Significance of immunoglobulin G4 (IgG4)-positive cells in extrahepatic cholangiocarcinoma: Molecular mechanism of IgG4 reaction in cancer tissue

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Significance of IgG4-positive cells in extrahepatic cholangiocarcinoma: Molecular mechanism of IgG4 reaction in cancer tissue

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ABSTRACT

IgG4 reactions consisting of marked infiltration by IgG4-positive plasma cells in affected organs is found in cancer patients as well as patients with IgG4-related diseases. Notably, extrahepatic cholangiocarcinomas accompanying marked IgG4 reactions clinicopathologically mimic IgG4-related sclerosing cholangitis. A regulatory cytokine, IL-10, is thought to induce the differentiation of IgG4-positive cells. In this study, to clarify the mechanism of the IgG4 reaction in extrahepatic cholangiocarcinoma, we investigated non-professional antigen-presenting cells (APCs) generating IL-10-producing regulatory T cells (anergy T cells) and Foxp3-positive regulatory cells producing IL-10. Immunohistochemistry targeting IgG4, HLA-DR, CD80, CD86, and Foxp3 was performed using 54 cholangiocarcinoma specimens from 24 patients with gallbladder cancer, 22 with common bile duct cancer, and 8 with cancer of the Papilla of Vater. Moreover, a molecular analysis of Foxp3 and IL-10 was performed using a cultured human cholangiocarcinoma cell line. Consequently, 43% of the cholangiocarcinomas were found to be abundant in IgG4. Those expressing HLA-DR, but lacking costimulatory molecules (CD80 and CD86), and those expressing Foxp3 detected by an antibody recognizing the N terminus, accounted for 54% and 39% of cases, respectively. Moreover, the number of IgG4-positive cells was larger in these cases than in other groups. In cultured cells, the presence of a splicing variant of Foxp3 mRNA and the expression of IL-10 were demonstrated. In conclusion, extrahepatic cholangiocarcinoma is often accompanied by the significant infiltration of IgG4-positive cells. Cholangiocarcinoma cells could play the role of non-professional APCs and Foxp3-positive regulatory cells, inducing IgG4 reactions via the production of IL-10 indirectly and directly, respectively.

INTRODUCTION

Biliary tract cancers can be anatomically divided into intrahepatic and extrahepatic cholangiocarcinomas, the latter including hepatic hilar cancer, common bile duct cancer, gallbladder cancer, and cancer of the Papilla of Vater. The biological behavior and carcinogenesis of each cancer differ, but the histology of most biliary tract cancers is the same as that of ordinary adenocarcinomas. In addition to neoplastic lesions, several types of cholangitis causing biliary stenosis are important in the differential diagnosis of biliary diseases. Particularly, primary sclerosing cholangitis and a complication of IgG4-related systemic diseases, IgG4-related sclerosing cholangitis, clinicopathologically mimic extrahepatic cholangiocarcinomas.

IgG4 is a minor immunoglobulin subtype composing 3-6% of all the IgG circulating in adults,[1] but is important for a systemic disorder, IgG4-related disease, that features elevated serum IgG4 levels and abundant infiltration with IgG4-positive plasma cells in affected organs.[1-3] Moreover, IgG4-related cholangitis and pancreatitis (autoimmune pancreatitis, type 1) are characterized by sclerosing lesions (storiform fibrosis) and cholangiocarcinomas and pancreatic cancer usually accompany some degree of desmoplastic change and also, in some cases of pancreatic cancer, IgG4 reactions.[4] Therefore, a pathological examination is necessary to differentiate IgG4-related diseases from tumors in pancreatico-biliary lesions. We have already reported that extrahepatic cholangiocarcinomas also accompany various degrees of IgG4 reactions assumed to be associated with the evasion of immune surveillance (Kimura et al, in submission). However, the mechanisms inducing IgG4 reactions in cholangiocarcinoma tissue are still unknown.

IL-10, a regulatory cytokine mainly produced by Foxp3+ regulatory T cells (Treg cells), Th2 cells, and IL-10-producing regulatory T cells, is thought to induce the differentiation of IgG4-positive plasma cells or favor B cell switching to IgG4 in the presence of IL-4.[5,6] The

expression of Foxp3 and IL-10 has been demonstrated, in several carcinoma tissues and cultured cancer cell lines, suggesting that cancer cells themselves induce the Treg cell-like immuno-regulatory milieu to evade immuno-surveillance.[7-10].

MHC class II-positive cells lacking the costimulatory molecules CD80 (B7-1) and CD86 (B7-2) induce anergy to native T cells. Among T cell subsets, T regulatory type 1 cells (Tr1 cells) characterized by the production of IL-10 are induced by immature dendritic cells (DCs).[11] Moreover, costimulation-dependent T cell clones stimulated without provision of the costimulatory signal were demonstrated not to be proliferative, but to differentiate into IL-10-producing anergic T cells in primary biliary cirrhosis.[12] In addition to immunocompetent cells such as DCs, non-immunocompetent cells including carcinoma and normal epithelial cells have been demonstrated to express MHC class II, indicating an ability for antigen presentation, but these MHC class II-positive epithelial cells are usually called non-professional APCs, differing from professional APCs such as DCs. Several studies have suggested that antigen presentation by MHC class II-positive epithelial cells that lack costimulation signals, such as keratinocytes and pancreatic islet cells, would favor the generation of anergic T cells.[13-15]

It is clinicopathologically important, but practically difficult, to differentiate between IgG4-related sclerosing cholangitis and extrahepatic cholangiocarcinoma. In this study, we retrospectively evaluated IgG4-positive plasma cells in extrahepatic cholangiocarcinomas and mechanisms in terms of cholangiocarcinoma cells as non-professional APCs and regulatory cells. This study should help to clarify the pathological significance of IgG4 reactions in cholangiocarcinomas and also IgG4-related diseases.

MATERIALS and METHODS

Patients and tissue preparations: Formalin-fixed and paraffin-embedded sections of 54 surgically resected specimens from 24 gallbladder cancers, 22 common bile duct cancers, and 8 cancers of the Papilla of Vater (Average age 74y.o, male/female=29/25) were obtained from the registry of liver diseases in the Department of Pathology, Kanazawa University School of Medicine. Each cholangiocarcinoma was classified histologically a well- (including papillary), moderately, or poorly differentiated adenocarcinoma, based on the predominant histologic grade. Special histological types such as adenosquamous carcinoma and mucinous carcinoma were not included in the present study. Serial sections (4μm) were prepared from each formalin-fixed, paraffin-embedded block.

Immunohistochemistry: The deparaffinized and rehydrated sections were microwaved in citrate buffer (pH6)(for CD80, CD86) or EDTA buffer (pH9)(for Foxp3) for 20 min in a microwave oven. Following the blocking of endogenous peroxidase activity, these sections were incubated at 4°C overnight with antibodies against IgG4 (mouse monoclonal; diluted 1:200; Southern Biotech, Birmingham, AL, USA), Foxp3 (reacts with the C-terminus, mouse monoclonal, 5μg/ml, Abcam, Tokyo, Japan), Foxp3 (reacts with the N-terminus, rat monoclonal, 2.5μg/ml, eBioscience, San Diego, CA), HLA-DR (mouse monoclonal, 0.5μg/ml, Dako Japan, Tokyo), CD80 (rabbit monoclonal, 1:200, Epitomics, Burlingame, CA), and CD86 (rabbit monoclonal, 1:250, Abcam, Tokyo, Japan) and then at room temperature for 1hr with anti-mouse, anti-rabbit, or anti-goat immunoglobulin 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 antibodies were replaced with an isotype-matched, non-immunized immunoglobulin as a negative control of the staining procedures.

Histological examination: In addition to the histological observations by HE staining, the distribution of the immuno-positive cells was examined. In a primary survey, we examined all

tumorous areas in each specimen and, for counting IgG4-positive mononuclear cells, selected three representative areas containing IgG4-positive plasma cells, and expressed results as the mean number of immuno-positive cells in high power fields (HPFs). Because ≥10 IgG4-positive cells/HPF is proposed according to HISORt criteria published for autoimmune pancreatitis,[16,17] the cases with ≥10 and <10 IgG4-positive cells/HPF on average were evaluated as IgG4-rich and -poor cases, respectively. For the expression of Foxp3, HLA-DR, CD80 and CD86, positive carcinoma cells were evaluated as positive (distinct expression) or negative (no or faint expression) according to the staining intensity.

Cultured cells: Two commercially available cell lines, HuCCTl and MCF7 (positive control of IL-10)[10], were obtained from Health Science Research Resources Bank (Osaka, Japan). The cell lines were derived from cholangiocarcinoma and breast cancer cells, respectively.

Reverse transcription (RT)-PCR: The cell lines were cultured in flasks with a standard medium for 48 hrs. Cultured cells were collected from the flasks or plates with a cell scraper for determination of the baseline mRNA expression of Foxp3 and IL-10 by RT-PCR. Lymph node tissue was also used as a positive control for Foxp3 mRNA. Briefly, total RNA was isolated from each sample with the RNeasy^{TM} Total RNA System (QIAGEN, Hilden, German) and treated with RNase-Free DNaseI. For RT, 1μg of total RNA, M-MLV RTase (ReverTra Ace, Toyobo, Tokyo, Japan) and oligo-dT primers were used. PCR amplification was performed using DNA polymerase (Takara EX Taq, Takara, Tokyo, Japan) and specific primers for human mRNA sequences (Table 1). The GAPDH mRNA was used as a house-keeping gene. Following PCR, an annealing of primers for 1min and an extension at 72^{o}C for 2min (the annealing temperature and cycle number are shown in Table 1), PCR products were subjected to agarose gel electrophoresis.

Enzyme-linked immunosorbent assay (ELISA): Approximately 1x10^{4} HuCCT1 cells per well in 96-well plates were cultured for 24 hours; then supernatants were tested for human IL-10 by

ELISA (R&D).

***Statistical Analysis*:** Data were analyzed using the Welch's t-test; $p<0.05$ was considered statistically significant.

RESULTS

Infiltration of IgG4-positive cells in extrahepatic cholangiocarcinoma: Immunohistochemistry revealed that IgG4-positive plasma cells were scattered within and around cancerous nests to various degrees in most cases (Fig.1). In the cases with marked infiltration, the IgG4-positive cells were prominent with intermingling of other inflammatory cells. Fig.1C shows the number of IgG4-positive cells/HPF in extrahepatic cholangiocarcinomas from common bile ducts, gallbladder, and the Papilla of Vater, but there was no significant difference in IgG4-positive cell counts among anatomical locations of extrahepatic cholangiocarcinomas. Therefore, they were integrated as shown in Fig.1D. Consequently, the combined quantitative evaluation revealed that 23 (43%) of 54 cholangiocarcinoma patients had ≥10 IgG4-positive cells/HPF. There was no correlation between the density of IgG4-positive cells and any clinicopathological factor including age, gender, anatomical location (common bile ducts, gallbladder, and the Papilla of Vater), or the histological differentiation (well, moderate, and poor) of extrahepatic cholangiocarcinoma.

***Cholangiocarcinoma cells as non-professional APCs and their association with IgG4 reactions*:** Representative images of immunostaining are shown in Fig.2. Expression of HLA-DR was found in some infiltrating immunocompetent cells. Moreover, HLA-DR-positive cholangiocarcinoma cells were also found in 33 of 54 cases. HLA-DR expression in tumor cells showed an uniformity and metastatic foci in lymph nodes as well as main tumors expressing HLA-DR. In contrast, the expression of costimulatory molecules (CD80 and CD86) was mostly faint or absent. Only 4 cases were clearly positive for CD86 in cholangiocarcinoma cells and all of them

were positive for HLA-DR. No cases evidently expressed CD80. Cholangiocarcinoma cells expressing HLA-DR, but lacking costimulatory molecules (CD80 and CD86) were found in 29 of 54 cases (54%) and suggested to act as non-professional APCs inducing IL-10-producing anergy T cells. The relation between IgG4 reactions and HLA-DR & costimulatory molecules in cancer cells is shown in Fig.3. In cases of positivity for HLA-DR and negativity for costimulatory molecules, the number of IgG4-positive cells was significantly higher than in cases of negativity for HLA-DR and of positivity for both HLA-DR and costimulatory molecules.

Cholangiocarcinoma cells as a regulatory cells: Immunohistochemistry using the antibody reacting with the C-terminus of Foxp3 detected only mononuclear cells (Treg cells), but the antibody reacting with the N-terminus highlighted cholangiocarcinoma cells as well as Treg cells (Fig.4A). The cytoplasm as well as nucleus of tumor cells was positive in several cases. However, because Foxp3 is a transcription factor, the nuclear pattern was evaluated as functional expression. Consequently, 21 of 54 (39%) cholangiocarcinomas tested positive for Foxp3 by the antibody reacting with the N-terminus. The relation between the IgG4 reaction and Foxp3 expression in cholangiocarcinoma cells is shown in Fig.5. In cases of positivity for Fopx3, the number of IgG4-positive cells was significantly higher than in cases of negativity for Foxp3.

RT-PCR analysis demonstrated that a cholangiocarcinoma cell line, HuCCT1, expressed the mRNA of Foxp3, but close examination using 4 sets of primers corresponding to exons 1, 3, 10+11+12, and 12 revealed a lack of exon 3 (Fig.6), suggesting the presence of a splicing variant of Foxp3 in cholangiocarcinoma cells. Moreover, RT-PCR and ELISA revealed that HuCCT1 cells expressed IL-10 mRNA (Fig.6) and protein in the culture medium at 7.8 - 15.6 pg/ml.

DISCUSSION

IgG4 is important to the pathogenesis of IgG4-related diseases. However, patients with pancreatic adenocarcinomas accompanying IgG4 reactions and/or elevated serum IgG4 levels[4,18-20] and with pancreatic and biliary cancers arising from IgG4-related diseases[20-22] have been reported, though a cause-and-effect relationship between IgG4 reactions and cancers has still to be demonstrated. Moreover, in IgG4-non-related diseases including primary sclerosing cholangitis, IgG4 reactions were found to various degrees.[23,24] Therefore, the presence of IgG4-positive cells is not a histological hallmark of IgG4-related diseases and IgG4 reactions are speculated to be non-specific in several pathological conditions including cholangiocarcinomas. The present study also demonstrated the presence of extrahepatic cholangiocarcinoma cases with abundant IgG4 reaction, though there was no significant difference in IgG4-positive cell counts among anatomical locations of extrahepatic cholangiocarcinomas (common bile ducts, gallbladder, and the Papilla of Vater). The significance and mechanisms of IgG4 reactions in cancers as well as IgG4-related diseases are still unknown, but we speculated that cancer cells directly participate in the histogenesis of IgG4 reactions. Because the regulatory cytokine IL-10 is known to induce the differentiation of IgG4-positive plasma cells or favor B cell switching to IgG4 in the presence of IL-4,[5,6] we noted the IL-10-related regulatory cytokine network around cholangiocarcinoma tissue in this study.

Immunohistochemistry for MHC class II (HLA-DR) and costimulatory molecules (CD80 and CD86) revealed that cholangiocarcinoma cells as well as professional APCs such as B cells and DCs expressed HLA-DR and CD86. The expression of CD80 was limited in some APCs and not found in cholangiocarcinoma cells. Consequently, cholangiocarcinoma cells expressing HLA-DR, but lacking costimulatory molecules (CD80 and CD86) were found in 54% of cases. These cancer cells could act as non-professional APCs, possibly generating IL-10-producing regulatory T cells (anergy T cells), and then an IL-10-predominant cytokine milieu could cause the induction of IgG4-positive cells.[5,6] In these phenotypic cases, the number of IgG4-positive cells infiltrating carcinoma tissue was higher

than in HLA-DR-negative cases and both HLA-DR- and CD86-positive cases, confirming this speculation. Cells positive for both HLA-DR and CD86 are suggested to play the role of professional APCs, as it was reported that MHC class II-positive thyroid epithelial cells could present antigens to T cells and activate autoreactive T cells.[25,26] Although further study is needed to clarify the functional mechanism of these cholangiocarcinoma cells as APCs, this study demonstrated that HLA-DR- and CD86-positive cancer cells were not associated with IgG4 reactions in cholangiocarcinoma tissue.

As to pathogenesis of IgG4 reactions in IgG4-related diseases, the participation of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells (Treg cells) which are capable of producing IL-10, has been speculated.[27] Foxp3 is thought to be the master transcription factor of Treg cells and, until recently, Foxp3 expression was thought to be restricted to the T-cell lineage. However, immunohistochemistry and flow cytometric analysis demonstrated that some carcinoma tissues and cultured cancer cell lines expressed Foxp3.[7-10] Immunohistochemistry using the antibody recognizing the N terminus, but not C terminus, of Foxp3 highlighted cholangiocarcinoma tissue in 39% of cases as well as Treg cell morphology, suggesting the presence of the splicing variant of Foxp3 in cholangiocarcinoma cells. Molecular analysis using a cholangiocarcinoma cell line demonstrated that the cells expressed mRNA of Foxp3, but lack Exon 3. This type of splicing variant has already been reported in a melanoma cell line and created a novel amino acid caused by a frame-shift at the C terminus.[9] This is why the antibody recognizing the C terminus of Foxp3 could not detect the variant of Foxp3 found in cholangiocarcinoma tissue. Although a functional analysis of this variant as a transcription factor is necessary, it has already been reported that Foxp3 expression is closely correlated with the expression of IL-10 in all Foxp3-positive cell lines.[10] The present study using a cholangiocarcinoma cell line also demonstrated cells express mRNA of IL-10 as well as Foxp3. Moreover, the IL-10 protein was detected in the culture medium by ELISA at a

concentration of 7.8 - 15.6 pg/ml, suggesting that the production of IL-10 was preserved with this splicing variant. The finding suggests that cholangiocarcinoma cells themselves function in immunosuppression similar to Treg cells via IL-10 production. This was supported by the present data that in Foxp3-positive cases, the number of IgG4-positive cells infiltrating cholangiocarcinoma tissues was higher than that in Foxp3-negative cases, albeit several negative cases still accompanied a significant IgG4 reaction (≥10 IgG4+ cells/HPF).

In this study, we demonstrated two different types of IgG4 reactions in cholangiocarcinoma tissues. Although statistical significance could be obtained in terms of cholangiocarcinoma as both non-professional APCs and IL-10-producing regulatory cells, some cases deviated from each mechanism. Therefore, as shown in Fig.7, we divided all cases into a "non IL-10-inducing group" and "IL-10-inducing group" and reevaluated the present results accordingly. The former (n=24) consisted of MHC class II-negative and Foxp3-negative cases and MHC class II-positive, costimulatory molecule (CD86)-positive, and Foxp3-negative cases; the latter (n=30) contained MHC class II-positive and costimulatory molecule-negative cases and Foxp3-positive cases. This combined analysis demonstrated that all cases in the "non IL-10-inducing group" except 2 were poor in IgG4 (<10 IgG4+ cells/HPF) and that the difference in IgG4 reactions between the "IL-10-inducing group" and "non-IL-10-inducing group" was significant, compared with that of the individual analysis in terms of non-professional APCs and IL-10-producing regulatory cells. This finding indicates that cholangiocarcinoma cells directly participate in the induction of IgG4 reactions via a IL-10-predominent cytokine milieu as non-professional APCs and/or regulatory cells. However, the presence of IgG4-rich cases belonging to the "non-IL-10-inducing group" suggests another possible mechanism inducing IgG4 reactions in cholangiocarcinomas. Further studies are mandatory to clarify the mechanism of IgG4 reactions.

In conclusion, the marked infiltration of IgG-positive cells is found in several cases of

cholangiocarcinoma, indicating that we should take into account the differentiation of IgG4-related diseases and cholangiocarcinoma. The IgG4 reactions in cholangiocarcinomas, moreover, are closely associated with the IL-10-predominant regulatory cytokine milieu caused by cancer cells themselves directly and indirectly. Because IL-10 plays a primary role in suppressing immune responses, IgG4 reactions in cholangiocarcinoma might reflect evasion from immune surveillance.

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FIGURE LEGENDS

Fig.1 IgG4-positive cells in extrahepatic cholangiocarcinomas. A: Gallbladder cancer. A papillary adenocarcinoma with prominent inflammatory cells was found. B: Immunohistochemistry for IgG4. Numerous IgG4-posituve cells are present in the inflammed stroma. The inset show a higher magnification. C: Number of IgG4-positive cells/high power field in common bile duct cancer, gallbladder cancer, and cancer of the Papilla of Vater. There was no significant difference in IgG4-positive cell counts among anatomical locations of extrahepatic cholangiocarcinoma. D: Number of IgG4-positive cells in cholangiocarcinoma. A quantitative evaluation revealed that 23 (43%), 16 (30%), and 5 (9%) of 54 cholangiocarcinoma patients had ≥10, ≥20, and ≥50 IgG4+ cells/HPF, respectively.

Fig.2 Immunohistochemistry for IgG4 (A and D), HLA-DR (B and E), CD80 (C), and CD86 (F). A-C: IgG4-rich case of gall bladder cancer. Numerous IgG4-positive cells are found within cancer tissue (A). In addition to infiltrating mononuclear cells, carcinoma cells also tested positive for HLA-DR (B, arrows). There are no tumor cells positive for CD80 (C). D-F: IgG4-poor case of common bile duct cancer. No IgG4-positive cells are found (D), but obvious expression of HLA-DR and CD86 in carcinoma cells is found (E and F).

Fig.3 Correlation between IgG4-positive cell counts and antigen-presenting-related molecules in cholangiocarcinoma. The number of IgG4-positive cells in the cholangiocarcinoma cases expressing HLA-DR, but lacking costimulatory molecules (CD80 and CD86), is significantly higher than those of negativity for HLA-DR and costimulatory molecules and of positivity for both HLA-DR and costimulatory molecules. Bars indicate mean±S.E.M.

*<0.05.

Fig.4 Foxp3 expression in cholangiocarcinoma. Immunohistochemistry using the antibody recognizing the C-terminus (A) and N-terminus of Foxp3 (B and C). The antibody reacting with the C-terminus detects only mononuclear cells (Treg cells) in the nuclear pattern (A). In contrast, the antibody reacting with the N-terminus highlights the nucleus and cytoplasm of cholangiocarcinoma cells as well as Treg cells (B, arrows), but the localized expression in the nucleus is also found in cholangiocarcinoma cells (C).

Fig. 5 Correlation between IgG4-positive cell counts and Foxp3 expression in cholangiocarcinoma. Nuclear expression of Foxp3 is found in 21 cases of cholangiocarcinoma and in these cases, the number of IgG4-positive cells was significantly higher than those of negativity for Foxp3. Bars indicate mean±S.E.M. *<0.05.

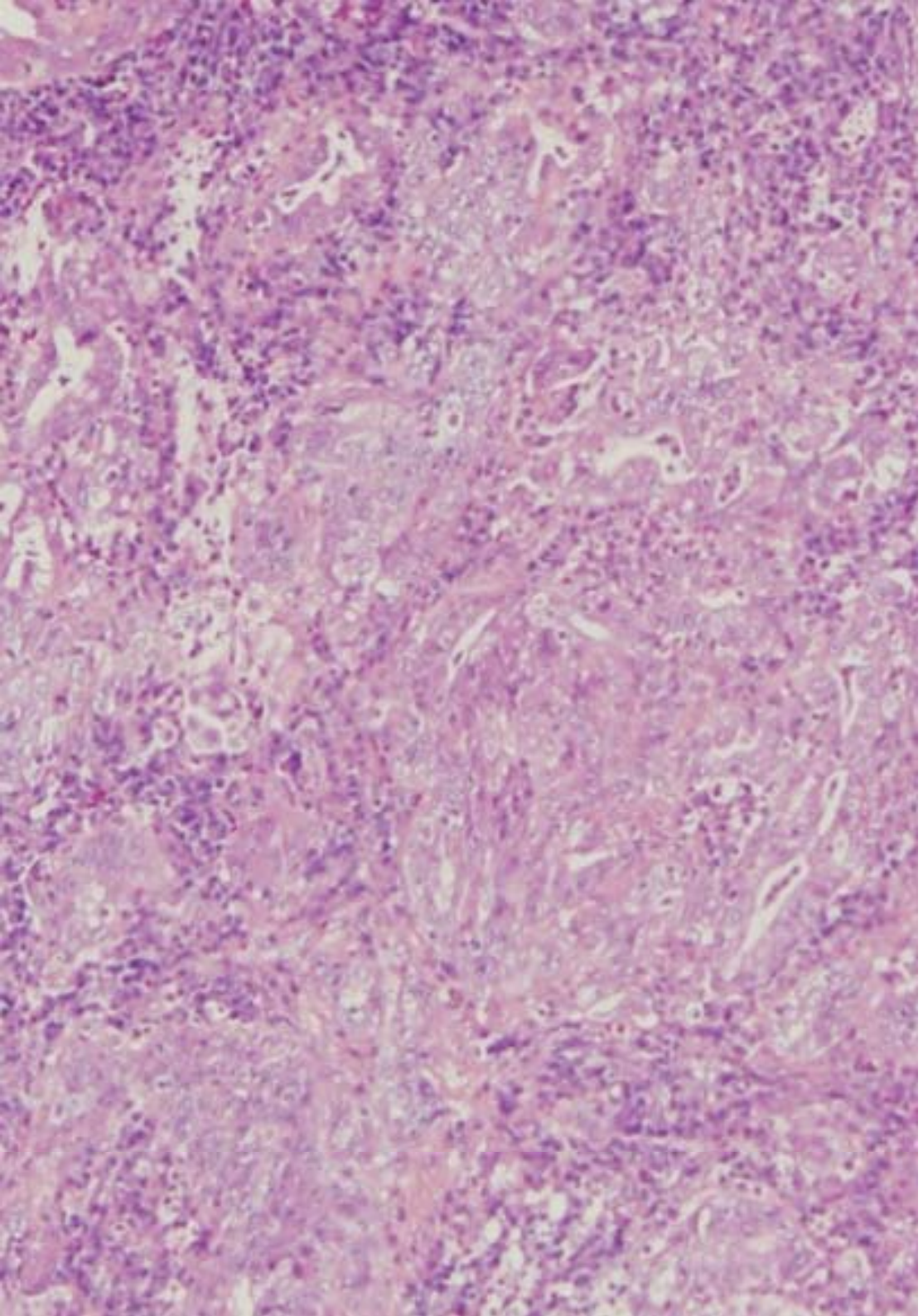
Fig. 6 Detection of Foxp3 and IL-10 mRNAs in a cultured cholangiocarcinoma cell line (HuCCT1). RT-PCR analysis using 4 sets of primers corresponding to exons 1, 3, 10-12, and 12 demonstrated that HuCCT1 expressed the mRNA of Foxp3, but lacked exon 3. Moreover, HuCCT1 expressed IL-10 mRNA. Each RT-PCR product gave bands of the appropriate molecular weight. MCF7 (breast cancer cell line) and lymph node (LN) was used as positive controls, and negative control (NC) was obtained by omitting reverse transcriptase for reverse transcription of HuCCT1.

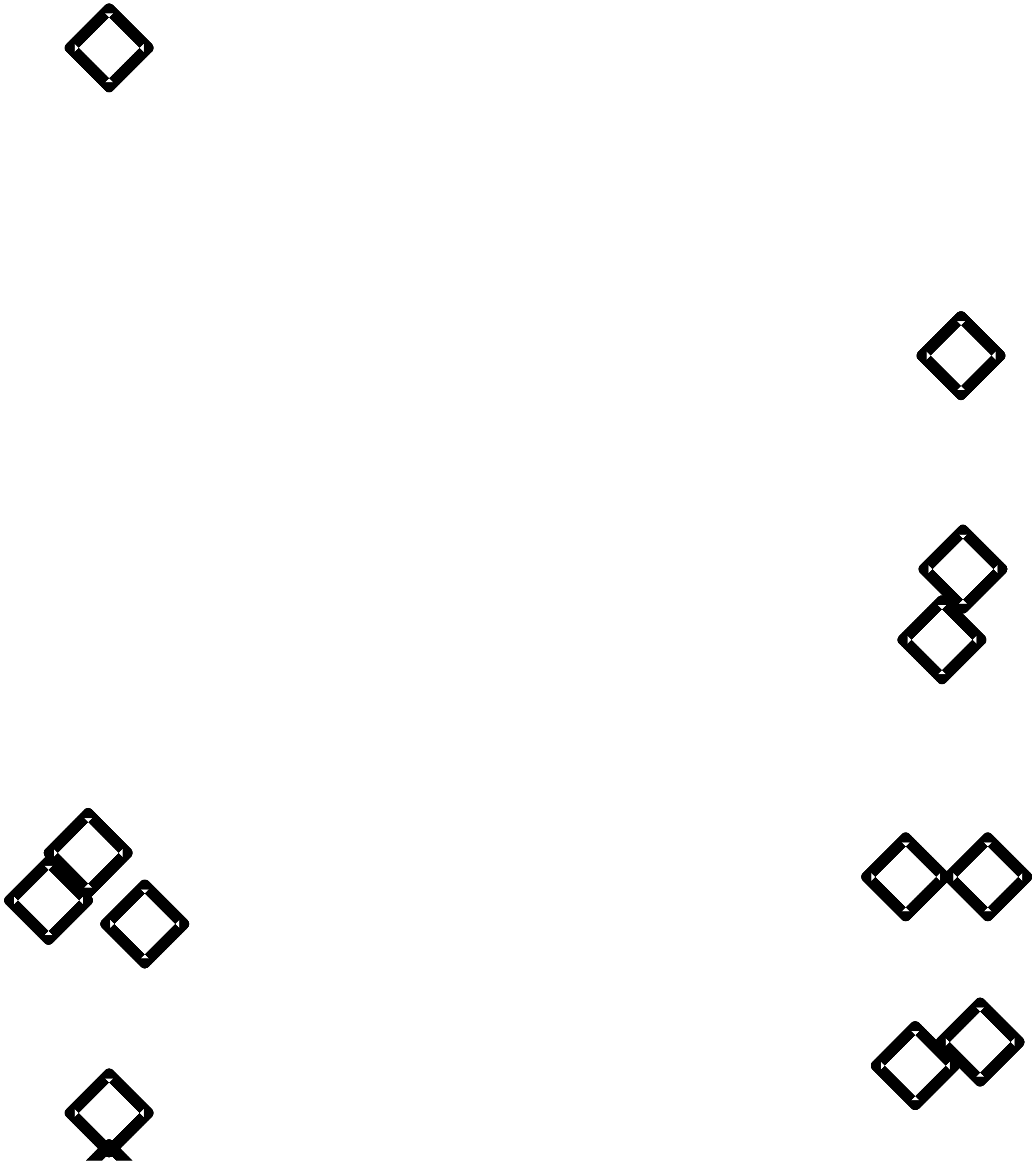
Fig.7 Correlation between IgG4-positive cell counts and IL-10-predominant milieu. All cases were divided into two categories. The "Non IL-10-inducing group" includes MHC class II

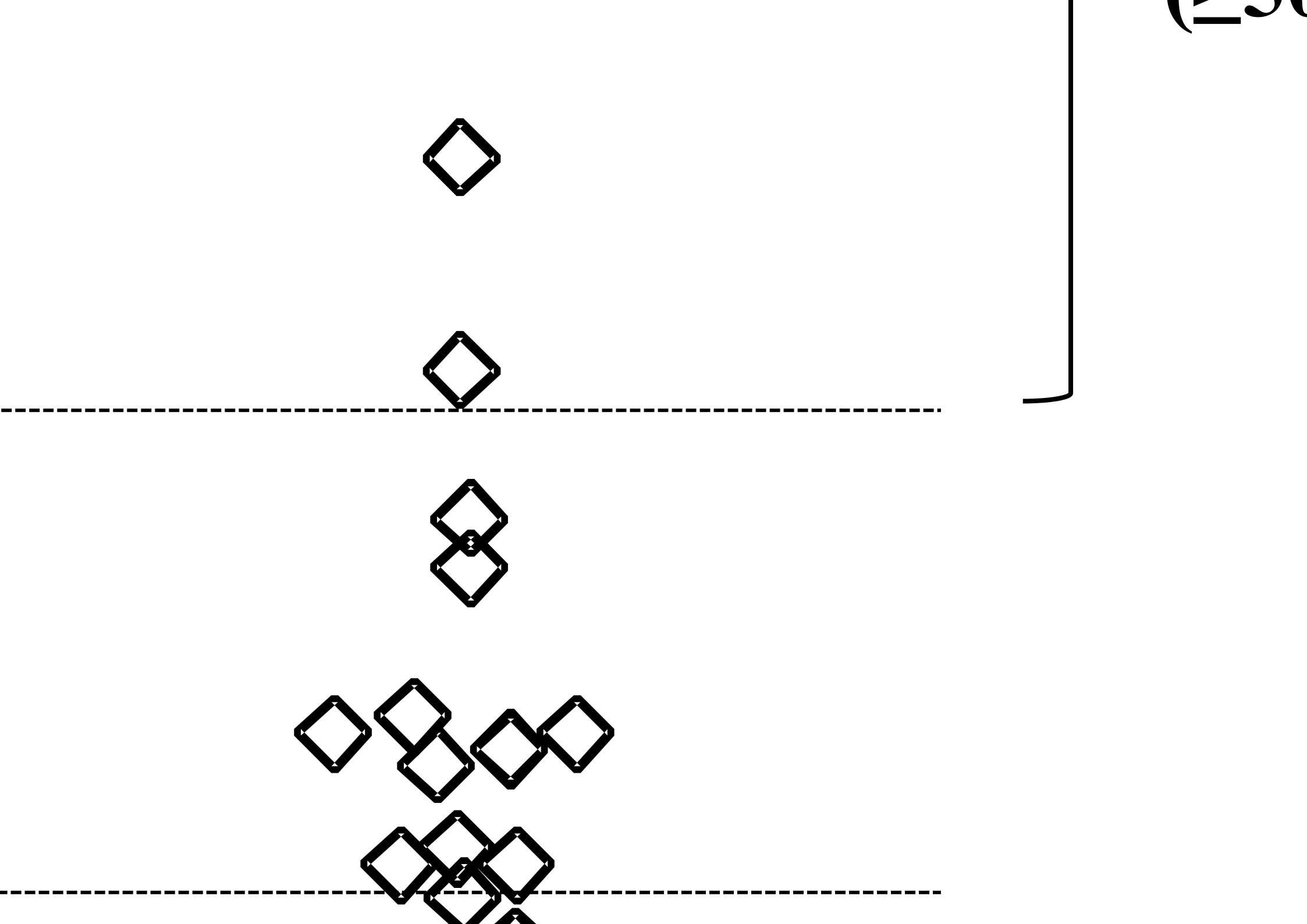
(HLA-DR)-negative and Foxp3-negative cases and MHC class II-positive, costimulatory molecule (CD86)-positive, and Foxp3-negative cases; the "IL-10-inducing group" includes MHC class II-positive and costimulatory molecule-negative cases and Foxp3-positive cases. All cases in the "non IL-10-inducing group" except 2 are <10 IgG4+ cells/HPF and the number of IgG4-positive cells in the "IL-10-inducing group" is significantly higher than those of the "non IL-10-inducing group". Bars indicate mean±S.E.M. *<0.05.

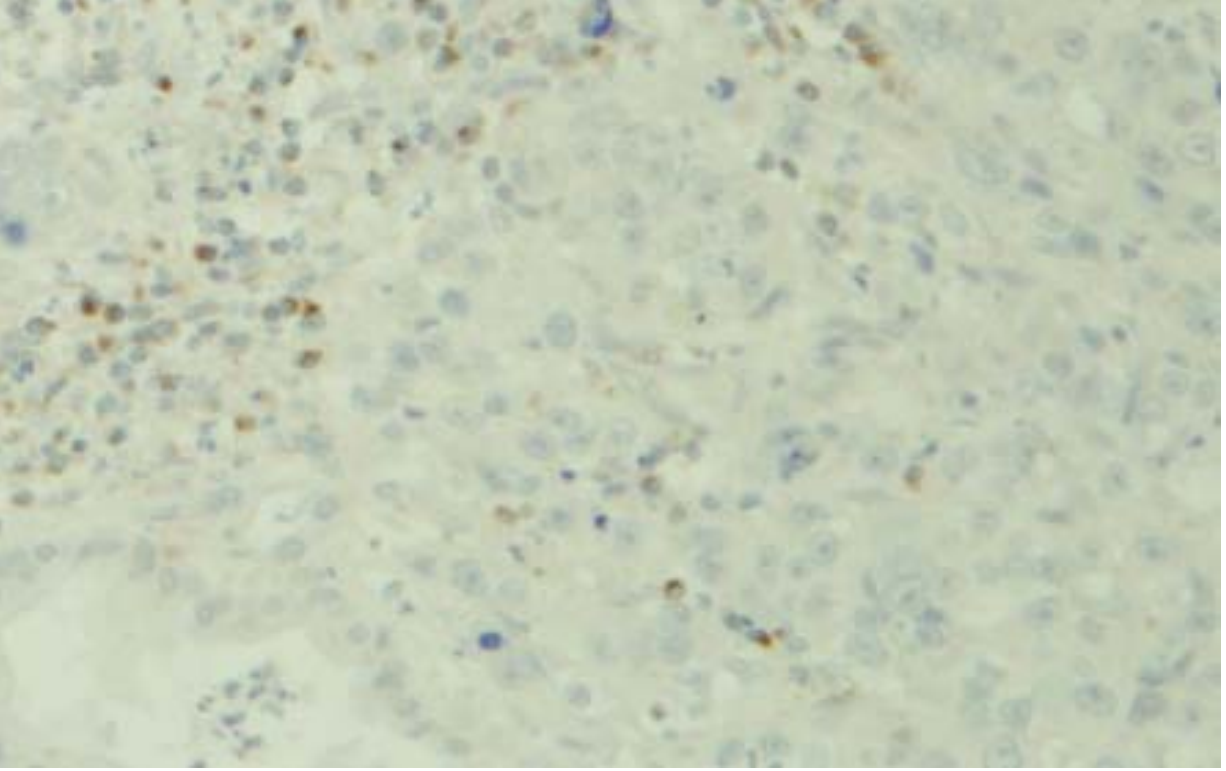
Table 1 Primers used for RT-PCR

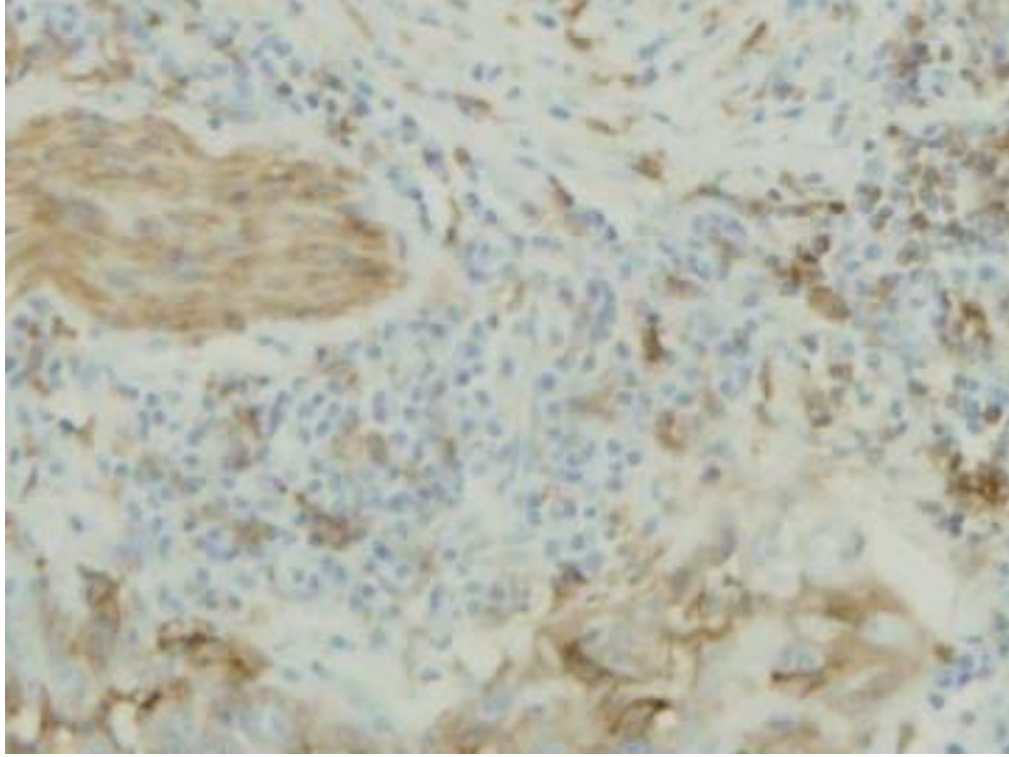
Transcript	Primers	Product size
Foxp3		
Exon 1	Forward 5’-ACCGTACAGCGTGGTTTTTC-3’	111 bp
	Reverse 5’-AGGCTTGGTGAAGTGGACTG-3’	
Exon 3	Forward 5’-TGCCTCCTCTTCTTCCTTGA-3’	125 bp
	Reverse 5’-GGAGGAGTGCCTGTAAGTGG-3’	
Exons 10&11&12	Forward 5’- CACAACATGCGACCCCCTTTCACC -3’	167 bp
	Reverse 5’- AGGTTGTGGCGGATGGCGTTCTTC-3’	
Exon 12	Forward 5’- CAGCTGCTCGCACAGATTAC -3’	91 bp
	Reverse 5’- TTGGGGTTTGTGTTGAGTGA-3’	
IL-10	Forward 5’- TGCAAAACCAAACCACAAGA -3’	325 bp
	Reverse 5’- GCATCACCTCCTCCAGGTAA-3	
GAPDH	Forward 5’-GCACCGTCAAGGCTGAGAAC-3’	142 bp
	Reverse 5’-ATGGTGGTGAAGACGCCAGT-3’	











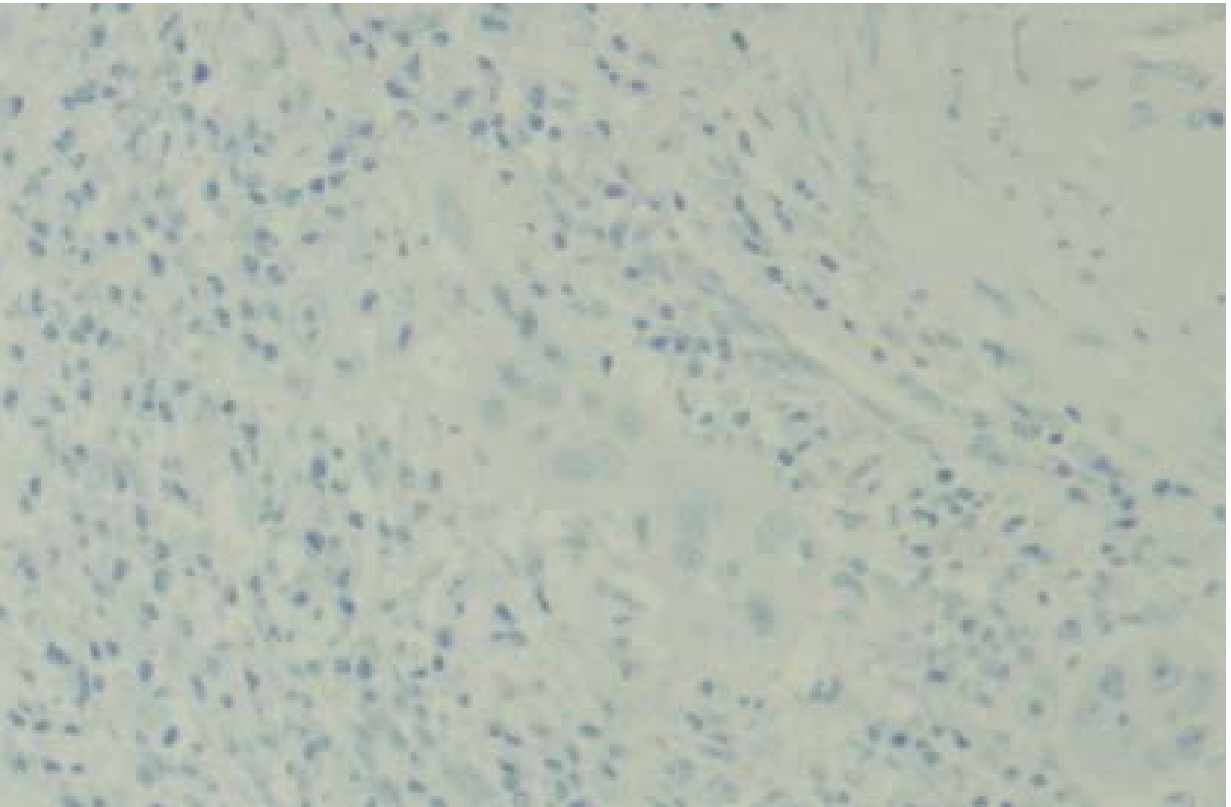
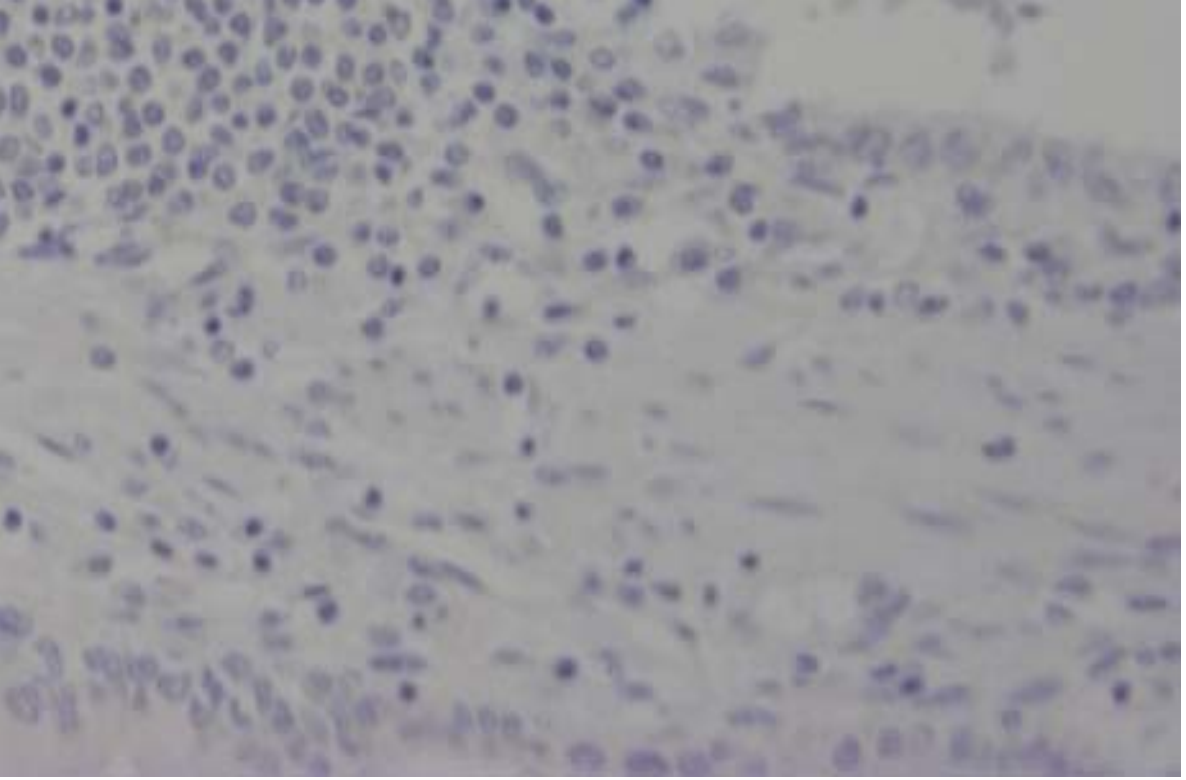
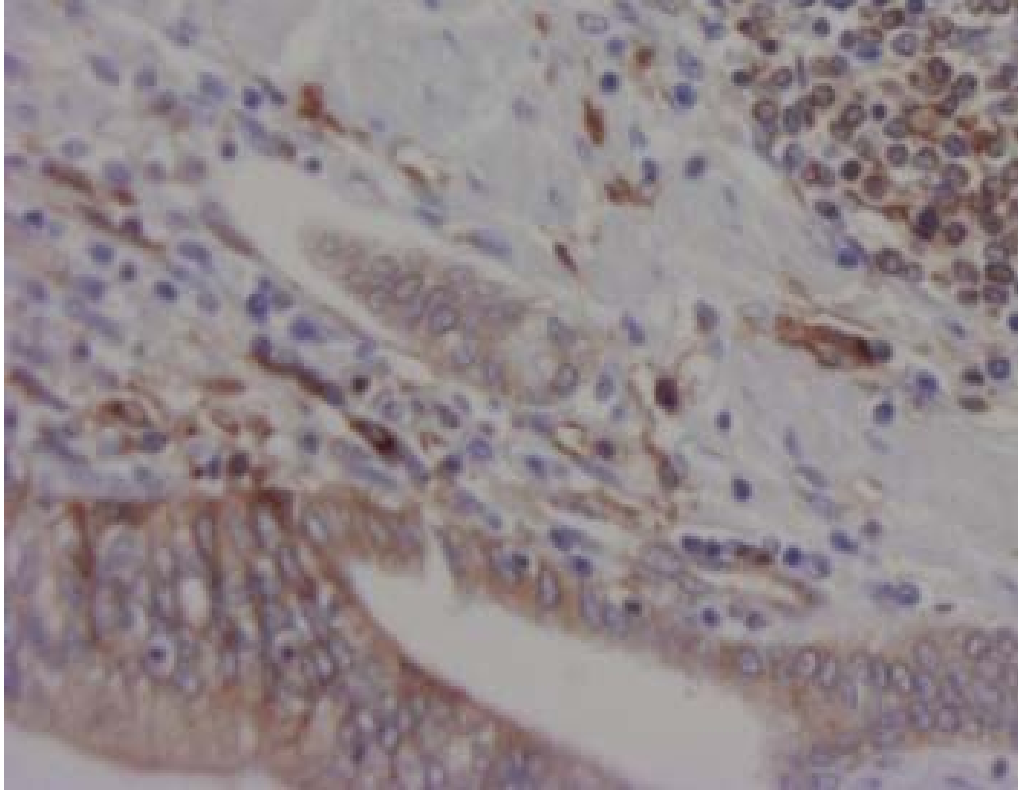


Fig.2





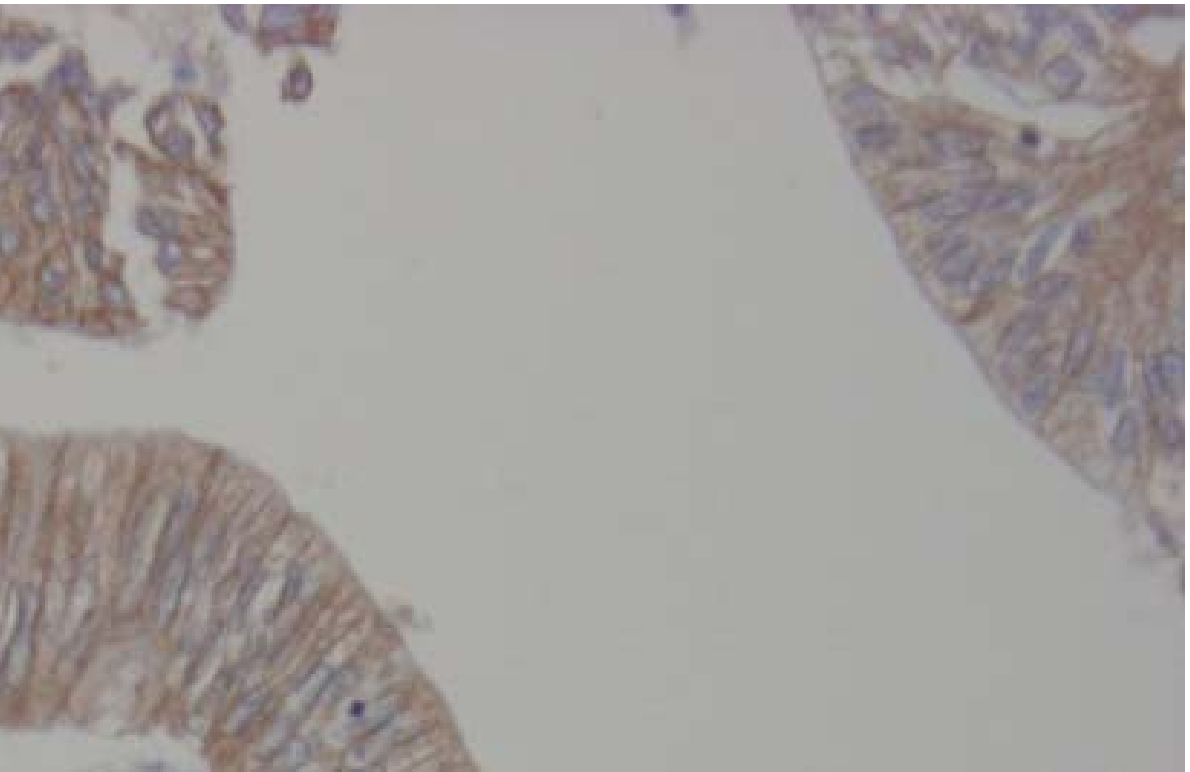


Fig.2

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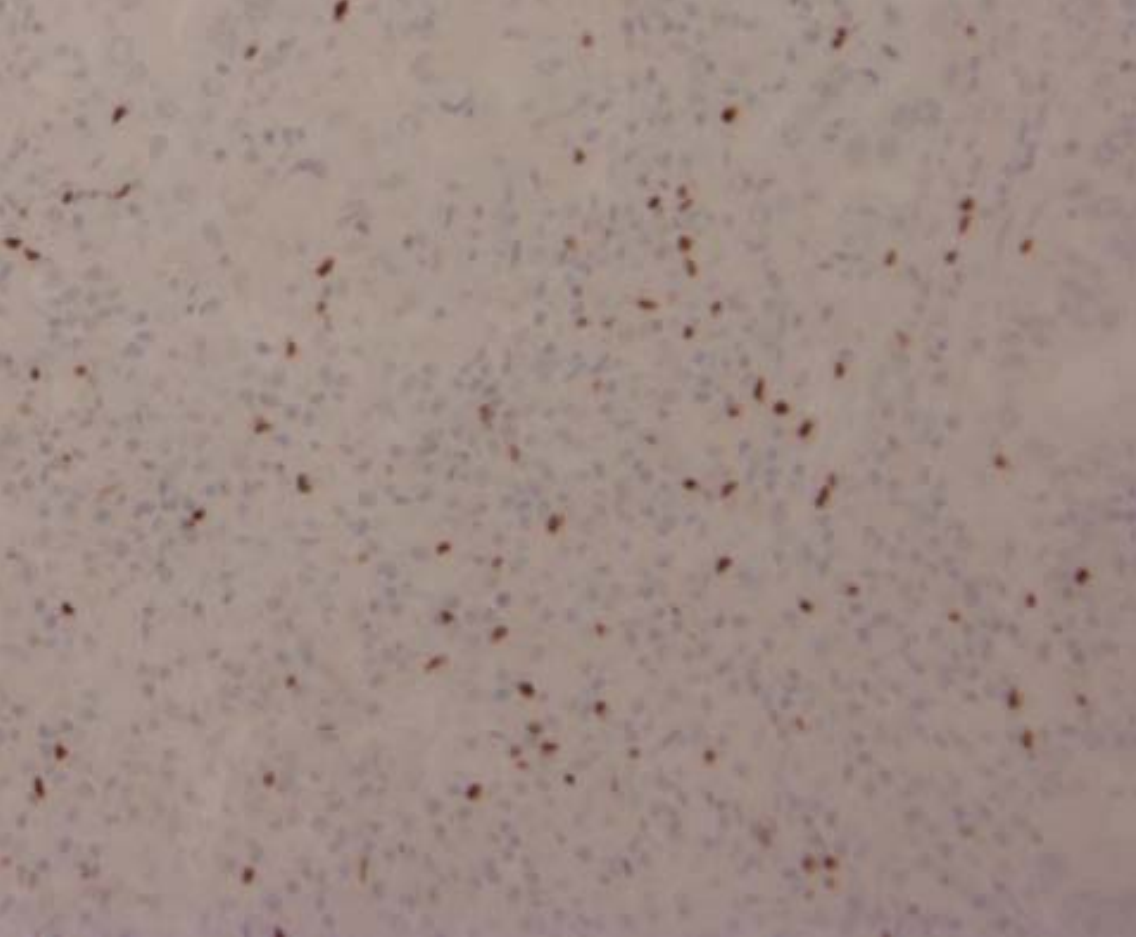
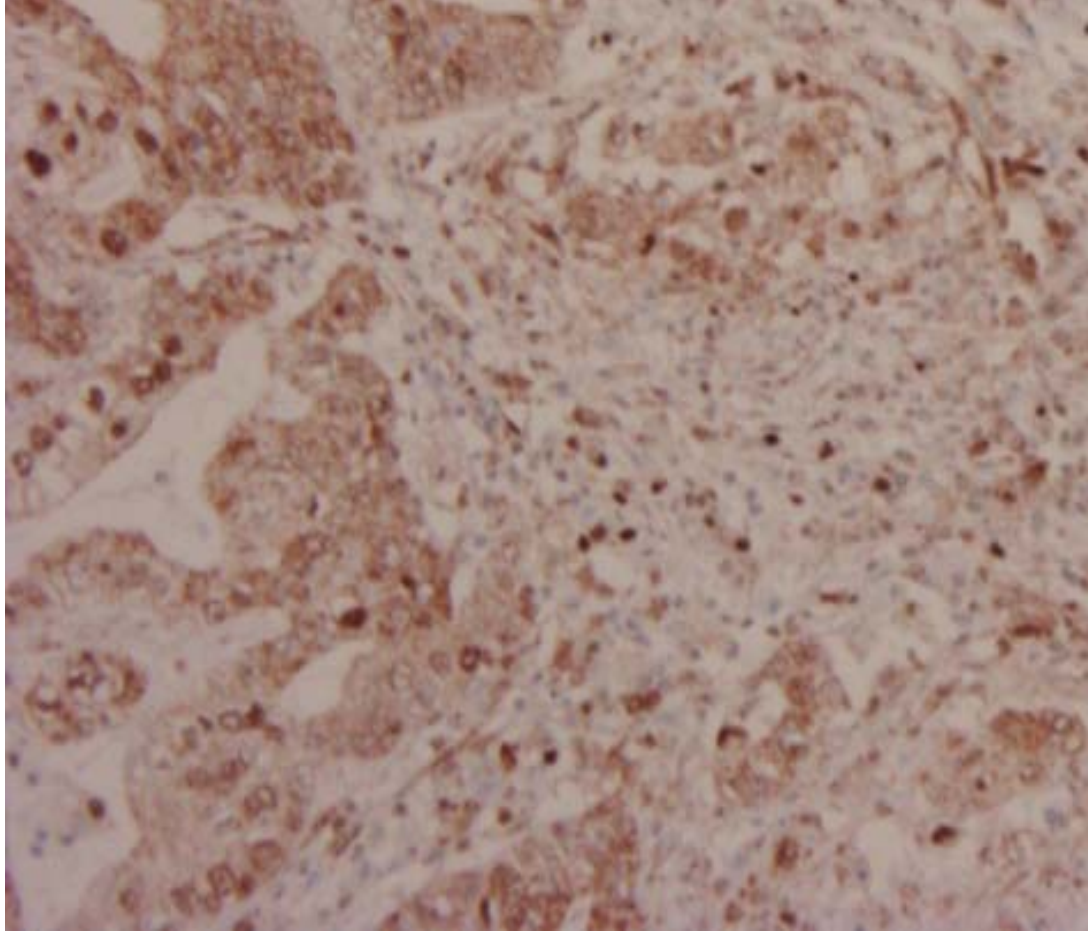




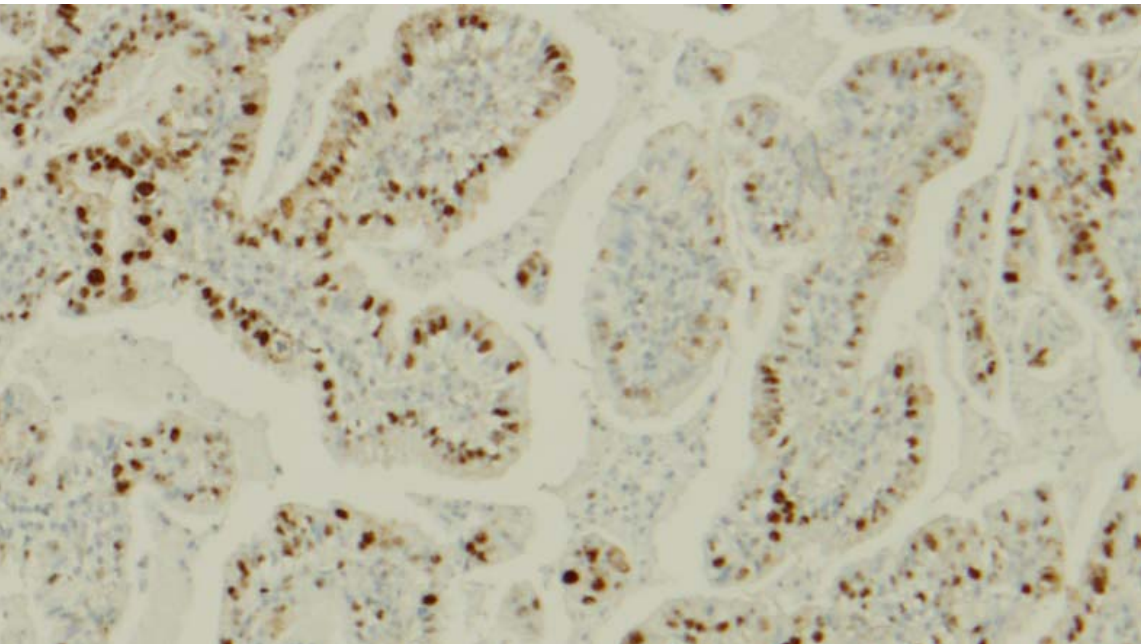
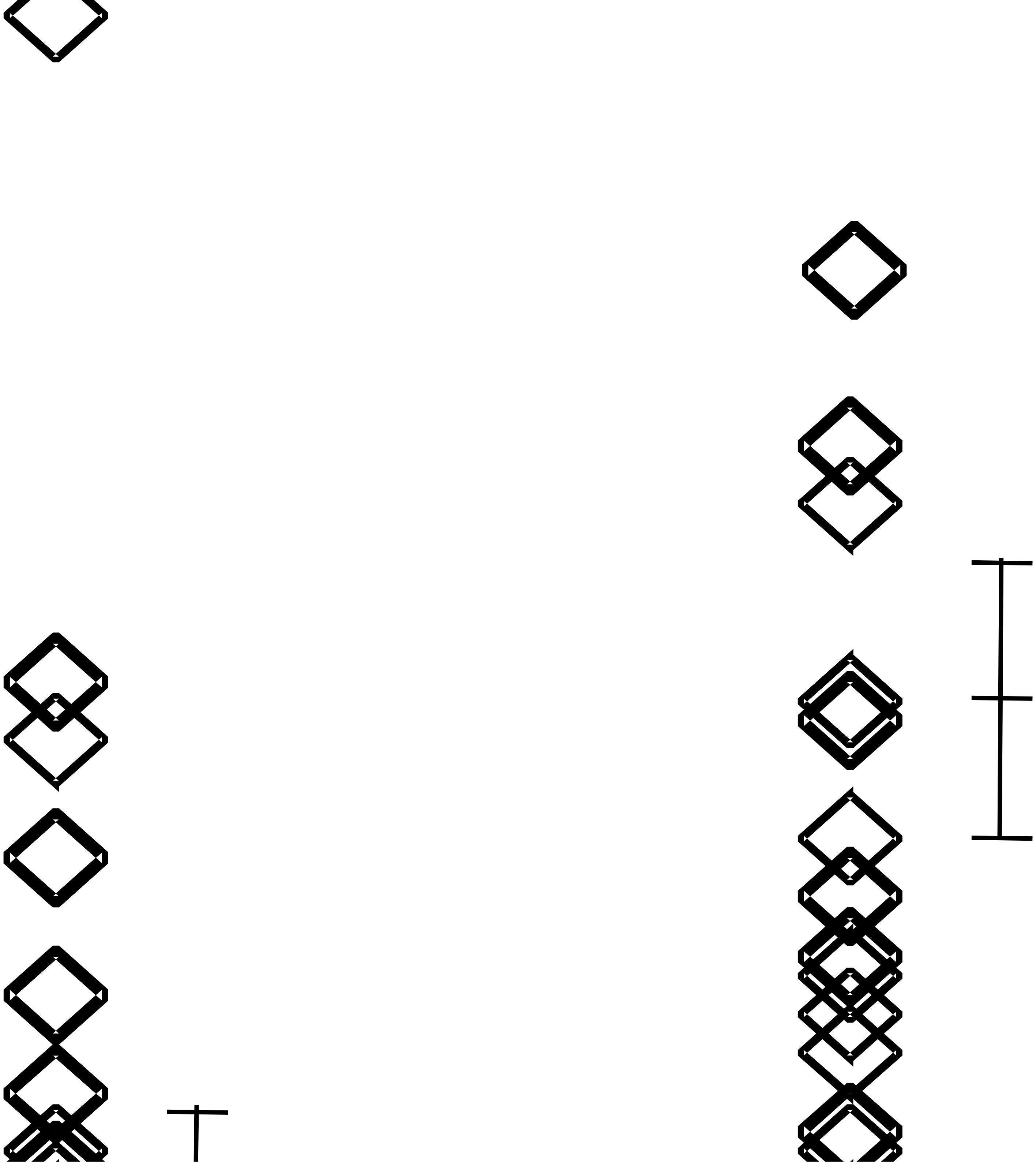


Fig.4



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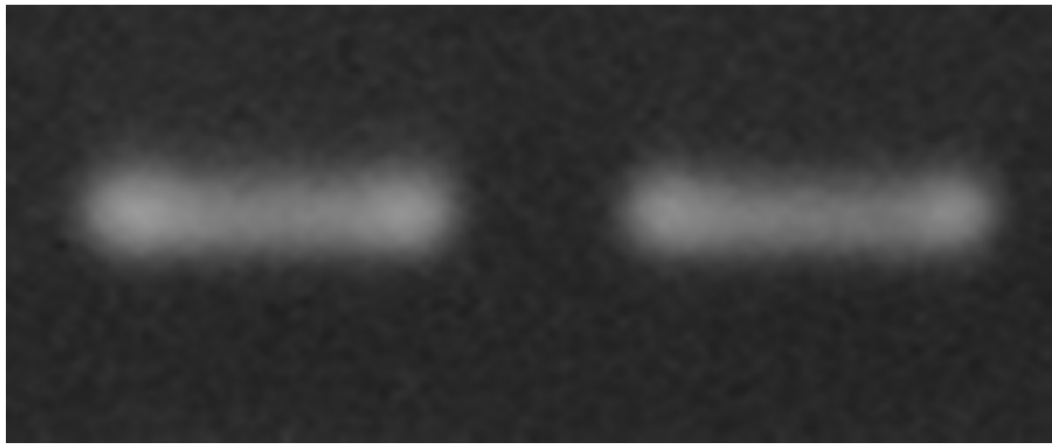

Exon 12

HuC MCF7 LN NC