (HGAC) tissues (n = 47 cases) and patient-matched adjacent nontumoral parenchyma (n = 20 cases) were evaluated by tissue microarray and immunohistochemistry. The HGAC tissues showed upregulated C5b-9 expression. Multinomial logistic regression and likelihood ratio testing showed that over-expression of C5b-9 in HGAC tissue was significantly correlated with clinical stage ($P = 0.007$) and tumor stage ($P = 0.005$), but not with tumor distant organ metastasis, lymphoid nodal status, sex, or age. Patients with late-stage gastric adenocarcinoma had a higher amount of tumor cells showing positive staining for C5b-9 than patients with early-stage disease. These results may help in diagnosis and assessment of disease severity of human gastric carcinoma.

Key Words: complement 5b-9, immunohistochemistry, tissue microarray, human gastric adenocarcinoma

Gastric carcinoma (GC) is one of the most prevalent cancer types in the world, with particularly high rates reported from countries in the Far East region, including Japan, Korea, and China.[1] The global rate indicates that risk of developing GC is high, reported as 1 in 115, and the survival rate is poor, with only 20% to 30% of cases surviving to the 5-year timepoint past diagnosis.[2] Al-

though radiotherapy and chemotherapy are well established and routinely applied treatments of GC, the median survival time is often < 1 year, due to the commonplace status of advanced metastatic disease.[3] As such, causal prophylaxis is a particularly attractive focus of research efforts aiming to improve the management of GC cases. As metastasis is more likely to occur in later stages of GC, early diagnosis is key to improving the efficiency and success rates of surgical removal. Unfortunately, early detection of GC remains a particular clinical challenge.[4] In our research efforts to identify useful diagnostic and/or prognostic biomarkers of GC, to aid in early detection, we turned our attention toward the terminal complement complex C5b-9, a component of the complement system, a major component of the innate immune response. The complement system is activated by endogenous ligands, such as apoptotic cells and tumor antigen that trigger proteolytic cleavage of complement components through the classical, lectin, and/or alternative pathways. Activation of the complement system leads to generation of the effector complement molecule C5, which is split into C5a and C5b by the C5 convertase enzyme; the C5b product then functions to recruit complement factors C6-C9 as the C5b-9 complex, also known as the membrane attack complex (MAC). The MAC is capable of forming a transmembrane pore in cells (including self-cells such as cancer cells and pathogen-infected cells) to induce cell death through Ca^{2+} (influx) overload.[5] This process has been extensively studied in the conditions of defense against viral infections,[6] clearance of immune complexes,[7–9] maintenance of tissue homeostasis,[9] and mediation of tumor-associated inflammatory signaling.[10,11]

Upregulated expression of C5b-9 has been noted on tumor cells in a multitude of various cancer types, such as cervical carcinomas[12,13] and ovarian cancers,[14-16] and the expression has been postulated as a reliable marker for complement activation in cancer conditions. Moreover, studies of cancers of the breast,[17] thyroid,[18] and colon,[19–21] have begun to elucidate the relationship between high C5b-9 expression levels and poor prognosis. C5b-9 expression was found to be localized mainly on the subepithelial and vascular basement membranes of thyroid carcinoma cells and shown to affect cell surface attachment to thyroid follicular cells,[18] suggesting a role in tumor cell detachment and metastasis. In addition, C5b-9 was found to act as a tumor promoter in a mouse model of colon cancer, with regulatory effects on multiple carcinogenesis-related

target genes and signaling pathways and the ability to induce the cell cycle upon activation of the phosphatidylinositol 3-kinase (PI3K)/Akt/FOXO1 and ERK1 pathways in a Gi protein-dependent manner.[5] To date, however, the mechanisms by which the complement system, in particular the C5b-9 complex, is involved in the development of the various forms of cancers or their outcomes remain to be fully elucidated.

The study described herein was designed to evaluate the immunoreactivity of C5b-9 in human GC to gain insights into the possible clinical significance of this factor as a diagnostic and/or prognostic biomarker. Surgically resected GC specimens and adjacent nontumoral parenchyma biopsies were examined by microarray and immunohistochemistry to determine the correlation of C5b-9 expression with pathologic characteristics of GC in a patient population.

MATERIALS AND METHODS

Case Selection

The study protocol was approved by the Institutional Ethics Board of Chongqing Cancer Hospital (Chongqing, China). All study participants provided written informed consent before enrollment and study participation.

A total of 47 patients diagnosed with gastric adenocarcinoma, who had not received neoadjuvant chemotherapy and were scheduled for surgical resection, were recruited from the Department of Gastroenterological Surgery (Chongqing Cancer Hospital) between 2010 and 2013. Surgical resection specimens of tumoral tissues [identified hereafter as HGAC (human gastric adenocarcinoma) tissues, n = 47 cases] and biopsies of tumor-adjacent nontumoral gastric mucosa (5 cm away from the tumor margin, n = 20 cases) were collected and used for clinical and experimental analyses. Samples of the specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin-eosin for pathologic analysis, including histologic subtyping and tumor-node metastasis staging (according to WHO classification).[22] The data were used to provide patient prognosis information and to guide treatment approach (treatment was independent of the study's objectives, design, or analysis).

Construction of Tissue Microarray

Core tissue biopsies (2 mm in diameter) were excised from the paraffin-embedded samples (hereafter referred to as donor blocks). After careful selection by evaluating the hematoxylin-eosin staining pattern, the donor blocks were arrayed precisely into a new paraffin block (hereafter referred to as tissue array blocks) using a precision instrument custom-built for this purpose (Beecher Instruments, Silver Spring, MD). Finally, the tissue array blocks were cut into 4-μm-thick sections and transferred to polylysine-coated slides (hereafter referred to as tissue microarrays).

Immunohistochemical Staining

The tissue microarrays, containing consecutive 4 μm paraffin-embedded sections, were deparaffinized, incubated with 3% H_2O_2 at room temperature for 15 minutes, washed with 0.01 MPBS, and incubated with the primary rabbit polyclonal C5b-9 antibody (dilution, 1:200; catalog number: ab55811; Abcam, Cambridge, UK) at 4°C for overnight. After 3 washes with PBS, the sections were coated with horseradish peroxidase–conjugated anti-rabbit secondary antibody (dilution, 1:200; catalog number: ZB2010; Zhongshan Golden Bridge Biotechnology, Beijing, China) and incubated at room temperature for 40 minutes. After 3 washes with PBS, the DAB substrate (Sigma, St Louis, MO) was added and allowed to react for 50 seconds at room temperature. All processed sections were counterstained with hematoxylin.

Imaging and Graphical Data Analysis

Images of the immunostained tissue microarrays (n = 3 for each case) were recorded with a DP70 digital camera (Leica, Wetzlar, Germany). The image analysis was carried out by an observer who worked in a double-blinded manner and calculated the average levels as follows. An area ratio was used to represent the relative positive area, as described in previous reports,[22–24] where < 5% positive area was scored as 0, 6% to 25% positive area was scored as 1, 26% to 50% positive area was scored as 2, 51% to 75% positive area was scored as 3, and > 75% positive area was scored as 4. The relative expression level was used to represent the immunostaining intensity of each sample, where light-brown staining in the positive area was scored as 1, brown staining in the positive area was scored as 2, and heavy-brown staining in the positive area was scored as 3. The final expression level was calculated as the sum of the area ratio and relative expression scores, where low-level expression was ≤2, moderate-level expression was 3 or 4, and high-level expression was ≥ 5. Statistical analysis was carried out using the multinomial logistic regression and likelihood ratio test using the SPSS analytical software, version13.0 (Chicago, IL).

RESULTS

Clinical Characteristics of HGAC

The 47 GC cases consisted of 18 females and 29 males, with the average age being 58 years old. There were 25 cases with poorly differentiated carcinomas, 11 with moderately differentiated carcinomas, and 11 with well-differentiated carcinomas. The lymph node status of the cases included 12 of N0, 18 of N1, and 17 of N2; no case was of N3 lymph nodal status. Only 3 of the cases showed distant organ metastasis.

C5b-9 Immunoreactivity in GC Tumors

The HGAC specimens showed remarkably higher levels of immunoreactivity for C5b-9 than the adjacent nontumoral parenchyma samples, and the immunopositivity was predominantly localized to the cytoplasm and the cytomembrane (Fig. 1). The HGAC specimens characterized as clinical stage IV exhibited the most robust C5b-9
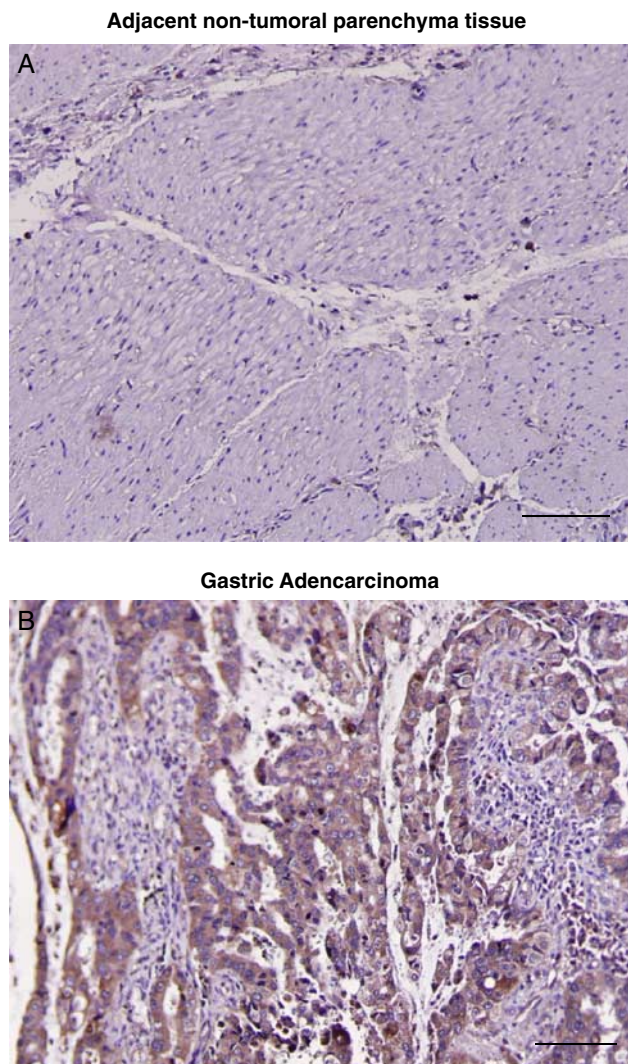
Adjacent non-tumoral parenchyma tissue

Gastric Adencarcinoma

FIGURE 1. Human gastric adenocarcinoma specimens show overexpression of C5b-9. Representative images of C5b-9 immunostained specimens from (A) tumor-adjacent non-tumoral parenchyma and (B) gastric adenocarcinoma. full color online

immunopositivity (Figs. 2J–L); the HGAC specimens of clinical stages III (Figs. 2G–I) and II (Figs. 2D–F) showed moderate levels of C5b-9 immunopositivity, whereas the HGAC specimens of stage I showed very little to no C5b-9 immunopositivity (Figs. 2A–C). When the immunopositivity of the 4 clinical stages were statistically analyzed, the difference between the serial hierarchy of clinical stage (IV > III > II > I) did not reach the threshold for significance; however, when the clinical stages were compared as groups of late-stage disease (IV + III) and early-stage disease (II + I), the immunopositivity was found to be significantly higher in the late-stage disease specimens.

Association of C5b-9 Expression With Clinicopathologic Characteristics of HGAC

Among the 47 HGAC specimens, 19 (40.4%) exhibited high levels of C5b-9 expression, 20 (42.6%)

exhibited moderate levels of C5b-9 expression, and 8 (17%) were little to no by IHC as compared with the levels detected in the adjacent nontumoral parenchyma samples. The total frequency of HGAC specimens showing C5b-9 overexpression was 83% (39/47). The C5b-9 expression levels were not significantly different among older and younger age groups (above 58 y old vs. 57 y old and below), males and females, lymph node status or distant organ metastasis. As shown in Table 1, multinomial logistic regression analysis and likelihood ratio testing showed that C5b-9 expression levels were significantly correlated with clinical stage ($P = 0.007$) and tumor stage ($P = 0.005$). As shown in Figure 3, the number of cells showing positive staining for C5b-9 was also significantly correlated with clinical stage and tumor stage, suggesting that C5b-9 may represent a useful biomarker for evaluating the progress and severity of HGAC.

DISCUSSION

To better understand the role of the complement system in GC, especially the contribution of C5b-9 to the disease's clinicopathologic characteristics, we conducted a tissue microarray–based analysis to investigate the tumor-related deposition of C5b-9 in a series of 47 cases. The expression level of C5b-9 was found to be higher in HGAC specimens than in adjacent nontumoral parenchyma, and this overexpression was found to be significantly associated with clinical stage and tumor stage. Specifically, the overexpression of C5b-9 was characterized as positively correlated with HGAC severity in late-stage patients, with no significant influence detected for HGAC in the early stages.

Among the 47 HGAC specimens examined in our novel approach of tissue microarray to detect tumor-related C5b-9 immunoreactivity, 83% showed overexpression of C5b-9 with higher levels of expression being observed in cases with later stages of disease. While the metastasis status was not correlated with C5b-9 expression, suggesting that this overexpression does not contribute to metastasis, it is possible that this complex plays a role in GC progression; however, the precise mechanisms by which C5b-9 may contribute to GC remain to be elucidated.

C5b-9 has been characterized previously as an antitumor molecule, demonstrated as capable of inhibiting tumor progression and inducing tumor cell lysis.[25,26] Previous studies using neoantigens and the tissue microarray approach to investigate the roles of C5b-9 in glioma,[27] lung adenocarcinoma,[28] medulloblastoma,[29] and stomach carcinoma[30] have demonstrated the presence of C5b-9 on tumor cells and in necrotic areas of tumors. The observed high levels of C5b-9 expression in cancer tissues have suggested that complement activation and the assembly of the terminal complement pathway may be ubiquitous in human tumors. However, none of the previous studies have investigated the relationship between C5b-9 and the severity of disease.
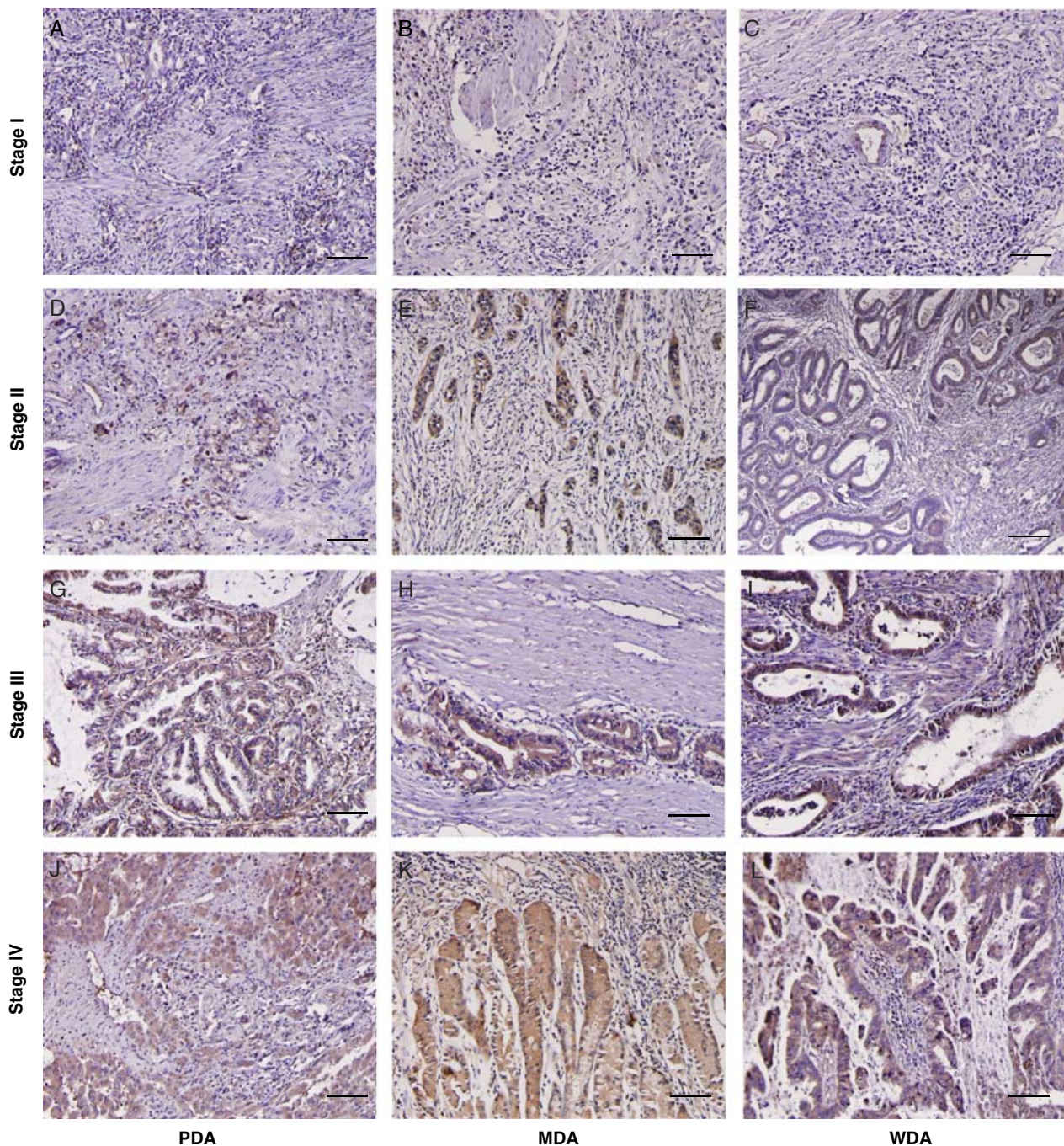
FIGURE 2. C5b-9 expression levels in human gastric adenocarcinoma specimens of various clinical stages. Representative images of C5b-9 immunostained GHAC specimens showing low expression in stage I (A–C) and stage II (D–F) cases but moderate expression (G–I) and high expression (J–L) in stage III and IV cases, respectively, regardless of differentiation. Bar = 100 μm. MDA indicates moderately differentiated adenocarcinom; PDA, poorly differentiated adenocarcinom; WDA, well differentiated adenocarcinom.

The complement system is also a contributing factor to autoimmune disease, and its deficiency has been implicated in the development and various manifestations of systemic lupus erythematosus, rheumatoid arthritis, and vasculitides.[31] In particular, studies of C5b-9 in autoimmune diseases have shown that formation of the MAC (C5b-9) can promote the pathogenic processes by directly inducing cell lysis. Interestingly, a previous study of complement system in hepatitis carried out in our laboratory using the mouse hepatitis virus 3–induced fulminant hepatitis model and human specimens from fulminant hepatitis cases showed high levels of activated C5 due to excessive C5b-9 in the diseased liver tissue,[32] indicating a pathogenic role for the C5b-9 complex in acute disease

TABLE 1. Statistical Relation of C5b-9 Expression With Clinicopathologic Features of HGAC

Features	C5b-9 Expression			Total	Significance
	High	Moderate	Low		
Adenocarcinoma					
Total	19	20	8	47	0.072
PDA	14	6	5	25	
MDA	2	7	2	11	
WDA	3	7	1	11	
Sex					0.114
Female	10	7	1	18	
Male	9	13	7	29	
Distant organ metastasis					0.452
Yes	2	1	0	3	
No	17	19	8	44	
Age (y)					0.095
Younger adult (≤57 y)	13	8	5	26	
Older adult (> 58 y)	6	12	3	21	
Tumor stage					**0.005**
T1	0	0	0	0	
T2	0	7	3	10	
T3	14	10	5	29	
T4	5	3	0	8	
Lymphoid nodal status					0.065
N0	1	7	4	12	
N1	10	6	2	18	
N2	8	7	2	17	
N3	0	0	0	0	
TNM clinical stage					**0.007**
I	0	4	3	7	
II	1	6	1	8	
III	12	7	4	23	
IV	6	3	0	9	

Multinomial logistic regression and likelihood ratio testing was performed. Bolded font indicates significant correlation ($P < 0.01$).

HGAC indicates human gastric adenocarcinoma; MDA, moderately differentiated adenocarcinom; PDA, poorly differentiated adenocarcinom; WDA, well differentiated adenocarcinom; TNM, tumor-node metastasis.
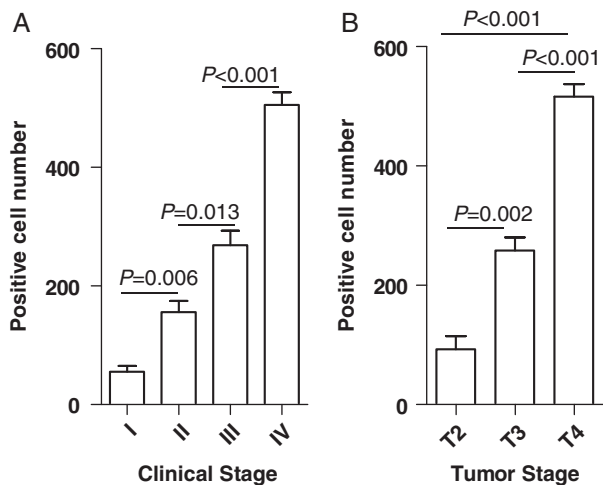

FIGURE 3. Number of C5b-9 immunopositive cells is significantly correlated with human gastric adenocarcinoma clinical stage (A) and tumor stage (B).

conditions. The mechanisms by which C5b-9 contributes to the pathogenic processes of autoimmune diseases may overlap with those of carcinomas, particularly considering the known roles of inflammation factors and signaling pathways in tumorigenesis and progression.

Although the data obtained from the research study described herein suggest a link between complement activation and gastric adenocarcinoma progression, the precise role of the complement cascade remains unclear. Certainly, the pathogenic mechanism will involve dynamic and complex interactions between inflammatory factors and carcinoma factors; this type of knowledge about the complement pathways in HGAC is expected to provide useful insights to support the development of novel therapeutic agents to defend against GC development and/or progression.

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