Increased numbers of IgG4-positive cells in tumors are associated with the progression of gastric cancer.

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Abstract

IgG4-related disease is a newly defined disease characterized by elevated serum IgG4 levels and infiltration of affected organs by IgG4-positive plasma cells. Recently, IgG4 was reported to be closely related with malignancies. To assess the relationship between IgG4 and the progression of gastric cancer, this study immunohistochemically assayed gastric cancer tissue samples for number of IgG4-positive cells using an anti-IgG4 antibody. In addition, pre- and postoperative serum concentrations of IgG4 were measured using an enzyme-linked immunosorbent assay. The number of CD138-positive plasma cells was significantly lower, while the number of IgG4-positive cells was significantly higher, in gastric tumor samples than in non-cancerous gastric mucosa. The number of IgG4-positive cells showed significant correlations with gross tumor appearance, tumor depth, lymph node metastasis, venous invasion, and lymphatic invasion. Prognosis was significantly poorer in patients with high numbers of IgG4-positive cells than in those with low numbers. Multivariate analysis indicated that both the number of IgG4-positive cells and the depth of tumor invasion were independently prognostic of survival in these patients. In conclusion, the number of IgG4-positive cells in gastric cancer tissue is closely associated with the progression of gastric cancer.

Keywords:

B cell, Gastric cancer, IgG4, prognosis

Introduction

Cancer immunotherapy is now receiving considerable attention because of the success of immune-checkpoint inhibitors, such as antibodies to programmed cell death protein 1 and cytotoxic T-lymphocyte antigen-4, in the treatment of various tumor types [1-3]. These successes have indicated that effective immunity to cancer cells can be induced even in cancer patients. Many cancer cells express tumor antigens, rendering them susceptible to recognition and lysis by T cells [4]. However, spontaneous rejection of established cancers is rare, because cancers frequently use physiological immunosuppressive mechanisms to escape from host immunity, a phenomenon known as “tumor immune evasion”.

Cancers have been reported to evade host immune systems via several mechanisms, including decreased expression of human leukocyte antigen [5], secretion of immunosuppressive cytokines including interleukin (IL)-10 and transforming growth factor-beta by cancer cells and cancer stromal cells [6], and up-regulation of immune checkpoint molecules such as programmed cell death-1 and T cell immunoglobulin and mucin domain-3 [7, 8]. Regulatory T cells (Treg), described as CD4+CD25+Foxp3+ cells and constituting around 10% of peripheral CD4+ T cells, are crucial for maintaining immune self-tolerance and homeostasis, and are involved in mechanisms of immune suppression [9, 10]. Treg cells utilize a wide range of suppressive mechanisms, including the secretion of immunosuppressive cytokines, such as IL-10 and transforming growth factor-beta, and the expression of cytotoxic T-lymphocyte antigen -4 [11-13]. Treg cell populations are increased in blood and tumor tissue of cancer patients and are closely correlated with the progression of various types of cancer via immunosuppression [14-16].

Recently, B cells as well as T cells were shown to be involved in tumor immune evasion. B cells respond to stimuli by differentiating, undergoing class switching, and producing antibodies of specific classes and subclasses [17]. Human B cells produce four subclasses of

immunoglobulin G (IgG), IgG1, IgG2, IgG3, and IgG4; these subclasses differ in biological function and the ability to activate immune system components [18, 19]. IgG4 is a minor immunoglobulin subtype, accounting for 3–6% of the total IgG circulating in normal subjects [20]. IgG4 shows relatively poor ability to bind complement components and Fc receptors and to activate effector cells. IgG4 has been associated with allergen-specific IgE responses in the physiopathology of allergic disorders [21].

IgG4 has recently attracted worldwide attention because of the identification of IgG4-related disease (IgG4-RD). IgG4-RD is characterized by elevated serum IgG4 levels, infiltration of affected organs by IgG4-positive plasma cells and a variable degree of fibrosis with a characteristic "storiform" pattern [22-24]. However, the relationship between IgG4 and malignancy remains largely unexplored, although IgG4+ cells were reported to infiltrate extrahepatic cholangiocarcinomas and pancreatic cancers [25]. Abnormal serum titers of IgG4 have been observed in patients with melanoma [26], and IgG4 was recently reported to significantly impair the potency of tumoricidal IgG1 in a human melanoma xenograft mouse model [17]. Serum IgG4 was shown to correlate inversely with survival in patients with melanoma [17]. Furthermore, IgG4 has been recently reported to be a predictor of risk for disease progression in melanoma [27]. Taken together, these findings suggest that IgG4 may be related to a previously unexplored aspect of tumor-induced immune escape.

Gastric cancer is one of the most common cancers in Asia and has the second highest mortality rate among all cancers worldwide [28]. Despite the expression of tumor rejection antigens such as melanoma antigens 1–3 [29] and the presence of tumor-specific cytotoxic T cells [30], the immune system fails to mount immune responses against gastric carcinoma cells, similar to findings in other cancers. However, the mechanisms by which gastric cancer cells overcome antitumor immunological responses are poorly understood. *Helicobacter pylori* infection has been reported associated with both the pathogenesis of

gastric cancer and that of the IgG4-RD autoimmune pancreatitis [31-33]. Moreover, assessment of 108 Japanese patients with IgG4-RD pancreatitis identified 18 cancers in 15 patients, with gastric cancer being the most common tumor type [34]. With regard to the correlation between IgG4-RD and malignancy, Asano also reported a close association between IgG4-RD and malignancy formation within 12 years after diagnosis, particularly during the first year with gastric cancer being the second common tumor type [35]. These findings indicate that IgG4 antibodies may be closely associated with gastric cancer. This study therefore evaluated the presence of IgG4-positive cells in tumor tissue and the relationship between IgG4-positive cells and gastric cancer progression.

Materials and methods

Patients and normal donors

This study enrolled 195 patients pathologically diagnosed with gastric adenocarcinoma and treated at Tottori University Hospital. None of these patients received radiotherapy, chemotherapy, or other medical interventions before surgery. Of these 195 patients, 131 (96 males and 35 females) were assessed immunohistochemically and 137 (96 males and 41 females, of mean age 65.1 ± 11.4 years) were evaluated by enzyme-linked immunosorbent assay (ELISA). Healthy controls for ELISA included 26 age-matched subjects (17 males and 9 females, of mean age 67.2 ± 10.3 years). All subjects provided informed consent for blood and tissue donations. Clinicopathological findings were generally determined according to the 14th edition of the Japanese Classification of Gastric Carcinoma [36].

Immunohistochemistry

Tissue samples were fixed in formalin and embedded in paraffin. Serial sections were cut at 4 μm, dewaxed, deparaffinized in xylene, and rehydrated through a graded alcohol series. For retrieval of IgG4, the tissue sections were incubated with Histofine protease based solution (Nichirei) for 7 min; for retrieval of CD138, the sections were boiled for 15 min in a microwave oven in citrate buffer (pH 9.0). The samples were incubated in 3 % hydrogen peroxidase for 10 min to block endogenous peroxidases, and in Block Ace (DS pharma Biomedical) for 20 min to prevent nonspecific antigen binding. The slides were subsequently incubated with the primary antibodies, mouse monoclonal anti-IgG4 (Nichirei, Japan) and mouse monoclonal anti- CD138 (Nichirei), for 1 h at room temperature. Secondary antibody binding was detected with Histofine MAX-PO (Nichirei), and the sections developed with Histofine DAB Solution (Nichirei) and counterstained with Mayer's hematoxylin.

Each slide was dehydrated through a graded alcohol series and covered with a coverslip. The presence of cells positive for IgG4 and CD138 on each slide was determined in a blinded manner. Cells in parts of each tumor where IgG4- and CD138-positive cells are observed most frequently were counted in a ×400 high-power field.

Immunofluorescence staining

Immunofluorescence double staining for IgG4 together with CD138 was performed on 2μm thick sections of snap-frozen tissue. After cutting, the sections were air-dried for 30 min and fixed in 4% formaldehyde for 15 min. They were rinsed three times for 5 min in phosphatebuffered saline (PBS). The incubation with mouse monoclonal anti-IgG4 (Nichirei) for 1 h at room temperature followed by incubation with rabbit polyclonal anti-CD138 (Bioss antibodies, MA, USA, working dilution 1:100) for overnight at 4℃ was preceded. IgG4 and

CD138 were detected indirectly, using antibodies mentioned above, followed by incubation for 90 min in the dark with Goat anti-Mouse IgG conjugated with Alexa Fluor 488 and Goat anti-Rabbit IgG conjugated with Alexa Fluor 594 (Thermo fisher scientific, MA, USA, working dilution 1:200). These slides were then rinsed three times for 5 min in PBS. All slides were mounted with ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (Thermo fisher scientific) and examined using fluorescence microscopy (BX53, Olympus, Tokyo, Japan).

Measurement of serum IgG4

Serum samples were collected from the patients prior to and 1 month after operation. Concentrations of IgG4 were measured by ELISA using a Human IgG4 Platinum ELISA kit (eBioscience, CA, USA).

Statistical analysis

Between group differences were analyzed by paired *t* tests or Mann–Whitney *U* tests. DSS was calculated according to the Kaplan–Meier method and compared using the log-rank test. Patients who died of causes other than gastric cancer were considered lost to follow up at the time of death. The Youden index was calculated using the receiver operating characteristic (ROC) analysis to determine an optimal cutoff value for the number of IgG4-positive cells for survival analysis. Multivariate analysis of factors prognostic of DSS was performed using a Cox's proportional hazards model and a stepwise procedure. Statistical significance was defined as $p < 0.05$. Statistical analyses were performed with GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and Stat View 5.0 for Windows (SAS Institute, Cary, NC, USA) software.

Results

Plasma cells and IgG4-positive cells in non-cancerous gastric mucosa and gastric cancer

We first determined the numbers of plasma cells, defined as CD138-positive cells, and IgG4-positive cells in the tissue of non-cancerous gastric mucosa and gastric cancer. The numbers of plasma cells were significantly lower (Fig. 1), while the number of IgG4-positive cells was significantly higher (Fig. 2), in gastric cancer tissue than in the non-cancerous gastric mucosa. Immunohistochemistry of serial sections and immunofluorescence staining indicated that IgG4-positive cells in gastric cancers were also CD138-positive, indicating that most IgG4-positive cells were plasma cells (Fig. 3a, b, c).

In assessing the correlations between cell numbers and clinicopathological variables, we found that the number of CD138-positive cells in tumor tissue was positively correlated with differentiation ($p = 0.005$). In contrast, the number of IgG4-positive cells in gastric cancers was correlated with gross tumor appearance ($p = 0.0099$), tumor depth ($p < 0.0001$), lymph node metastasis ($p = 0.0006$), venous invasion ($p = 0.0018$), and lymphatic invasion ($p = 0.0002$) (Table 1).

Prognostic significance of the number of IgG4-positive cells in gastric cancer tissue

We next investigated the prognostic significance of the number of IgG4-positive cells in tumor tissue. Of the 131 patients assessed for this variable, 128 underwent R0 resection and were included in further analysis. ROC analysis found that 10 cells per high-power field (HPF) had the highest Youden index (sensitivity + specificity – 1) and was an optimal cutoff value for the number of IgG4-positive cells in gastric cancers (AUC 0.718, 95% CI 0.608-0.829) (Fig. 4). Using this cutoff, the 128 patients were classified into two groups, with 71 (55.5%) having <10 (low) and 57 (44.5%) having ≥10 (high) IgG4-positive cells per HPF. The 5-year DSS rates of these two groups differed significantly (90.3% vs.

43.4%, $p = 0.0003$) (Fig. 5a). Using a Cox proportional hazards model and a stepwise procedure, we found that a high number of IgG4 positive cells was independently prognostic of poorer survival, as was greater depth of tumor invasion (Table 2). Since the depth of invasion was also an independent prognostic indicator by multivariate analysis, we have analyzed the prognosis of gastric cancer patients based on the number of IgG4 positive cell and the depth of invasion. The 5-year survival rate of patients who had advanced gastric cancer with high IgG4 cells, either advanced gastric cancer with low IgG4 cells or early gastric cancer with high IgG4 cells, and early gastric cancer with low IgG4 cells were 35.3%, 79%, and 100%, respectively and the difference was statistically significant ($p<0.0001$).

Serum concentrations of IgG4 in gastric cancer patients and healthy controls

We also compared pre- and post-operative serum IgG4 concentrations in gastric cancer patients with IgG4 concentrations in healthy controls. Preoperative serum IgG4 concentrations were significantly lower in advanced gastric cancer patients ($n = 71$) than in healthy controls ($n = 26$) (65.7 ± 62.2 vs. 90.2 ± 63.9 mg/dL, $p = 0.0453$) (Fig. 6). In contrast, preoperative serum IgG4 concentrations were similar in patients with early gastric cancer ($n = 66$) and healthy controls (n = 26) (90.1 ± 86.8 vs. 90.2 ± 63.9 mg/dL, $p = 0.57$). In patients with gastric cancer, the mean serum IgG4 concentration measured was significantly higher 1 month after surgery than preoperatively (112.5 ± 93.0 vs. 88.5 ± 82.7 mg/dL, $p < 0.0001$) (Fig. 7).

Discussion

This study has shown that IgG4-positive cells are abundant in gastric cancer tissue samples, although the number of plasma cells expressing CD138 was lower in gastric cancer tissue than in non-cancerous gastric mucosa. Immunohistochemistry and

immunofluorescence staining showed that IgG4-positive cells in gastric cancer tissue were also CD138-positive, indicating that most IgG4-positive cells were plasma cells. Furthermore, the number of IgG4-positive cells was closely related to the progression of gastric cancer, as shown by depth of tumor invasion and the presence of lymph node metastasis, lymphatic vessel invasion and blood vessel invasion. These results indicate that tumor-associated plasma cells were polarized to produce IgG4 and that the number of these cells was closely correlated with gastric cancer progression. In fact, our results indicated that the prognosis of gastric cancer patients with high IgG4 cells was significantly worse than those with low IgG4 cells. Furthermore, an addition of the depth of invasion to the number of IgG4 cells made it possible to predict the prognosis of gastric cancer patients more accurately.

The reasons for the abundance of IgG4-positive cells in gastric cancer tissue remain unclear. In this regard, we think that there are two possible explanations. One is that tumor microenvironment induce IgG4 positive cells in the tissue of gastric cancer. Another is that tumor microenvironment attract IgG4 positive cells to the gastric cancer cells from other places. With regard to the induction of IgG4 positive cells in the tissue of gastric cancer, IL-10, a regulatory cytokine produced mainly by Foxp3+ regulatory T cells (Treg cells), T helper 2 cells, and IL-10-producing Treg cells, has been reported to induce the differentiation of IgG4-positive plasma cells or, in the presence of IL-4, to favor B cell switching to IgG4 [37, 38]. Marked infiltration of IgG4-positive cells was recently reported in several cholangiocarcinomas, with the presence of these cells closely associated with the IL-10-predominant regulatory cytokine milieu generated by the tumors cells, both directly and indirectly [25]. We previously showed that gastric cancer cells express IL-10 and that serum IL-10 concentrations tended to be higher in gastric cancer patients than in normal controls [39, 40]. Furthermore, the numbers of Treg cells, the main source of IL-10, were reported increased in the blood and tumor tissue of gastric cancer patients [41]. Therefore,

IL-10 expression is likely associated with the increase in IgG4-positive cells in gastric cancer tissue observed in the current study.

Alternatively, cancer cells or gastric stromal cells may attract IgG4-positive plasma cells. Interactions between the chemokine CCL1 and the chemokine receptor CCR8 may be involved in the recruitment of IgG4-positive cells in patients with IgG4-related type 1 autoimmune pancreatitis and primary sclerosing cholangitis [42]. In this regard, we have preliminary data determined by immunohistochemistry indicates that gastric cancer cells express CCL1 (data not shown). The current study showed that the preoperative serum concentration of IgG4 was significantly lower in patients with advanced gastric cancer than in healthy controls. Moreover, serum IgG4 concentrations were significantly higher in gastric cancer patients after than before surgery, with the postoperative concentration almost equivalent to that in normal controls. These results support the hypothesis that gastric cancer and/or gastric stromal cells attract IgG4-positive cells from the peripheral blood to cancer site. Further investigations are needed to clarify the mechanisms underlying the abundance of IgG4-positive cells in gastric cancer tissue.

The function of IgG4 in tumor tissue is poorly understood. Because IL-10 plays primary roles in both suppressing immune responses and generating IgG4-positive plasma cells, the IgG4 reactions observed in the current study may reflect evasion of immune surveillance. An engineered tumor antigen-specific IgG4 was found to be ineffective in triggering effector cell-mediated tumor killing in vitro [17]. Furthermore, IgG4 significantly impaired the potency of tumoricidal IgG1 in a human melanoma xenograft mouse model [17]. The current study found that an increased number of IgG4-positive cells in gastric cancer tissue was significantly associated with poor prognosis in patients with gastric cancer. Moreover, multivariate analysis showed both the number of IgG4-positive cells in tumor tissue and the depth of tumor invasion were independently prognostic of DSS. Therefore, the

accumulation in gastric cancers of IgG4-positive cells may inhibit immune reactions against tumor cells, resulting in tumor progression. It was therefore of interest that the number of IgG4-positive cells was significantly higher in Borrmann 3 and 4 than in other gastric cancers. These cancers, especially Borrmann 4 gastric cancers, are characterized by rapid cancer cell infiltration and proliferation, accompanied by extensive stromal fibrosis. The increased proliferation of stromal fibroblasts within scirrhous gastric carcinoma lesions and peritoneal metastatic sites has suggested that stromal fibroblasts support the progression of this disease [43]. IgG4-RD has also been characterized by a variable degree of fibrosis [22-24]. Therefore, IgG4-positive cells in gastric cancer tissue may be involved in increased fibrosis, resulting in poorer prognosis. In fact, the number of IgG4-positive cells tended to be higher in scirrhous and intermediate type tumor than in medullary type tumor in the current study.

The close relationship between the number of IgG4-positive cells and poor patient prognosis suggests that IgG4-positive cells may be targets for the treatment of gastric cancer. Moreover, serum concentrations of IgG4 may be useful in predicting tumor recurrence and patient prognosis. Additional studies are needed to determine those possibilities.

To our knowledge, this study is the first to show that the numbers of IgG4-positive cells were increased in gastric cancer tissue and were associated with poorer prognosis in gastric cancer patients. Increases in the number of IgG4-positive cells may be one of the main mechanisms responsible for the progression of gastric cancer. Understanding the details of these mechanisms may lead to novel treatment approaches.

Conflict of interest statement: We declare that we have no conflict of interest.

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Table 1. Relationships between the numbers of CD138- and IgG4-positive cells and clinicopathological variables in patients with gastric cancer

Variables	CD138+ cells	P value	IgG4+ cells	P value
Age (years)		0.4987		0.197
<65	90.0±54.6		11.2±13.9	
≥65	82.8±47.8		14.0±15.0	
Gender		0.7511		0.459
Male	84.7±50.4		12.8±15.0	
Female	88.0±51.5		13.3±13.7	
Gross appearance		0.8628		0.0099
Borrmann 3–4	80.3±39.3		18.6±16.4	
Others	86.6±52.5		11.8±14.1	
Depth of invasion		0.7709		<0.0001
T1 (early)	87.4±53.5		8.6±12.3	
T2/3/4 (advanced)	84.0±48.1		16.5±15.5	
Lymph node metastasis		0.4474		0.0006
Absent	88.7±53.0		10.1±13.8	
Present	81.4±47.1		16.7±15.0	
Distant metastasis		0.1870		0.928
Absent	84.9±50.8		13.0±14.7	
Present	115.0±22.5		9.3±6.4	
Histology		0.0005		0.683
Differentiated	71.6±48.0		13.6±15.7	
Poorly differentiated	99.3±49.4		12.3±13.6	
Lymphatic involvement		0.4320		0.0018
Absent	79.3±47.1		8.0±11.6	
Present	88.3±51.9		15.1±15.3	
Vascular involvement		0.9711		0.0002
Absent	84.0±47.2		8.4±11.5	
Present	86.6±52.9		16.0±15.7	
Cancer stromal volume [a]		0.4492		0.0821
Med	81.0±55.7		9.0±11.0	
Int / Sci	86.9±49.1		14.1±15.4	

All results expressed as mean ± SD

[a] Cancer stromal volume: Medullary type (med), Scanty stroma; Scirrhous type (sci), Abundant stroma; Intermediate type (int), The quantity of stroma is intermediate between the two above types.

Table 2. Univariate and multivariate analyses of factors prognostic of disease-specific survival in patients with gastric cancer

Variables	Univariate analysis			Multivariate analysis		
	P value	HR [c]	95% CI [d]	P value	HR	95% CI
Age (years) (<65 vs ≥65)	0.4145	0.702	0.299-1.644			
Gender (Male vs Female)	0.0523	3.376	0.988-11.538			
Depth of invasion [a] (T1 vs T2/3/4)	0.0032	20.310	2.745-150.249	0.0104	14.237	1.869-108.478
Lymph node metastasis (Absent vs Present)	0.0002	6.268	2.350-16.716			
Histology [b] (Differentiated vs Poorly differentiated)	0.8207	1.096	0.495-2.427			
Lymphatic involvement (Absent vs Present)	0.0005	2.214	1.415-3.463			
Venous involvement (Absent vs Present)	0.0002	2.066	1.407-3.034			
IgG4-positive cells (<10 vs ≥10/HPF)	0.001	0.212	0.084-0.534	0.0468	0.385	0.150-0.987

[a] Depth of invasion: T1, tumor invasion of the lamina propria or submucosa; T2, tumor invasion of the muscularis propria or subserosa; T3, tumor penetration of the serosa; T4, tumor invasion of adjacent organs
[b] Histology: Differentiated, papillary or tubular adenocarcinoma; undifferentiated, poorly differentiated or mucinous adenocarcinoma, or signet ring cell carcinoma
[c] HR, hazard ratio
[d] CI, confidence interval

Figure legends

Figure 1. Numbers of CD138-positive cells in gastric cancer tissue and non-cancerous gastric mucosa. The number of CD138-positive cells was significantly lower in gastric cancers than in non-cancerous gastric mucosa ($p = 0.0233$).

Figure 2. Numbers of IgG4-positive cells in gastric cancer tissue and non-cancerous gastric mucosa. The number of IgG4-positive cells was significantly higher in gastric cancers than in non-cancerous gastric mucosa ($p < 0.0001$).

Figure 3. Immunohistochemical analysis of the expression of IgG4 and CD138 in serial sections of (a) non-cancerous gastric mucosa and (b) gastric cancer tissue.
(c) Immunofluorescence staining of IgG4 and CD138 in the gastric cancer tissue

Figure 4. Receiver-operating characteristic (ROC) curve for showing the optimal prognostic cutoff for the number of IgG4-positive cells per high power field.

Figure 5. (a) Kaplan–Meier analysis of the effects of number of IgG4-positive cells per high power field (HPF) on disease-specific survival (DSS) in patients with gastric cancer. The 5-year DSS rate was significantly lower in patients with ≥10 (high) than <10 (low) IgG4-positive cells/HPF (43.5% vs. 90.3%, $p = 0.0003$). (b) Kaplan–Meier analysis according to the combination of the number of IgG4-positive cells and the depth of invasion. The 5-year survival rate of patients who had advanced gastric cancer with high IgG4 cells, either advanced gastric cancer with low IgG4 cells or early gastric cancer with high IgG4

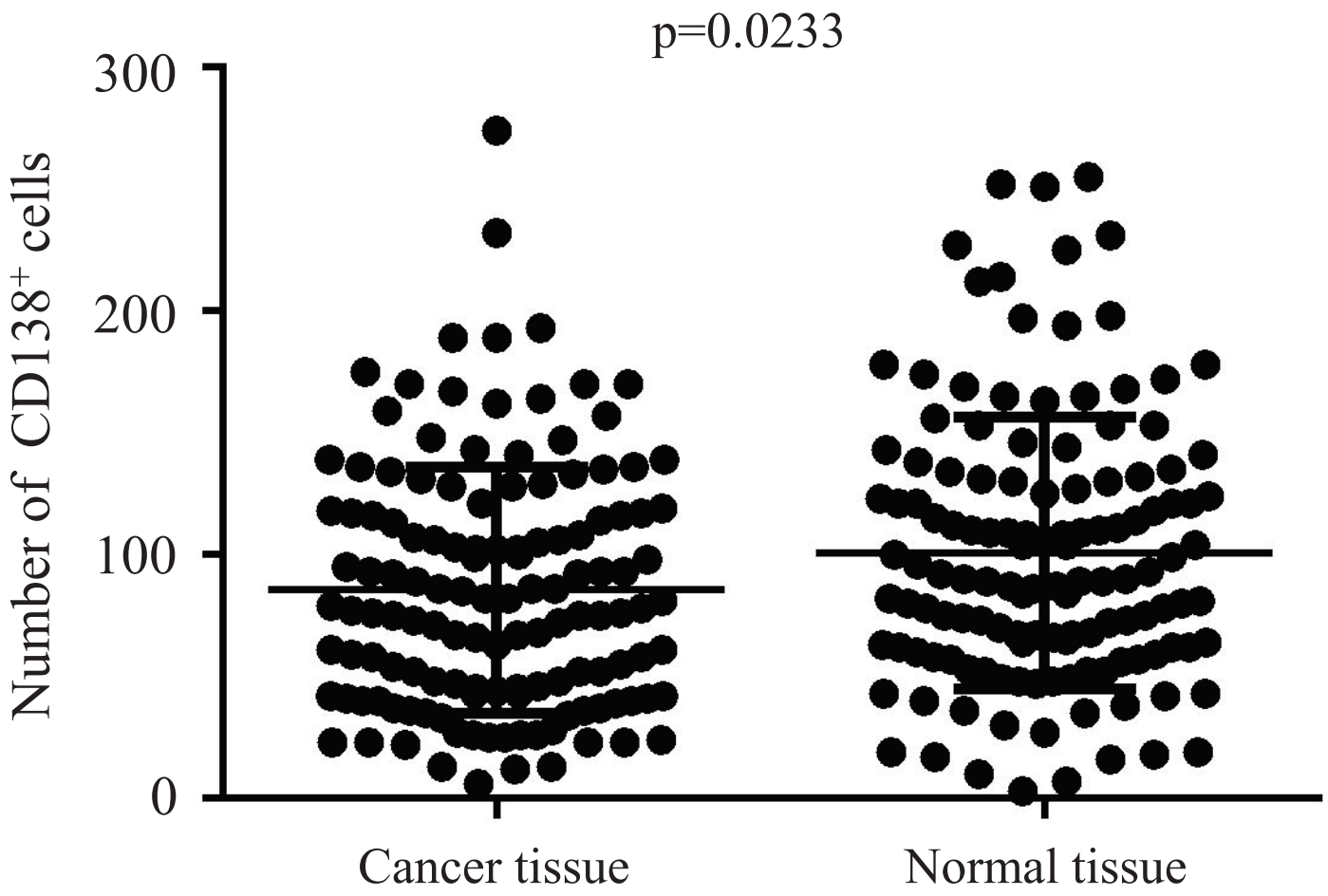
cells, and early gastric cancer with low IgG4 cells were 35.3%, 79%, and 100%, respectively and the difference was statistically significant ($p<0.0001$).

Figure 6. Preoperative serum concentrations of IgG4 in patients with early and advanced gastric cancer and healthy controls. The preoperative serum IgG4 concentrations in these three groups were 90.1 ± 86.8, 65.7 ± 62.2, and 90.2 ± 63.9 mg/dL, respectively, with the difference between advanced gastric cancer patients and healthy controls being statistically significant ($p = 0.0453$).

Figure 7. Comparison of pre- and postoperative serum IgG4 concentrations in gastric cancer patients. The mean IgG4 concentration was significantly higher postoperatively than preoperatively (112.5 ± 92.5 vs. 88.5 ± 82.3 mg/dL, $p < 0.0001$).

Figure

Miyatani et al. Figure 1



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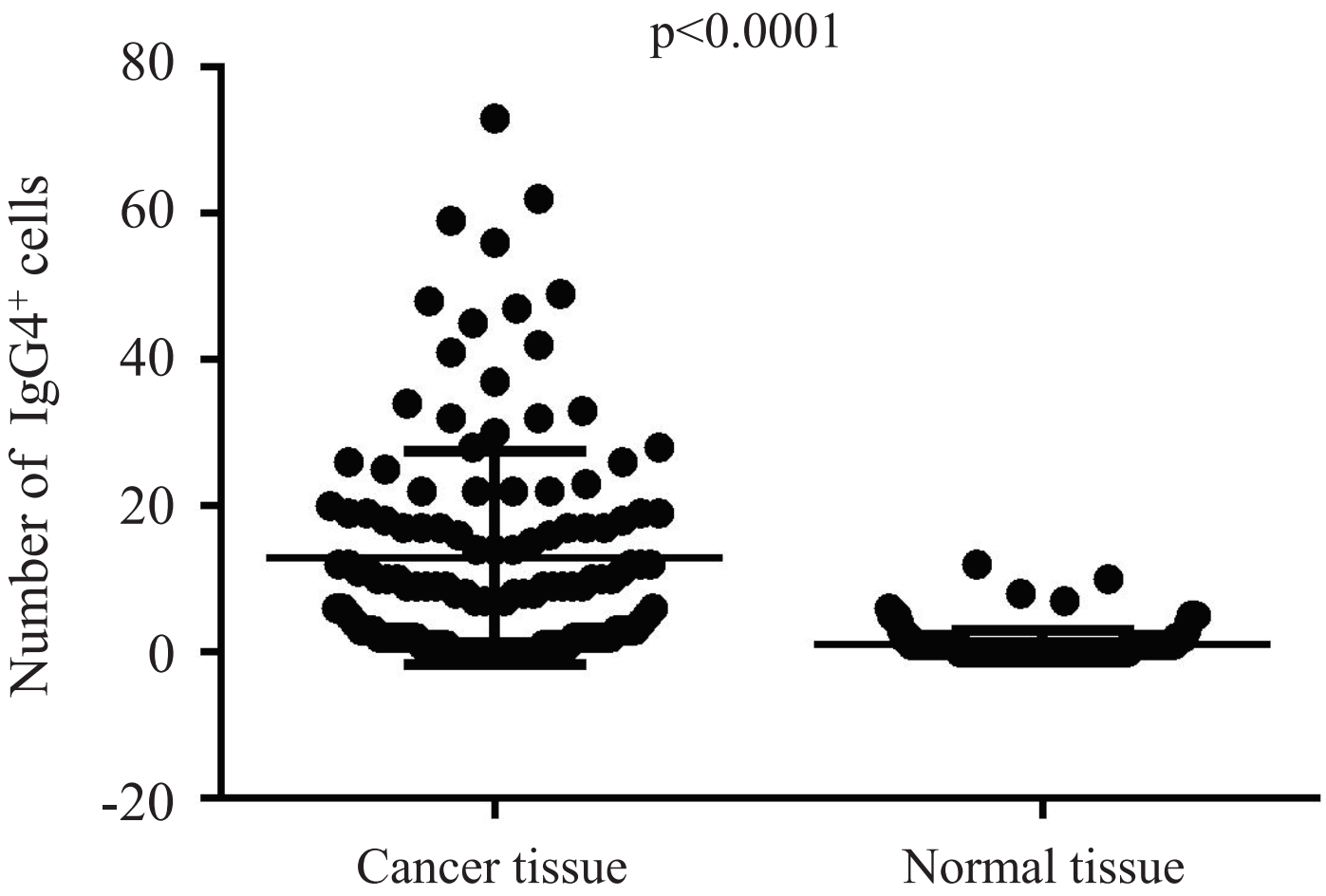

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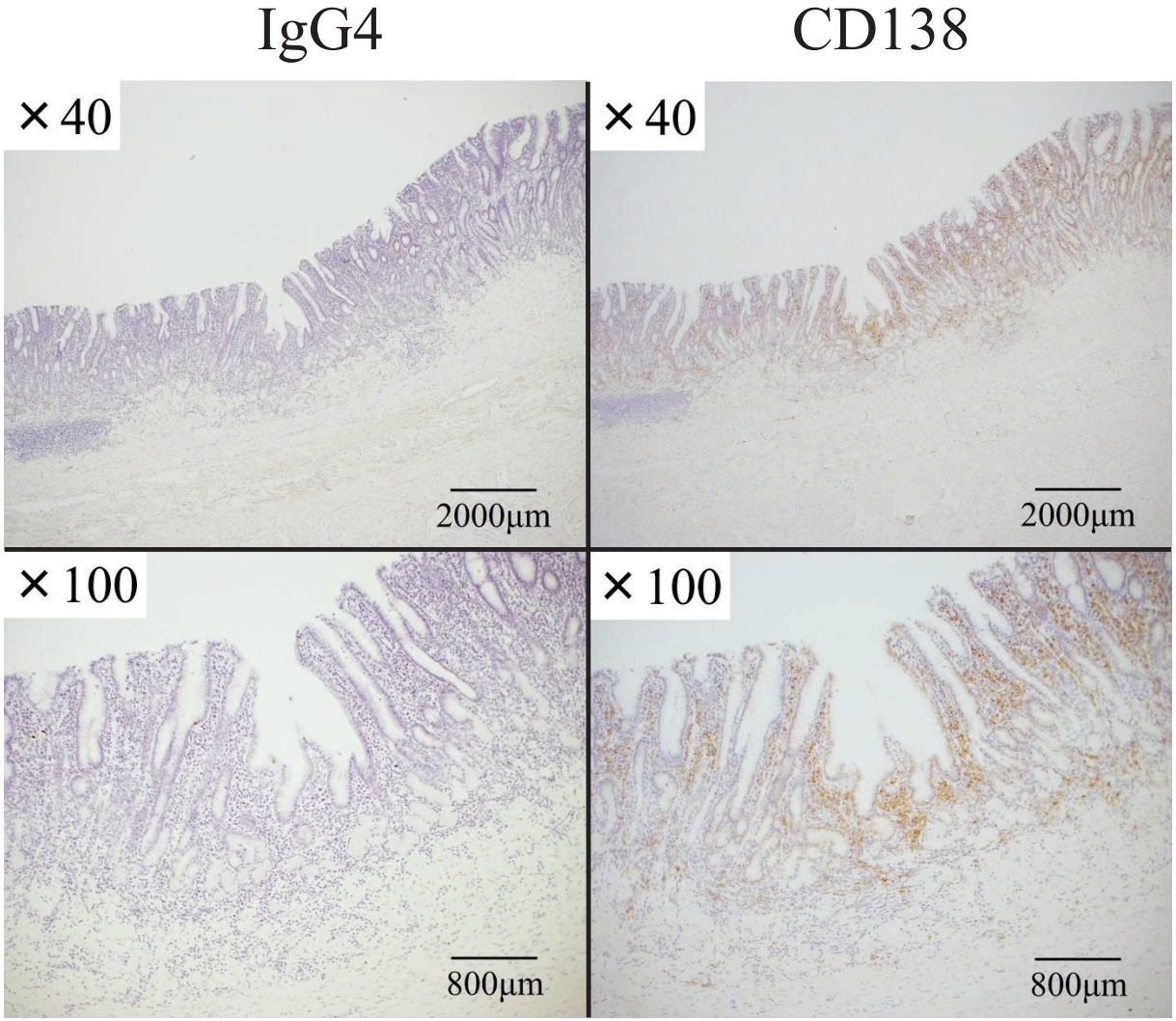

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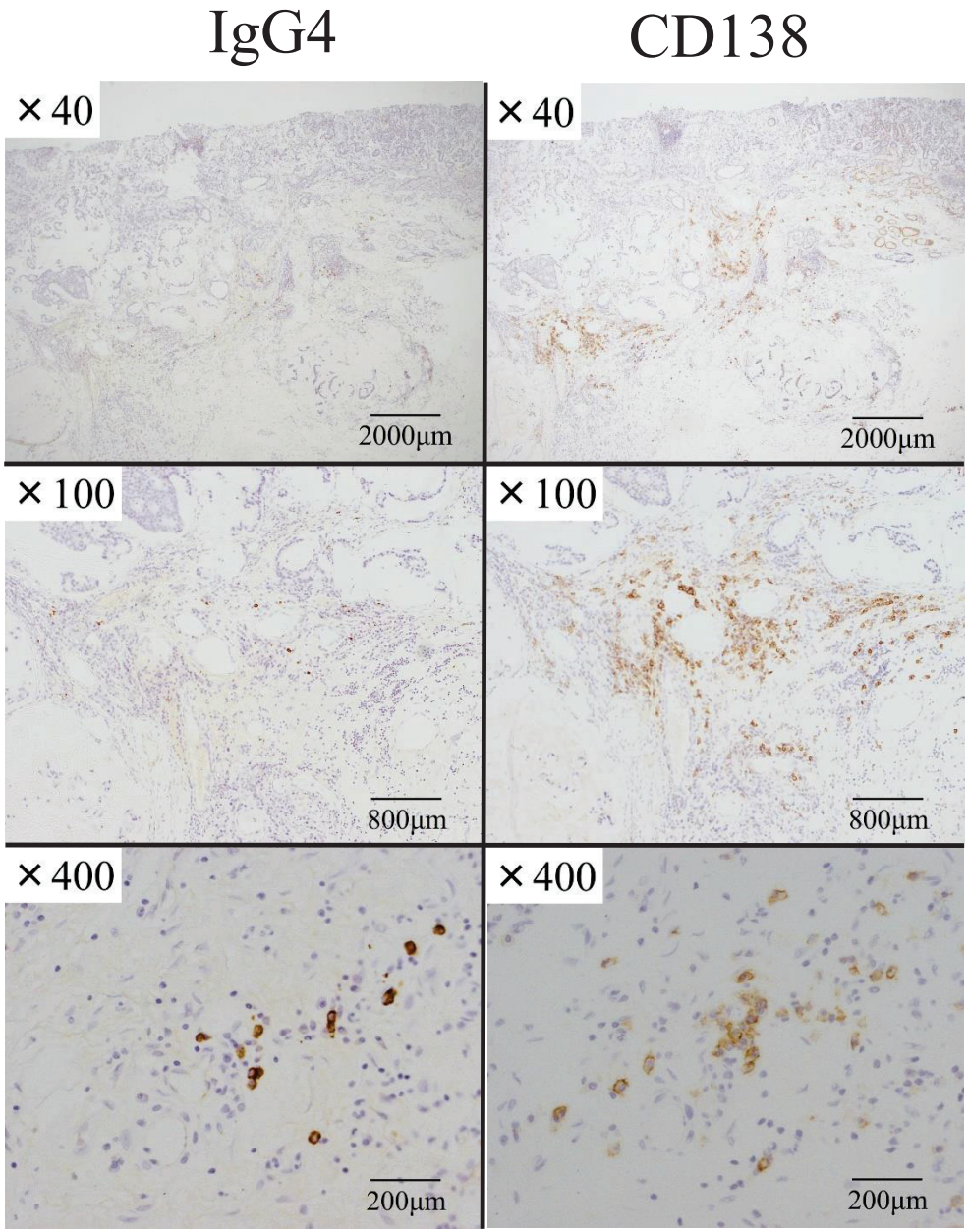
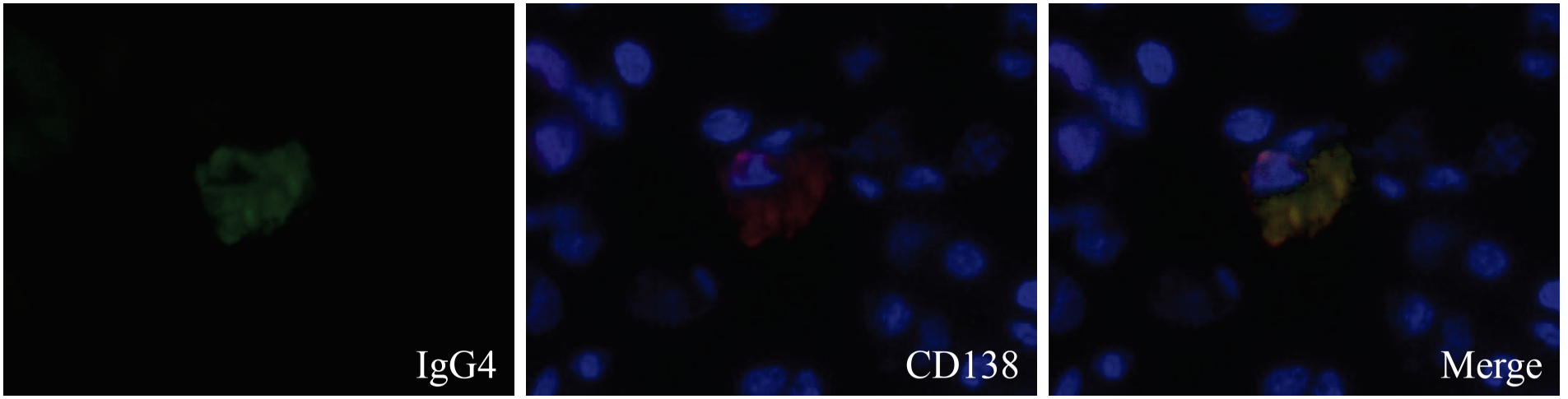


Miyatani et al. Figure 3c



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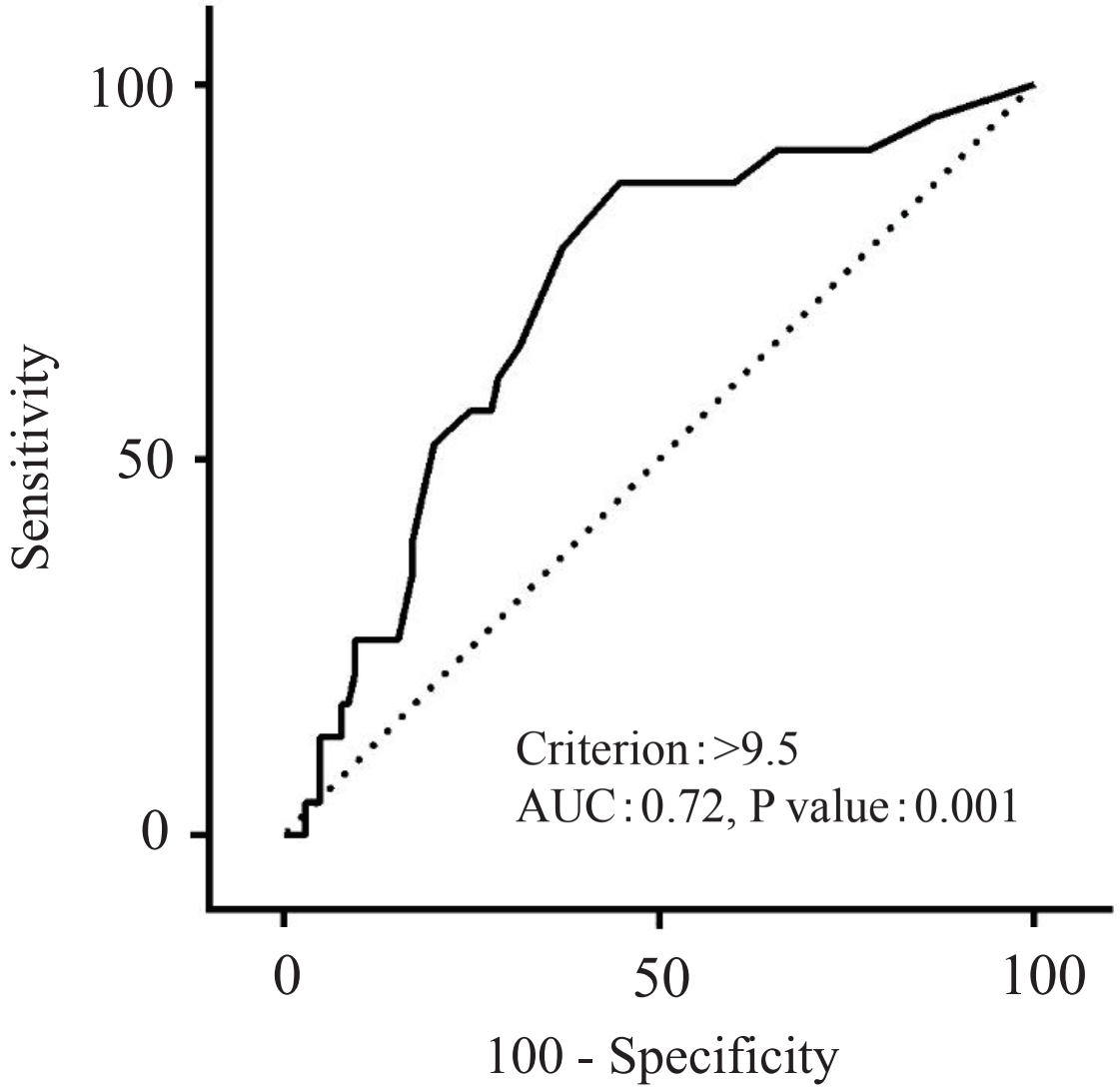

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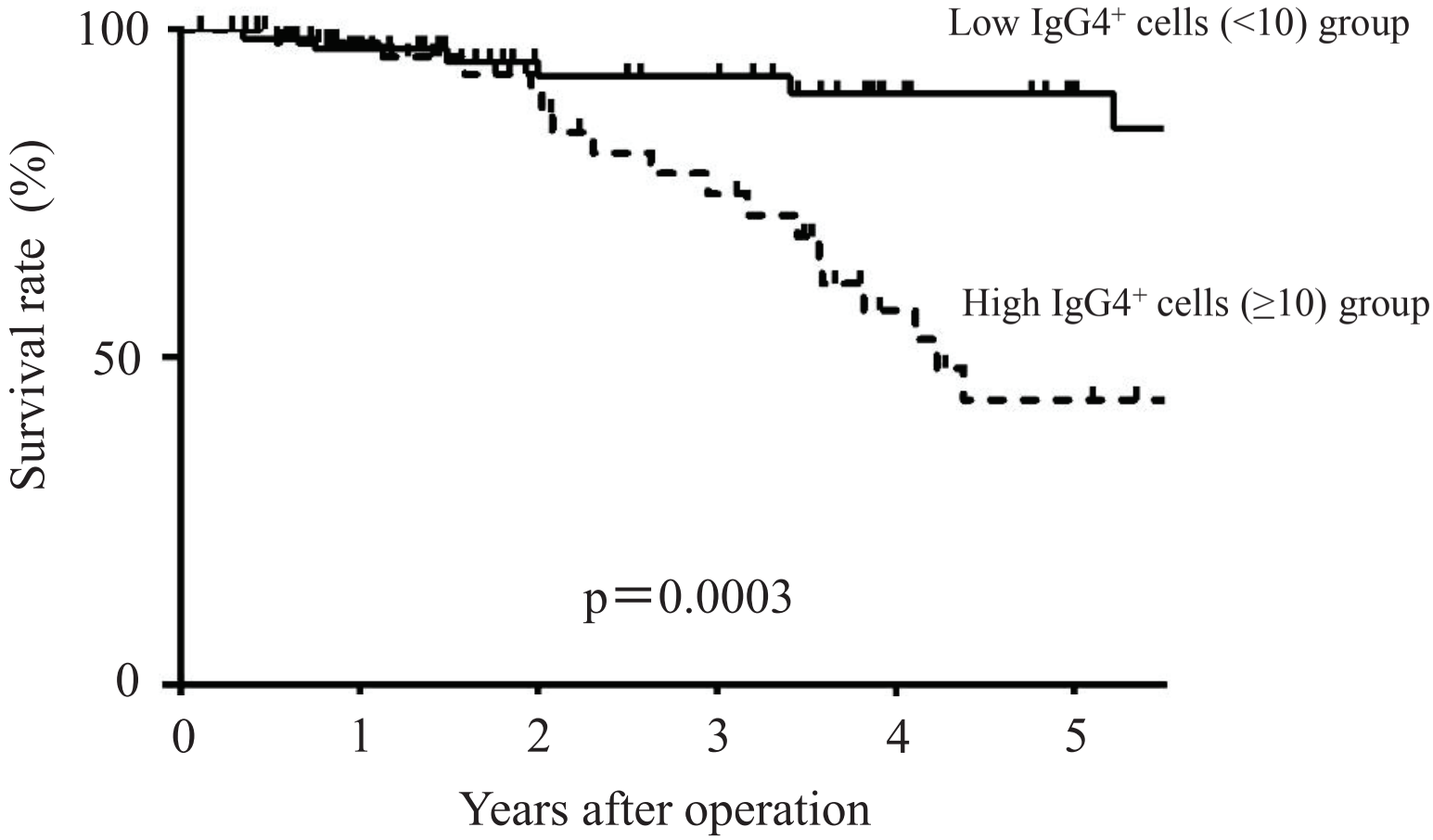

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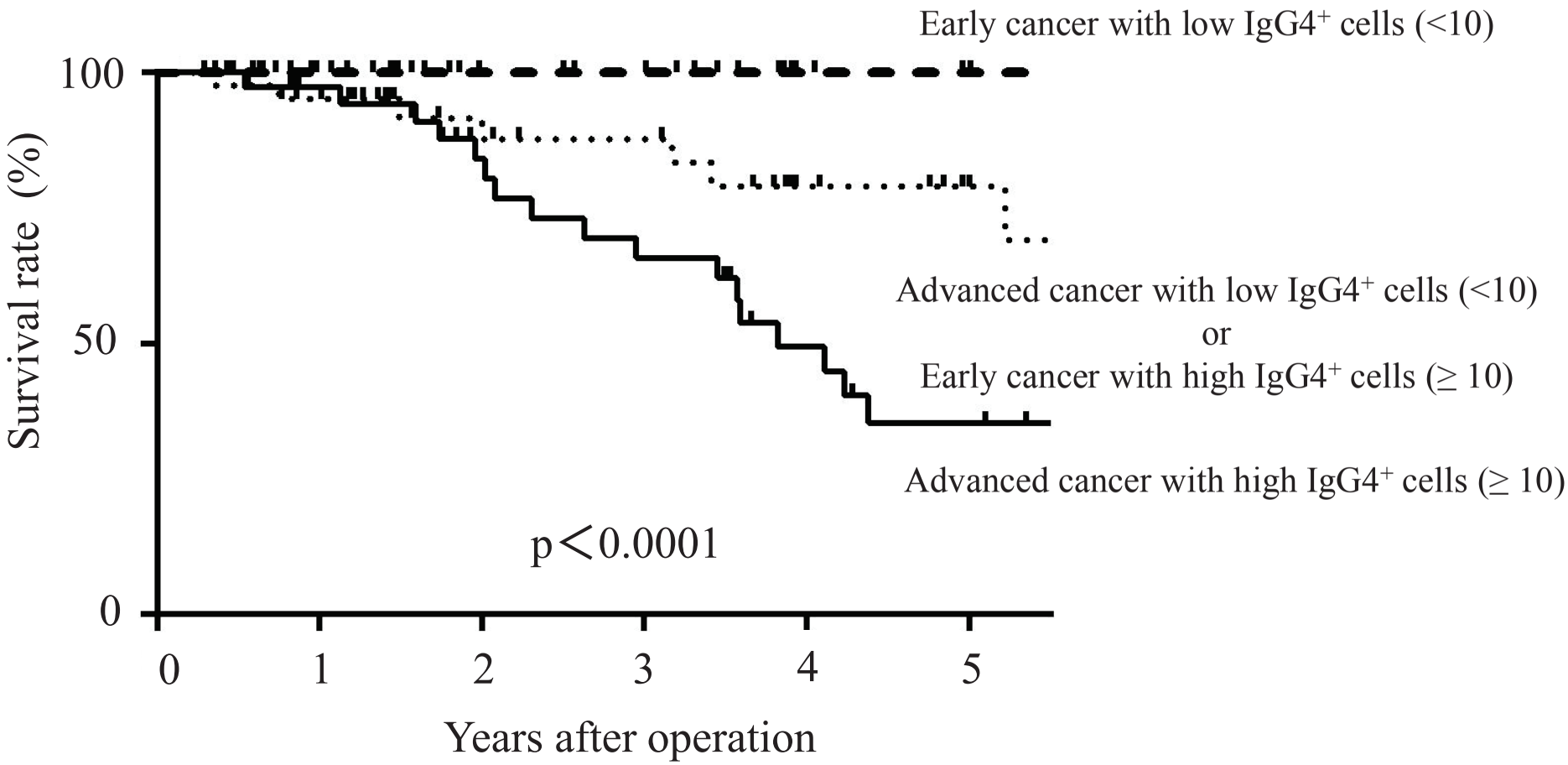
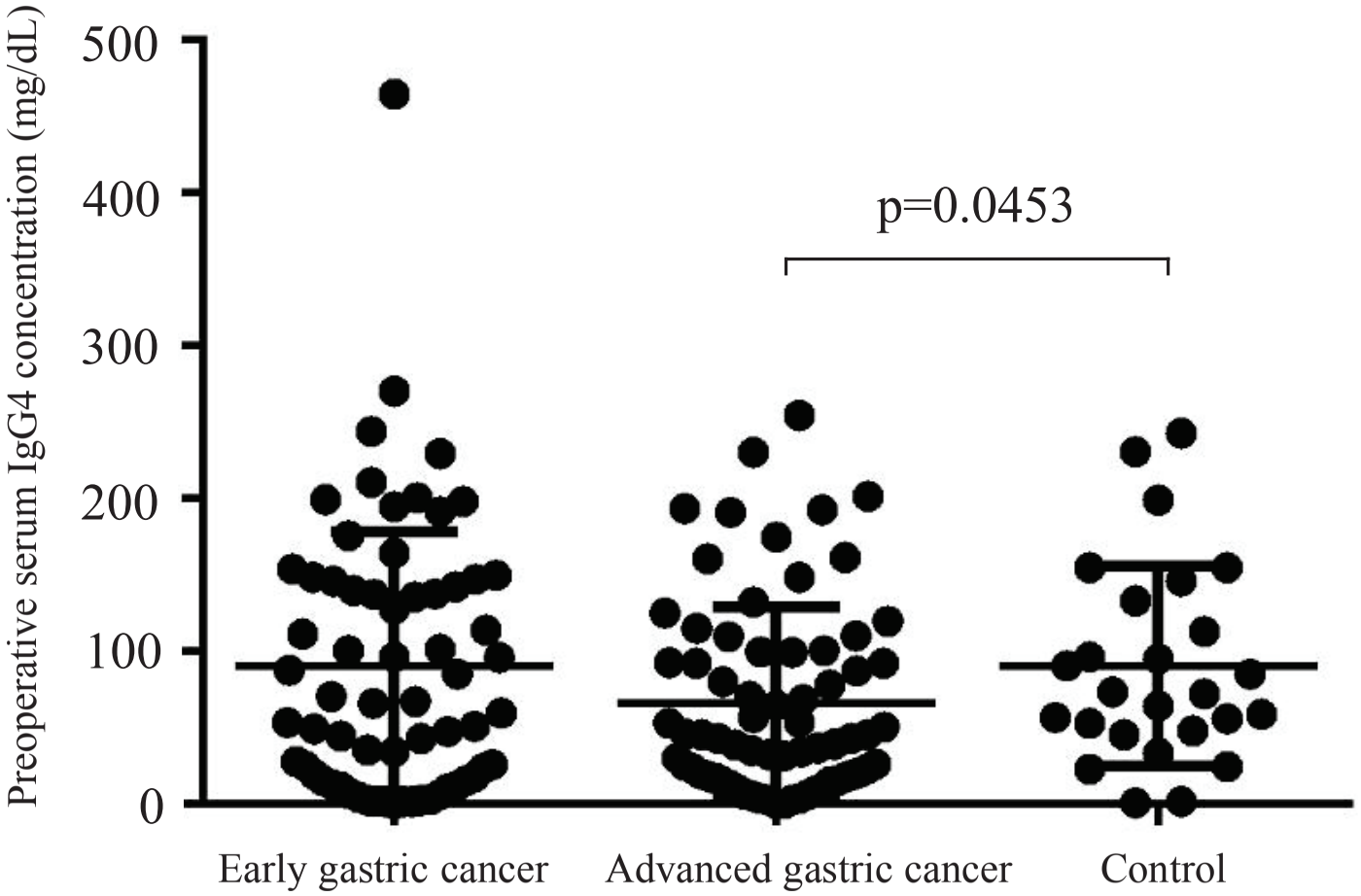


Miyatani et al. Figure 6



Miyatani et al. Figure 7