Pathological significance of IgG4-positive plasma cells in extrahepatic cholangiocarcinoma

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ABSTRACT

IgG4-related sclerosing cholangitis is histologically characterized by the infiltration of IgG4-positive plasma cells and sclerosing change. Moreover, several cases of carcinoma cases accompanied by IgG4-positive cells in tissue and increased serum IgG4 levels have been reported, but the association between cancer-associated immunity and an IgG4 reaction is still unclear. In this study, we examined the infiltration of IgG4-positive cells in extrahepatic cholangiocarcinoma and the pathological significance of the IgG4 reaction found in cancer tissues in terms of the evasion of immune surveillance by Treg cells. Immunohistochemistry for IgG4, Foxp3, CD4, and CD8 was performed using 68 surgical specimens from patients with extrahepatic cholangiocarcinoma and positive cells were investigated, particularly within and around cancerous tissues. Consequently, although IgG4+ cells were few (average, <10 cells/ high power field) in the majority of cases, ≥10 and ≥50 cells were found in 37% and 6% of cases, respectively. IgG4+ cells were predominantly found in the invasive front of carcinoma tissue. In these cases accompanying ≥10 IgG4+ cells, Foxp3+ Treg cells were also distinguishable and a positive correlation was found between the Foxp3+/CD4+ ratio and IgG4+ cell count, but few CD8+ cells invaded cancer cells (<10 cells). In conclusion, extrahepatic cholangiocarcinomas are often accompanid by the significant infiltration of IgG4+ cells and the IgG4 reaction showed a positive and negative correlation with Foxp3+ and CD8+ cells, respectively, suggesting the evasion of immune surveillance associated with CD8+ cytotoxic T cells via the regulatory function of Foxp3+ Treg cells.

INTRODUCTION

IgG4 is a minor immunoglobulin subtype composing 3-6% of all the IgG circulating in adults [1], but important for the formation of IgG4-related diseases that feature elevated serum IgG4 levels and abundant infiltration with IgG4-positive plasma cells in affected organs [1-3]. IgG4-related diseases incorporate various IgG4-associated inflammatory disorders including autoimmune pancreatitis (AIP), sclerosing cholangititis, sialoadenitis, retroperitoneal fibrosis, inflammatory abdominal aortic aneurism, intestinal pneumonia, interstitial nephritis, lymphadenopathy, and inflammatory pseudotumor [3-11]. Recently, cases of IgG4-related diseases were accumulated according to the widespread recognition of this disease entity and clinicopathological characterization for a differential diagnosis has been clarified. However, the pathological significance of increased serum IgG4 levels and marked infiltration of IgG4-positive plasma cells in target organs is still unknown.

IgG4-related diseases have varied clinical symptoms that may include features similar to malignant tumors. Because most IgG4-related diseases involving AIP are resolved by corticosteroid treatment, the diagnosis, particularly differentiation from malignant tumors, is very important [12-14]. Although using an upper normal limit for serum IgG4 of 135 mg/dL, Hamano et al [1], reported a diagnostic 95% sensitivity and 97% specificity (vs pancreatic cancer) for AIP, pathological examination is necessary to differentiate IgG4-related diseases from tumors in any organs. Moreover, several investigators have recently reported on patients with pancreatic cancer accompanying elevated serum IgG4 levels and some cases are speculated to arise from AIP [15-17]. Raina et al. [18] reported that as many as 7% of patients with pancreatic cancer have serum IgG4 levels above 135mg/dL and concluded that in patients with pancreatic mass

lesions and suspicion of cancer, an IgG4 level measuring between 135 and 200mg/dL should be interpreted cautiously and not accepted as diagnostic of AIP without further evaluation. Some kind of association between tumor immunity and IgG4 reaction has been assumed, but detailed information is not available.

As the pathogenesis of the IgG4 reaction in IgG4-related diseases, the participation of CD4+CD25+FOXP3+ regulatory T cells (Treg) and Th2-type helper T cells has been speculated [19]. Treg cells play a role in the progression of various malignant tumors, particularly in controlling the immune response against pancreatic ductal carcinoma from the premalignant stage to established cancer [20]. A high prevalence of Treg cells, moreover, seems to indicate a poor prognosis [20].

In this study, we retrospectively evaluated IgG4-positive plasma cells in extrahepatic cholangiocarcinomas including common bile duct cancers, gallbladder cancers, and cancers of the papilla of Vater and investigated the significance of the IgG4 reaction in cholangiocarcinoma from the point of view of tumor immune escape mediated by Treg cells.

MATERIALS and METHODS

Patients and tissue preparations: Formalin-fixed and paraffin-embedded sections of 68 surgically resected specimens from 39 patients with gallbladder cancers, 21 patients with common bile duct cancers, and 8 patients with cancer of the papilla of Vater (Average age 74 y.o, male/female=38/30) were obtained from the registry of liver diseases in the Department of Pathology, Kanazawa University School of Medicine. In 30 cases, follow-up data was also obtained in the present study. Each cholangiocarcinoma was classified histologically as well- (including papillary),

moderately, or poorly differentiated, based on the predominant histologic grade. Special histological types such as adenosquamous carcinoma and mucinous carcinoma were not included in the present study. Four micrometer-thick serial sections were prepared from each formalin-fixed, paraffin-embedded block. Each one was stained with Hematoxylin and eosin (H&E), and the others were used for immunohistochemistry.

Immunohistochemistry: The deparaffinized and rehydrated sections were microwaved in EDTA buffer for IgG4 and Foxp3, in buffer at pH 9 for CD4 and in citrate buffer for CD8 for 20 min in a microwave oven. Following the blocking of endogenous peroxidase, these sections were incubated at 4°C overnight with antibodies against IgG4 (mouse monoclonal; diluted 1:200; Southern Biotech, Birmingham, AL, USA), Foxp3 (mouse monoclonal; 10 μg/ml; Abcam, Cambridge, UK) , CD4 (mouse monoclonal; neat, Nichirei, Tokyo, Japan) or CD8 (mouse monoclonal; diluted 1:20, Dako, Tokyo, Japan), and then at room temperature for 1hr with anti-mouse immunoglobulins conjugated to a peroxidase-labeled dextran polymer (Simple Staining Kit; Nichirei). After a benzidine reaction, sections were counterstained lightly with hematoxylin. No positive staining was obtained when the primary monoclonal antibody was replaced with an isotype-matched, non-immunized immunoglobulin as a negative control of the staining procedures.

Histological examination: In addition to histological observation by H&E staining, the distribution of the immuno-positive cells was examined. In a primary survey, we examined all tumorous area in each specimen and, for counting IgG4+, Foxp3+, CD4+, or CD8+ mononuclear cells, selected three representative areas containing IgG4+ plasma cells, and expressed results as the mean number of each immuno-positive cell in high-power fields (HPFs). For semi-quantitative evaluation of

the IgG4 staining, the cases with ≥10 and □ 10 IgG4+ cells/HPF on average were evaluated as IgG4-positive and -negative cases, respectively. The ratio of Foxp3+ to CD4+ cells was calculated for the three selected area in each case and the average ratio (Foxp3/CD4) was compared between IgG4-rich and -poor cases, because the absolute number of Foxp3+ cells was prominently affected by the degree of infiltrating mononuclear cells. Moreover, the average number of CD8+ cytotoxic T cells (CTLs) within carcinoma cells was evaluated to estimate the degree of host immune response against cancer.

Statistical Analysis: Data were analyzed using tSpeason's correlation coefficient test. Survival curves to evaluate the association between prognosis and IgG4 reactions were calculated by the Kaplan-Meier method, and analyses were conducted with the log-rank test. A p-value of less than .05 was considered to be statistically significant.

RESULTS

Detection and distribution of IgG4+ cells in extrahepatic cholangiocarcinoma: Immunohistochemistry revealed IgG4+ plasma cells to be scattered within and around cancerous nests in most cases (Fig.1). Particularly, around nests and in the invasive area facing a non-cancerous biliary wall and surrounding fibroadipose tissue, these positive cells were prominent with some intermingling of other inflammatory cells (Fig.2). Moreover, one characteristic feature of IgG4-related diseases, the perineural infiltration of IgG4+ cells, was commonly seen in extrahepatic cholangiocarcinomas. In contrast, desmoplastic change and vascular invasion by cancer cells are usually seen in extrahepatic cholangiocarcinomas, but other features of

IgG4-related diseases, obliterative phlebitis caused by IgG4+ cells and storiform-type fibrosis, are rare.

A quantitative evaluation of IgG4+ cells revealed that 25 (37%), 19 (28%), and 4 (6%) of 68 cholangiocarcinoma patients had ≥10, ≥20, and ≥50 IgG4+ cells/HPF, respectively. There was no correlation between the density of IgG4+ cells and any clinicopathological factor including age, gender, anatomical location (common bile ducts, gallbladder, and the papilla of Vater), or the histological differentiation (well-, moderately, and poorly) of extrahepatic cholangiocarcinomas.

Association between IgG4 reaction and Foxp3+ Treg cells: The association between IgG4+ and Foxp3+ cells was evaluated in each case. Foxp3+ Treg cells were scattered in most cases with a marked IgG4 reaction (Fig.3). The relation between the IgG4 reaction and Foxp3+ Treg cells is shown in Fig.4. In IgG4-rich cases (≥10 IgG4+ cells/HPF), the ratio of Foxp3+/CD4+ cells correlated closely with the IgG4+ cell count, though it is varied in IgG4-poor cases (□ 10 IgG4+ cells/HPF).

Association between IgG4 reaction and CD8+ CTLs: CD8+ CTLs were scattered at various intensities in each case irrespective of within and around cancer nests. As a marker of immune activity against cancers, CTLs invaded cancerous nests resembling IELs, which are found in non-neoplastic biliary epithelial layers of biliary diseases such as primary biliary cirrhosis (Fig.5)[21] Consequently, patients with marked CD8+ CTLs showed scant IgG4 reaction (IgG4-poor cases) and all IgG4-rich cases had few CD8+ CTLs (□ 10 cells/HPF) (Fig.6)

Association between IgG4 reaction and patient's survival: After the surgical resection of extrahepatic cholangiocarcinomas, of the 30 patients with available outcome data, 21 died from recurrence of cancers. By the Kaplan-Meier estimator, the

overall survival curve for the 30 patients is shown in Fig.7 The patients with □20 IgG4+ cells/HPF had a better prognosis than those with ≥20 cells ($p<0.05$).

DISCUSSION

Elevated serum IgG4 levels and the infiltration of organs by numerous IgG4+ plasma cells are clinicopathological hallmarks of IgG4-related diseases. Moreover, obliterative phlebitis, storiform-type sclerosing fibrosis, and sometimes mass forming-type, are also characteristics of this disease category. It is clinically and pathologically important to differentiate IgG4-related diseases and tumors of affected organs. In particular, because desmoplastic change is a common feature of biliary and pancreatic cancers, IgG4-related diseases and cancers in these organs show similar radiological behaviors. Moreover, patients with pancreatic adenocarcinoma accompanying an IgG4 reaction and/or elevated serum IgG4 levels [16, 18, 22, 23] and with pancreatic and biliary cancers arising from IgG4-related disease [16, 24, 25] have been reported, though a cause-and-effect relationship between the IgG4 reaction and cancers has not been demonstrated. Therefore, the presence of IgG4+ cells is not a histological hallmark of IgG4-related diseases and the IgG4 reaction is speculated to occur non-specifically in carcinoma tissues. In this study, we retrospectively examined IgG4 reactions in cases of extrahepatic cholangiocarcinoma including common bile duct cancers, gallbladder cancer, and cancers of the papilla of Vater. Consequently, 10 or more IgG4+ cells/HPF were observed in 37% of cases and the cases with marked infiltration (≥50 IgG4-positive cells/HPF) and resembling IgG4-related diseases made up 6% of the total. For the pathological diagnostic criteria of IgG4-related disease, the essential number of IgG4+ cells varied from 5 to 50/HPF depending on the affected

organs, but in IgG4-related sclerosing cholangitis, ≥10 IgG4+ cells/HPF is proposed according to the HISORt criteria published for AIP [26, 27]. In our cases, therefore, several cancers accompanying remarkable IgG4-positive cells (≥50 cells/HPF) originated from the preceding IgG4-related disease, but follow-up data prior to discovery of cancers is needed to demonstrate this. Irrespective of the patients with cholangiocarcinoma and a marked IgG4 reaction and those arising from IgG4-related sclerosing cholangitis, the presence of adenocarcinoma should be taken into account in the pathological diagnosis of IgG4-related cholangitis, particularly using small specimens such as biopsy materials.

The present study demonstrated that a IgG4 reaction is often found to some degree in extrahepatic cholangiocarcinoma as well as IgG4-related diseases. Moreover, the perineural infiltration of IgG4+ cells which is a feature of IgG4-related cholangitis and AIP, was prominent in IgG4-rich cholangiocarcinoma cases. In contrast, patients with IgG4-related cholangitis are generally older men (85%) [26], but this male domination was not found in the IgG4 reaction of cholangiocarcinoma. Obliterative phlebitis and storiform fibrosis are also characteristic of IgG4-related diseases, but rare in cholangiocarcinoma. These histological features of IgG4-related diseases differing from cholangiocarcinoma are not located at the superficial biliary mucosa, unfortunately suggesting that these characteristic findings are not useful for a differential diagnosis using biopsy specimens. The IgG4 reaction is not a specific immunereaction of IgG4-related diseases, but the immunopathogenesis of IgG4 reactions should be different in IgG4-related diseases and cancers. Further study is necessary to clarify the histogenesis of IgG4 reactions.

IgG4 does not have the ability to activate complement and its physiological and

pathological significance are still unknown in healthy and IgG4-related diseased patients. As the pathogenesis of the IgG4 reaction in IgG4-related sclerosing cholangitis and pancreatitis, the participation of the Th2-type cytokine milieu and IL-10 produced by Treg cells is assumed to involve IgG4 class switching and/or the progressive proliferation/differentiation of IgG4+ plasma cells [10, 19, 28-30]. In the carcinogenesis of pancreatic cancer, the prevalence of Treg cells increases and that of cytotoxic CD8+ cells conversely diminishes in cancer tissues [20]. Moreover, the prevalence of Treg cells is negatively correlated with the prognosis of patients with pancreatic cancers [20]. These findings suggest that Treg cells play a role in controlling the immune response against pancreatic cancer, especially the evasion of tumor-associated immune surveillance. The present study using extrahepatic cholangiocarcinomas also demonstrated that in the IgG4-rich cases (≥10 IgG4+ cells/HPF), the number of IgG4 in cancer tissue positively correlated with the degree of Foxp3+ Treg cells and conversely the number of cytotoxic CD8+ CTLs was constantly small. Therefore, extrahepatic cholangiocarcinomas as well as pancreatic cancers could cause the evasion of immune surveillance via the regulatory function of Treg cells, involving the concomitant IgG4 reaction. Survival curves also indicated the patients with □ 20 IgG4positive cells/HPF to have a better prognosis than those with ≥20 cells. Although the role of IgG4+ plasma cells against cancer tissue is uncertain, the degree of IgG4+ plasma cell infiltration might be a pathological marker of prognosis in extrahepatic cholangiocarcinoma.

In conclusion, this study revealed that extrahepatic cholangiocarcinoma often accompanies the significant infiltration of IgG4+ cells, indicating that we should take into account the differentiation of IgG4-related diseases and cholangiocarcinoma, especially using small specimens such as biopsy materials. Moreover, the IgG4 reaction

in cholangiocarcinoma might be associated with the evasion of immune surveillance by CD8+ CTLs and tumor progression through Treg cells.

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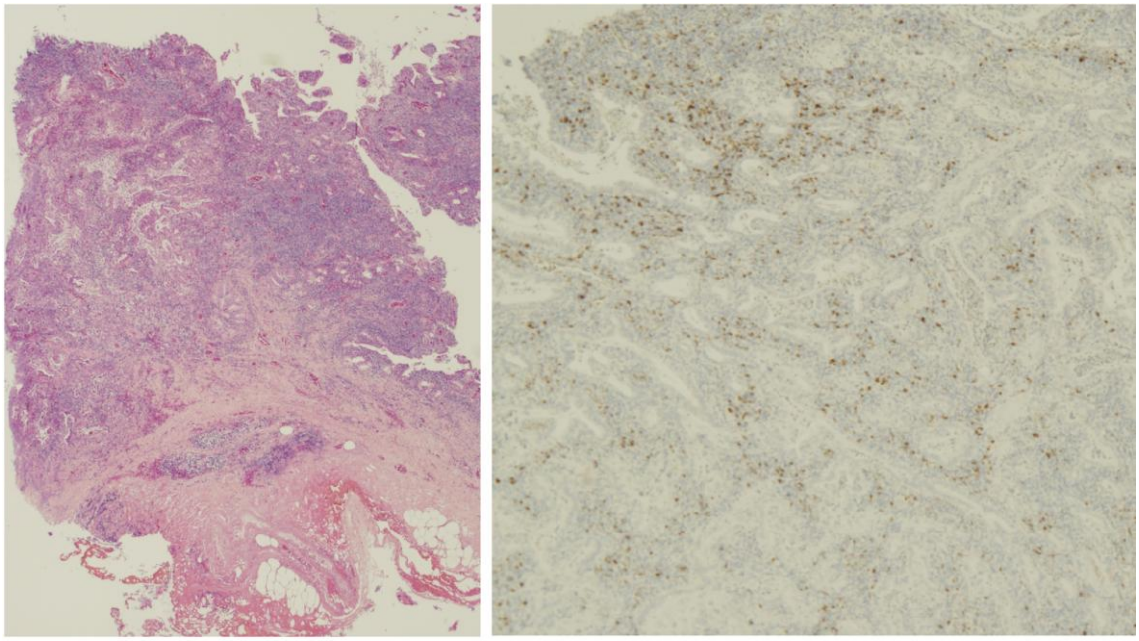
FIGURES and LEGENDS



A B

Fig.1

Fig.1 Common bile duct cancer. A: Papillary adenocarcinoma and prominent inflammatory cells are found. B: Immunohistochemistry of IgG4. Numerous IgG4+ cells were present in the inflammed stroma.

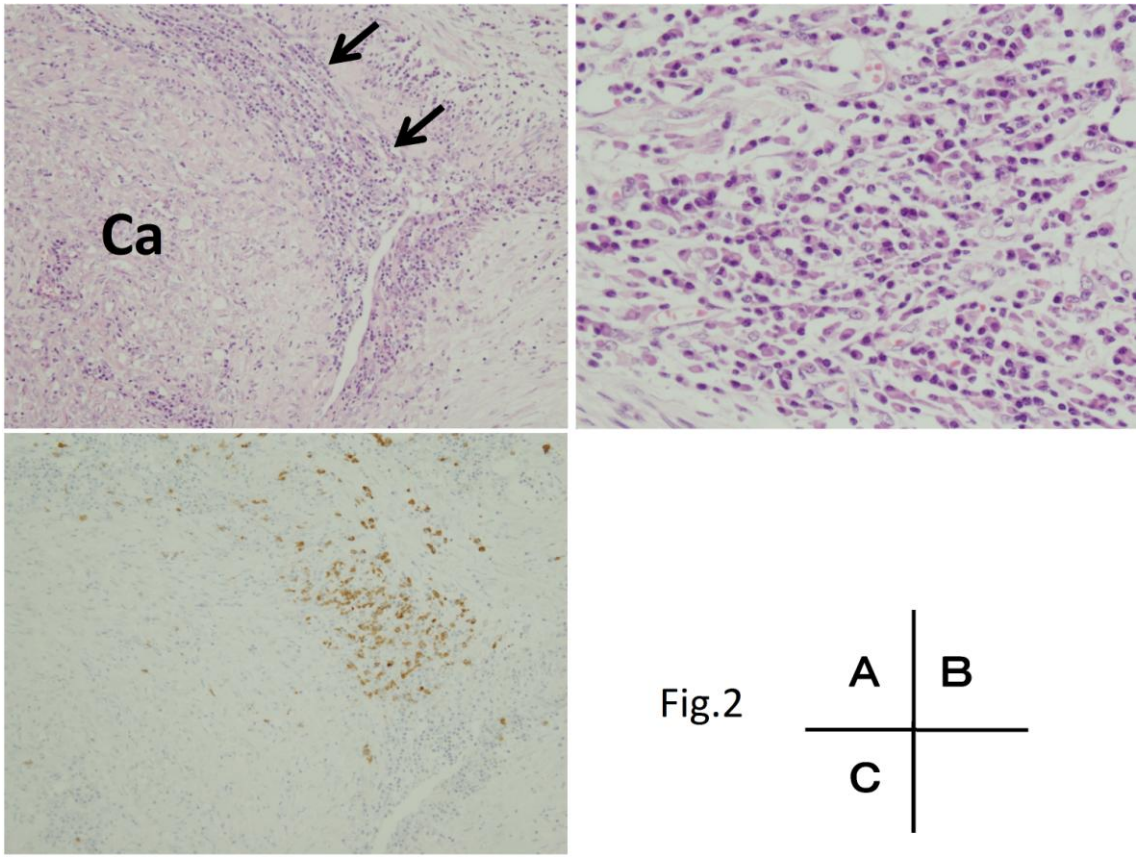


Fig.2

Fig.2 Common bile duct cancer. A: In a marginal zone (arrows) of poorly differentiated adenocarcinoma (Ca), many inflammatory cells are found. B: Higher magnification of the marginal zone. Inflammatory cells are mostly composed of plasma cells. C: Immunohistochemistry for IgG4. Many positive cells are scattered in the marginal zone.

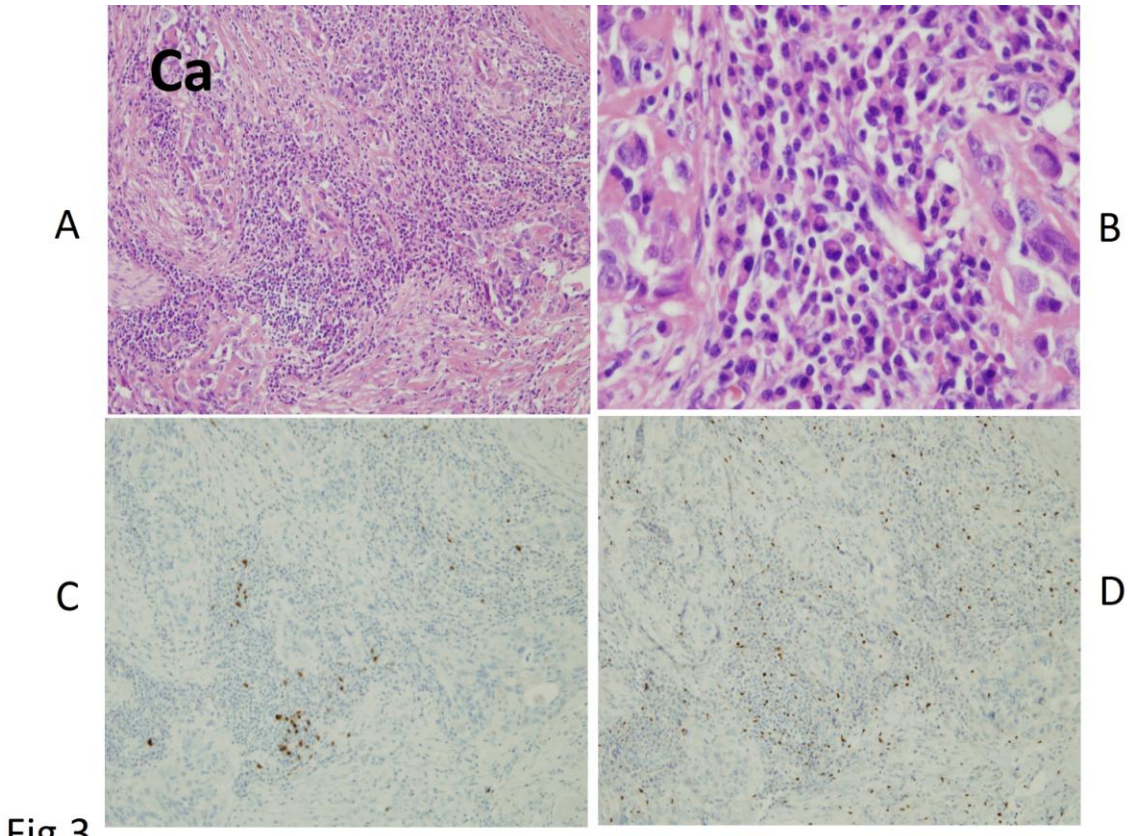


Fig.3

Fig.3 Gallbladder cancer. A: In an invasive area of poorly differentiated adenocarcinoma (Ca), numerous inflammatory cells are found. B: Higher magnification of the invasive area. Many plasma cells are found. C: Immunohistochemistry for IgG4. Several IgG4-positive cells are accumulated. D: Immunohistochemistry for Foxp3. Positive cells are diffusely scattered and slightly accumulated here and there.

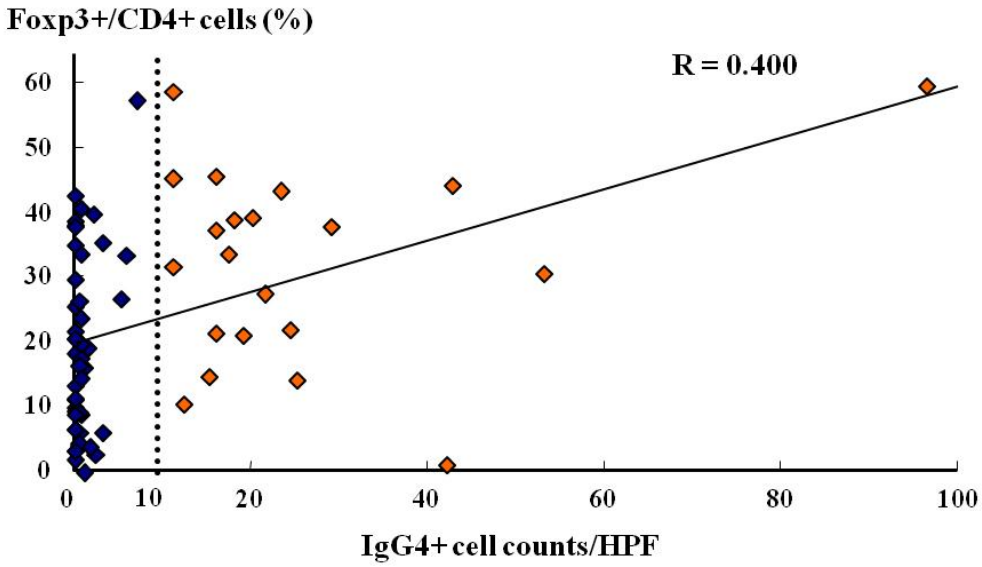


Fig.4

Fig.4 Relationship between IgG4+ cells and Treg cells in extrahepatic cholangiocarcinoma. In cases with ≥10/HPF IgG4+ cells (IgG4-rich cases), there is a good correlation between IgG4+ and the ratio of Foxp3+/CD4+ cells (R = 0.400).

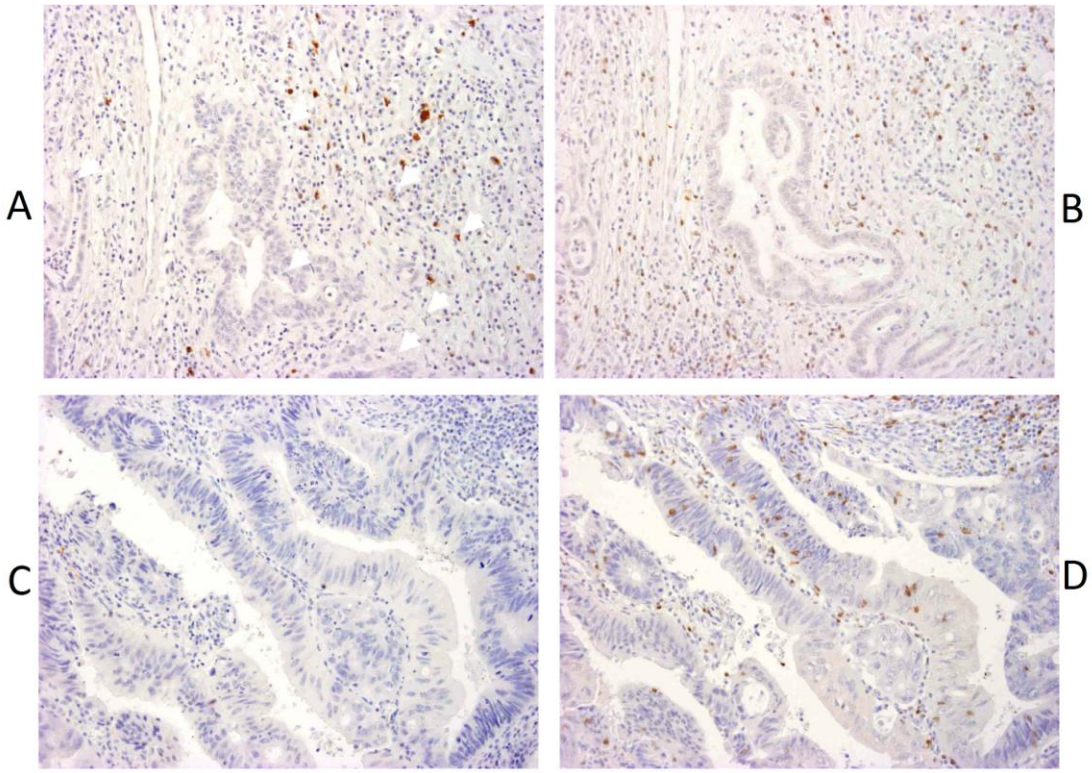


Fig.5

Fig.5 Comparison of CD8+ cytotoxic T cells in IgG4-rich (A and B) and -poor (C and D) cases of extrahepatic cholangiocarcinoma. Immunohistochemistry for IgG4 (A and C) and CD8 (B and D). In IgG4-rich cases, CD8+ as well as IgG4+ cells are found around adenocarcinoma tissue, but no CD8+ cells are in cancer cells lining the adenocarcinoma. In contrast, many CD8+ cells invade within cancer cells lining adenocarcinoma in IgG4-poor cases.

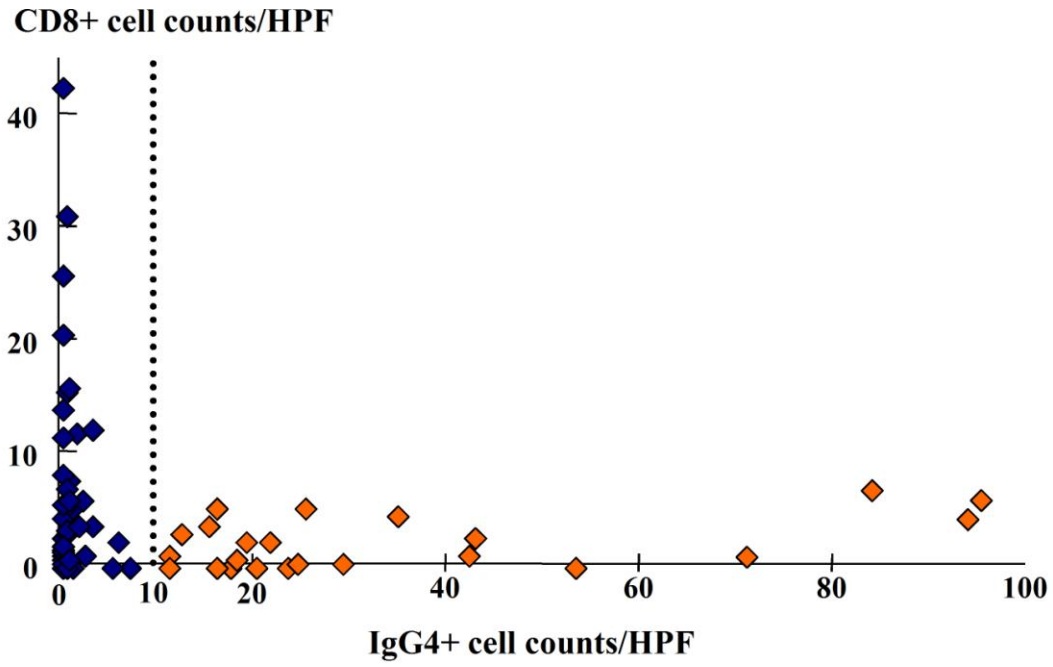


Fig.6

Fig.6 Relationship between IgG4+ and CD8+ cytotoxic T cells in extrahepatic cholangiocarcinoma. In all cases with ≥10/HPF IgG4+ cells (IgG4-rich cases), the number of CD8+ cells is less than 10.

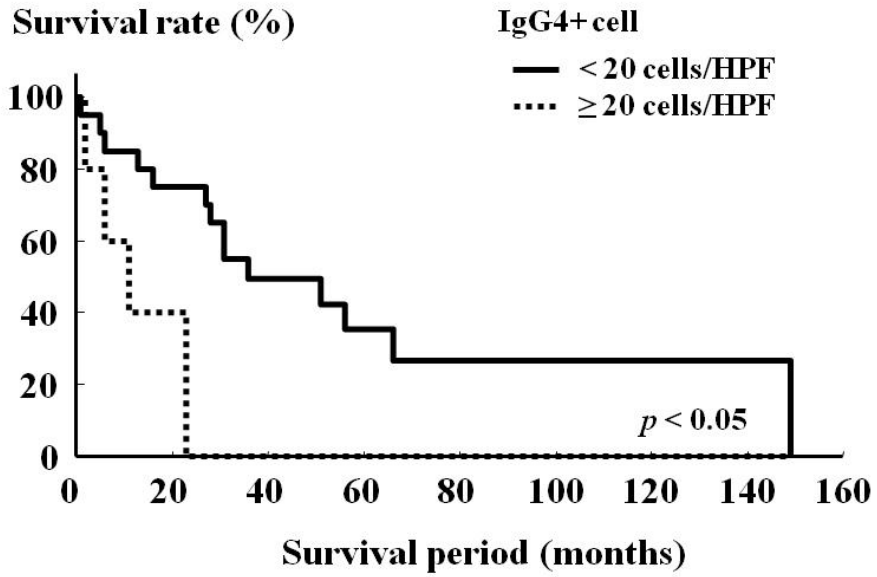


Fig.7

Fig.7 Survival curve of patients with extrahepatic cholangiocarcinoma. Kaplan-Meier plots of the postoperative survival period were carried out in two groups of patients, □ 20 and ≥20 IgG4+positive cells/HPF. Log-rank analysis of the postoperative survival periods indicated that cancer patients with □ 20 IgG4+ cells/HPF had better prognosis than those with ≥20 cells ($p<0.05$).