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FOXP3+ Regulatory T Cells and Tumoral Indoleamine 2,3-Dioxygenase Expression Predicts the Carcinogenesis of Intraductal Papillary Mucinous Neoplasms of the Pancreas

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Key Words
Regulatory T cells · Intraductal papillary mucinous neoplasms · Indoleamine 2,3-dioxygenase

Abstract
Background and Aims: FOXP3+ regulatory T cells (Tregs) play a central role in self-tolerance and suppress the effective antitumor immune response. A recent study revealed that indoleamine 2,3-dioxygenase (IDO)-mediated tryptophan depletion was able to affect local tumor-infiltrating lymphocytes. The aim of this study was to investigate the clinical significance of the tumor-infiltrating Tregs and tumoral IDO expression during the progression of intraductal papillary mucinous neoplasms (IPMNs) of the pancreas. ***Methods:*** We investigated the prevalence and localization of FOXP3+ Tregs, CD8+ lymphocytes, and IDO expression in IPMNs by immunohistochemistry. We recruited 39 cases with IPMNs (IPMA: adenoma, n = 11; IPMB: borderline malignancy, n = 9; IPMC: noninvasive carcinoma, n = 7; I-IPMC: invasive IPMC, n = 12). ***Results:*** The prevalence of Tregs increased step by step during the carcinogenesis of IPMNs (Kruskal-Wallis test: $p < 0.0001$). IDO expression in the tumor was observed in 5 cases with IPMNs (IPMC, n = 1; I-IPMC, n = 4). IDO expression in the tumor was positively correlated with the prevalence of Tregs in IPMNs. ***Conclusions:*** FOXP3+ Tregs play a role in controlling the immune surveillance against IPMNs at the premalignant stage. IDO expression in the tumor is one of the late-stage phenomena of multistage carcinogenesis of IPMNs.

Introduction

Intraductal papillary mucinous neoplasms (IPMNs) of the pancreas are characterized by the intraductal proliferation of neoplastic mucinous cells, which was papillary growth in the large pancreatic duct leading to cystic dilatation of the pancreatic ducts [1, 2]. Similar to the well-defined adenoma-carcinoma sequence in colorectal cancer, IPMNs progress from intraductal papillary mucinous adenoma (IPMA) to borderline IPMN (IPMB), then to intraductal papillary mucinous carcinoma (IPMC) [3]. According to the WHO classification, IPMC is classified as either 'noninvasive' or 'invasive' [4]. In the past, many

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reports revealed that a lot of the genetic mutations and protein expression of the tumor as well as the microenvironment of the tumor were gradually changing according to the carcinogenesis stage of the IPMNs [2, 5–8]. It is challenging to predict which cases would rapidly progress from IPMA and IPMB to IPMC, eventually invasive IPMC (I-IPMC), and which cases were likely to have recurrence after the resection.

Regulatory T cells (Tregs) are one of the unique T cell subsets that play important roles in immunological self-tolerance. They have a functionally immunosuppressive property that inhibits effector cells from acting against self in autoimmune diseases or tumor immunity [9, 10]. Tregs can inhibit immune responses mediated by CD4+CD25– and CD8+ T cells in vitro in a contact-dependent manner through inhibition of the production of interleukin-2, and express high-level intracellular CTLA-4, and do not proliferate in vitro following T cell receptor stimulation [11]. Tregs secrete transforming growth factor (TGF)-β1 and interleukin-10 and may express membrane-bound TGF-β1, the levels of which are increased upon stimulation in vitro. Recent murine studies indicate that FOXP3 (murine counterpart is Foxp3, a member of the forkhead or winged helix family of transcription factors) is thought to be the most reliable marker for Tregs [12, 13]. Many studies in murine models have shown that the depletion of Tregs can amplify antitumor immunity [14]. Moreover, in humans, many studies have revealed that the population of Tregs in tumor-infiltrating lymphocytes is significantly higher than in normal tissues in several malignancies [15, 16]. Recent studies in humans showed that induced FOXP3+ Tregs in vitro did not always have a suppressive function but primary FOXP3+ Tregs in vivo were functionally suppressive and did not express effector cytokines. FOXP3 remains an accurate marker to define primary Tregs in humans [17].

As seen in recent studies, indoleamine 2,3-dioxygenase (IDO)-expression antigen-presenting cells potently suppress host antitumor T cell responses and induce tolerance to tumor-derived antigens [18, 19]. More recently, it has been postulated that IDO+ cells might promote the development of Tregs in tumor-draining lymph nodes [20, 21]. IDO is a catalyzing enzyme of tryptophan along the kynurenine pathway [22]. The activity of IDO in the mouse placenta has an important role in preventing the rejection of allogenic fetuses [23]. IDO seems to block the proliferation of alloreactive T lymphocytes by the local depletion of tryptophan. Furthermore, it has been found that the IDO is produced by tumor cells in order to resist attacks by the host immune system [24], and that some human tumors (uterine cervical cancer, colorectal cancer and pancreas cancer) also constitutively express IDO [25–27]. But the relationship between tumoral IDO and Tregs remains unclear. The aim of this study was to investigate the clinical significance of Tregs and IDO in the tumor microenvironment during the progression and multistage carcinogenesis of IPMNs. We also investigated the relationship between IDO expression in the tumor and tumor-infiltrating lymphocytes, especially Tregs.

Table 1. Characteristics of patients with IPMNs

Total cases	39
Age, years (median, range)	64 (47–78)
Gender, male/female	30/9
Pathological diagnosis[1]	
IPMA/IPMB/IPMC/I-IPMC	11/9/7/12
Tumor location, head/body/	
tail/two or three lesions	20/9/3/7
Tumor size, mm (median, range)	32 (2–80)
Mucus nodule, mm (median, range)	
(presence/absence)	15 (2–40) (29/10)
Main pancreatic duct diameter, mm	
(median, range)	8 (2–35)
Cystic dilated branch diameter, mm	
(median, range)	34 (10–75)
CEA, ng/ml (median, range)	2.8 (1–63)
CA19-9, ng/ml (median, range)	24 (1–10,000)

Pancreas ductal carcinomas, 8 cases. Non-neoplastic pancreas lesions, 6 cases.

[1] Classified according to WHO classification (IPMA = IPMN with low-grade dysplasia; IPMB = IPMN with moderate dysplasia; IPMC = noninvasive IPMC; I-IPMC = invasive IPMC).

Methods

Patients and Samples

Thirty-nine patients with IPMNs who underwent surgical treatment at Yokohama City University Hospital between 1993 and 2007, as well as 8 patients with pancreatic ductal carcinoma and 6 patients with non-neoplastic pancreas lesions (cholangiocarcinoma) were included in the study. All the patients had not received any prior therapy. All patients with IPMNs and PDCs received standard therapy appropriate for their clinical stages. Tumors were classified according to the WHO classification, the International Union Against Cancer tumor node metastasis classification, and the classification of pancreatic carcinoma of the Japan Pancreas Society. All patients had complete medical records and had been followed by the tumor registers for survival and outcome. The latest survival data were collected on September 30, 2008. The mean follow-up was 44.9 months for patients with IPMNs. The clinicopathologic features of the patients are summarized in table 1.

Immunohistochemical Analysis

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections as described previously [28]. We resected 4-μm-thick sections of representative blocks with monoclonal antibodies against the following: CD8 (4B11; 1:40) from AbD Serotec Laboratories, Ltd. (MorphoSys, UK), and FOXP3 (236A/E7; 1:50) from Abcam Laboratories, Ltd. (Cambridge Science Park, UK), and IDO (IDO-MCA; 1:400) from Oriental Yeast Co., Ltd. (Shiga, Japan). Briefly, the sections were deparaffinized and rehydrated. After blocking of endogenous peroxidase with methanol containing 0.3% H_2O_2, the sections were autoclaved at 121C for 10 min in citrate buffer (10 mmol/l sodium citrate; pH 6.0) for antigen retrieval. After blocking with normal goat serum, the sections were reacted overnight with appropriately diluted primary antibodies. The sections were then reacted sequentially with biotin-conjugated anti-mouse IgG antibodies (Vector Laboratories, Burlingame, Calif., USA) and Vectastain Elite ABC reagent (Vector Laboratories). For staining CD8 and IDO, the EnVision+ Polymer system (DAKO, Glostrup, Denmark) was used as the chromogen, and the nuclei were counterstained with hematoxylin. Serial sections were prepared from each paraffin block. The first section was stained with HE and the second, third, fourth, and fifth sections were subjected to immunohistochemistry to detect the CD8, FOXP3 and IDO antigens.

Quantification Method

CD8+ and FOXP3+ lymphocytes were counted in the corresponding visual fields. Quantitative evaluation of the lymphocytes was carried out by analyzing five different high-power fields (×40 objective and ×10 eyepiece). The numbers of CD8+ and FOXP3+ lymphocytes were calculated for each case. IDO expression levels were classified semiquantitatively based on the percentage of tumor cells that stained with IDO and the staining intensity previously reported [29]. The percentage positivity was scored as 0 if <5% of the cells were stained (negative), 1 if 5–30% (sporadic), 2 if 30–70% (focal), and 3 if it was >70% (diffuse), whereas the staining intensity was scored as 0 if there was no staining, 1 if cells were weakly stained, and 2 if strongly stained (equal to the positive control level). The final IDO expression score was defined as follows: IDO– if the sum of the percentage positivity score and the staining intensity score was 0–1, IDO1+ if the sum was 2–3, and IDO2+ if the sum was 4–5. In this scoring system, IDO expression in the tumor stromal cells was not considered because IDO immunostaining in nontumor cells was not remarkable or absent in all the cases examined. In each case, five different areas were evaluated, and the mean of the results was considered to be the final IDO expression score. The scoring procedure was carried out by two independent observers (N.K., K.K.) without any knowledge of the clinical data.

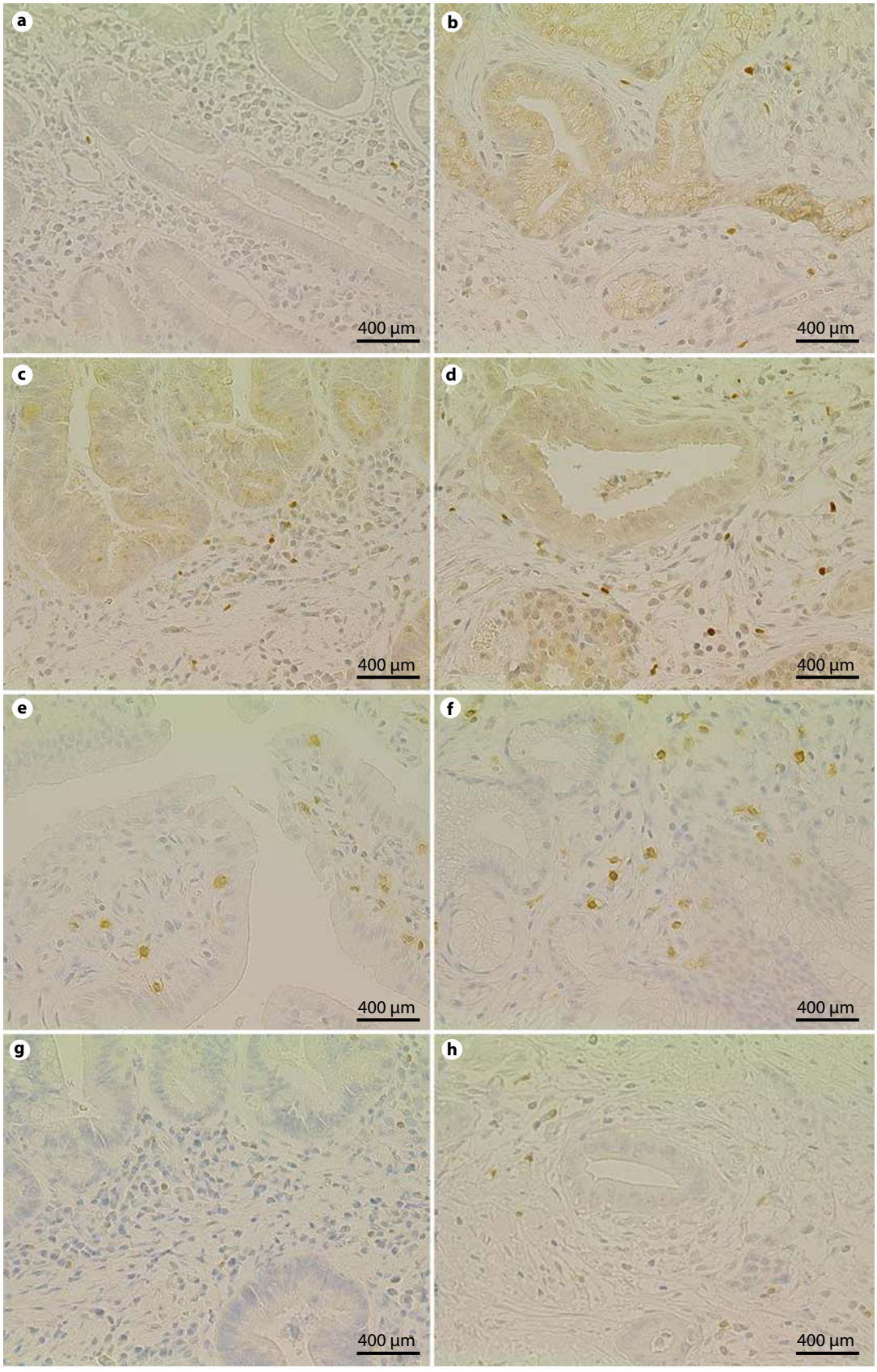
Statistical Analysis

Values were expressed as mean ± SD. Statistical analyses were performed with StatView-J 5.0 software (Abacus Concepts, Berkeley, Calif., USA). Associations among the variables were assessed by the χ^2, Student's t, Mann-Whitney U, and Kruskal-Wallis tests. If there was evidence of non-normality, the Mann-Whitney U test or the Kruskal-Wallis test was used to test the difference in medians among the groups. Survival rates were calculated by the Kaplan-Meier method. Differences between survival curves were analyzed by the log-rank test. To assess the correlation between survival time and multiple clinico-pathological parameters, multivariate analyses were performed by the Cox proportional hazards regression model. Differences were considered significant when $p < 0.05$.

Results and Discussion

FOXP3 immunoreactivity was detected at high levels and was localized to the nuclei of the lymphocytes (fig. 1a–d). The absolute numbers of FOXP3+ Tregs that had infiltrated I-IPMC was significantly higher than that of Tregs in healthy pancreas ($p < 0.0001$) (fig. 2a). Among the IPMN (IPMA, IPMB, IPMC, and I-IPMC), the absolute numbers of FOXP3+ Treg was significantly higher in the progression during multistage carcinogenesis of IPMNs (Kruskal-Wallis test $p < 0.0001$). The absolute numbers of CD8+ iELs and CD8+ T cells in IPMNs were significantly higher than that of CD8+ iELs and CD8+ T cells in healthy pancreas ($p < 0.0001$) (fig. 2b, c). Among the IPMN, the absolute number of CD8+ iELs and CD8+ T cells was significantly lower in the progression during multistage carcinogenesis of IPMNs (Kruskal-Wallis test $p < 0.0001$, $p = 0.0014$). We classified two groups (FOXP3 high or FOXP3 low) according to the absolute number of FOXP3+ Tregs. We also classified two groups (CD8+ iELs high or low and CD8+ T cells high or low). FOXP3 high was positively correlated with clinical subclassification (main duct type), tumor size, diameter of mucus nodule, maximum size of main pancreatic duct and histological grade ($p = 0.0414$, $p = 0.0066$, $p = 0.0015$, $p = 0.0004$, $p < 0.0001$) (table 2). The FOXP3 high group was inverse correlated with the absolute number of CD8+ iELs and CD8+ T cells ($p < 0.0001$, $p = 0.0012$). Overall survival and disease-free survival were analyzed in these patients. 34 of 39 (87.2%) patients survived >5 years after surgical resection and 31 of 39 (79.5%) patients had no recurrence >5 years. Every patient of the FOXP3 low group or the CD8+ T cells high group survived >5 years (fig. 3a, c).

IDO immunoreactivity was detected at variable levels and was localized to the cytoplasm of tumor cells (fig. 1i–k). In contrast, IDO immunoreactivity in the tumor stroma was very faint or absent. Based on the IDO expression score in the tumor, all cases were classified into two groups: high IDO expression (IDO2+) and no or low IDO expression (IDO– or IDO1+). Of the 39 cases, high IDO expression was found in 5 cases (4 cases were I-IPMC and 1 case was IPMC), whereas IDO– and IDO1+ tumors were found in 34 cases. IDO immunoreactivity in the



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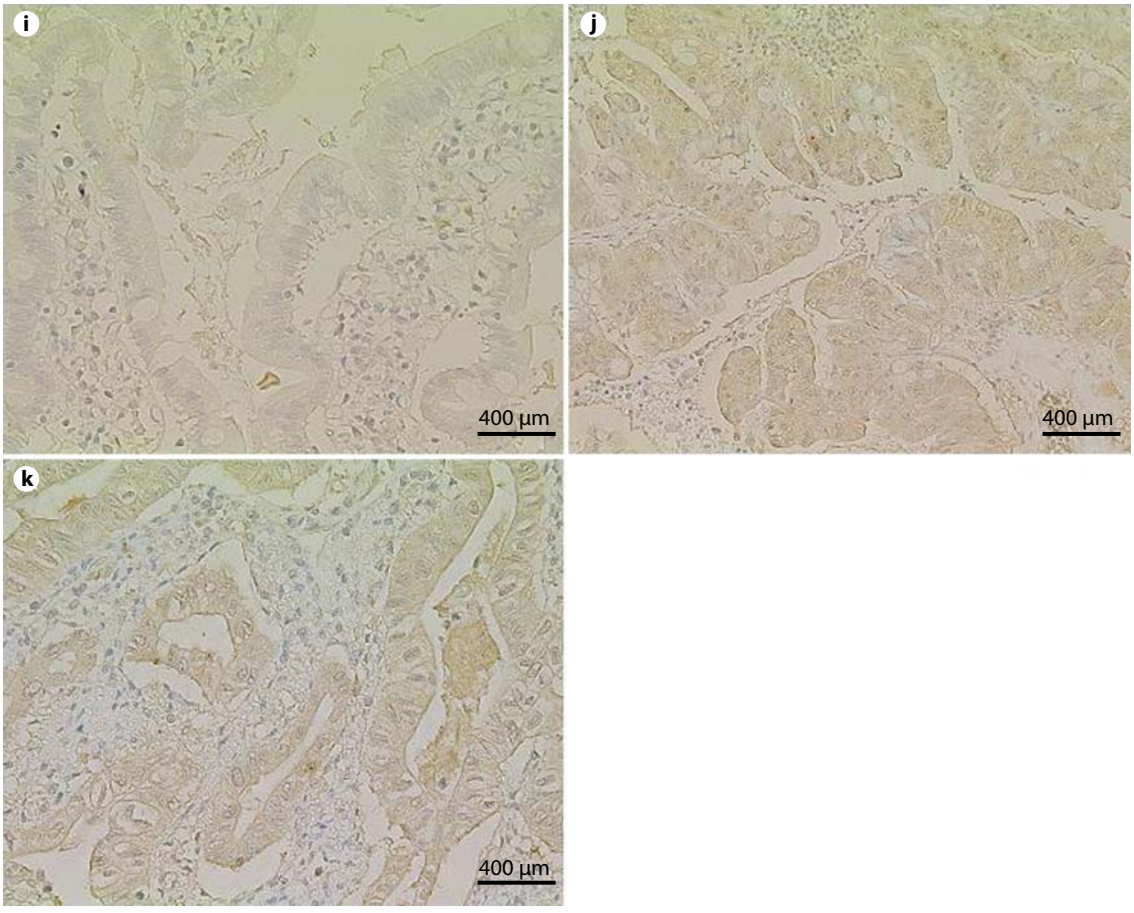

Fig. 1. Increased population of Tregs in tumor stroma corresponding to the progression of IPMNs and PDCs. Immunostaining with FOXP3: **a** adenoma, **b** borderline malignancy, **c** noninvasive carcinoma, **d** invasive carcinoma. Decreased population of CD8+ T cells in tumor stroma and intra-epithelial lymphocytes. Immunostaining with CD8: **e** adenoma, **f** borderline malignancy, **g** noninvasive carcinoma, **h** invasive carcinoma. IDO protein expression in the tumor of IPMN shown by immunohistochemistry. **i** IDO protein did not express in IPMA. **j** IDO protein expressed in I-IPMC. **k** PDC.

nontumoral healthy pancreatic lesion was absent. On the other hand, high IDO expression was found in 7 cases (87.5%) in the PDCs. Low IDO expression was 1 case in PDC. We analyzed the clinicopathological features of IPMNs and tumoral IDO expression. High IDO expression was positively correlated with tumor size, maximum size of dilated branch, TNM stage and tumor markers (CEA, CA19–9) ($p = 0.0258$, $p = 0.0478$, $p = 0.0427$, $p = 0.0265$, $p = 0.0075$) (table 3). IDO high or IDO low was correlated with overall survival and disease-free survival of IPMNs ($p = 0.0015$, $p = 0.0078$) (fig. 3d).

We analyzed IDO expression in the tumor correlated with the number of FOXP3+ Tregs, CD8+ T cells and CD8+ iELs in the tumor stroma. High IDO expression was positively correlated with the number of FOXP3+ Tregs ($p = 0.0066$) but high IDO expression was not correlated with the number of CD8+ iELs in the tumor stroma and CD8+ T cells ($p = 0.4946$, $p = 0.1606$) (table 3).

The idea of generating cytotoxic T lymphocytes that have antitumor activity has been the focus of many clinical trials aimed at delivering effective immunotherapy to cancer patients [30]. It is now clear that although various vaccination methods are capable of inducing tumor antigen-specific T cells in the circulating blood, these immunological responses are not correlated with clinical responses. Mechanisms of immunoevasion at the effector phase are currently under investigation with an increasing focus on the tumor microenvironment [31]. In the

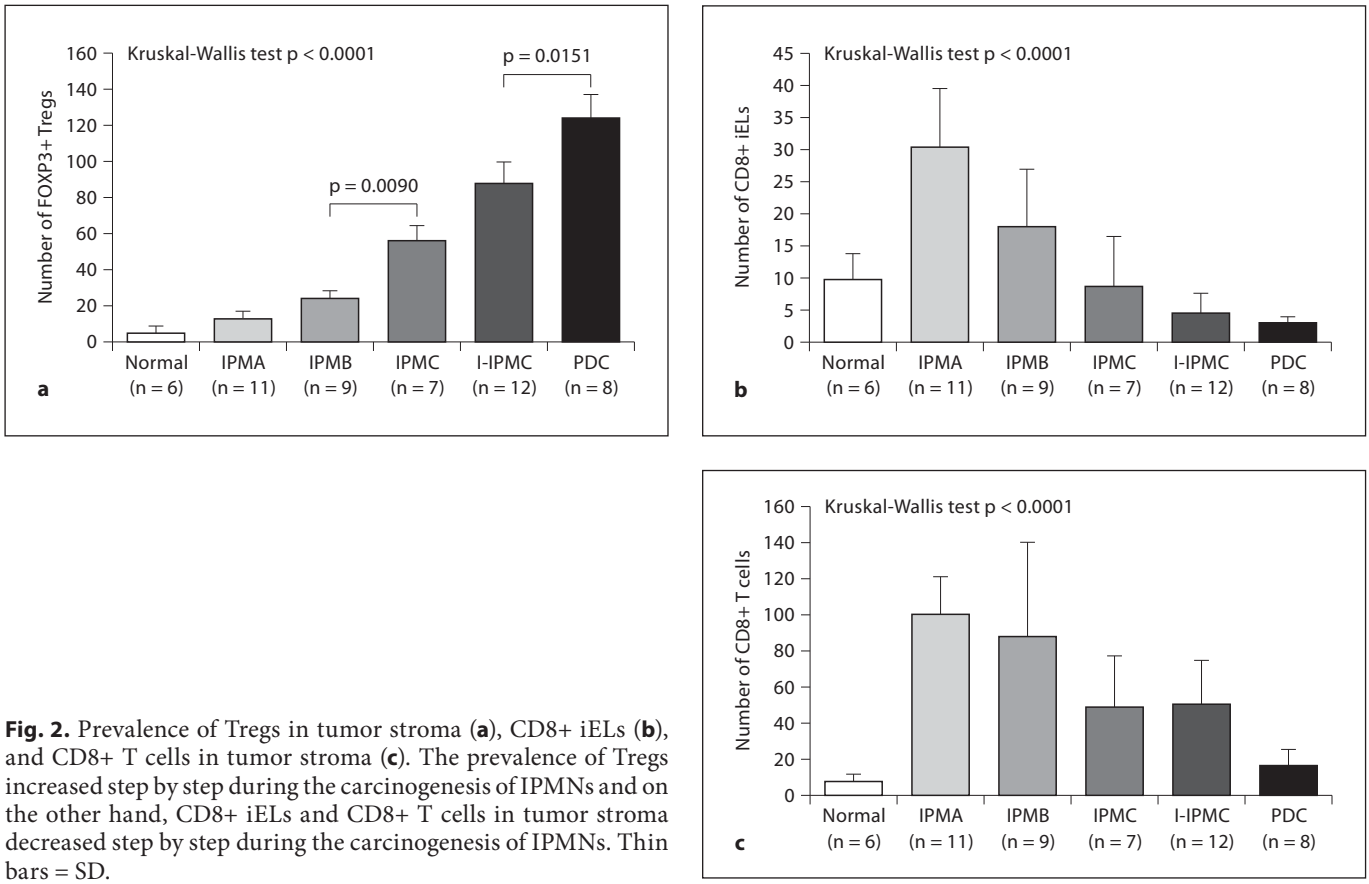

Fig. 2. Prevalence of Tregs in tumor stroma (**a**), CD8+ iELs (**b**), and CD8+ T cells in tumor stroma (**c**). The prevalence of Tregs increased step by step during the carcinogenesis of IPMNs and on the other hand, CD8+ iELs and CD8+ T cells in tumor stroma decreased step by step during the carcinogenesis of IPMNs. Thin bars = SD.

Table 2. Correlation between clinicopathologic findings and prevalence of Tregs

	High Tregs	Low Tregs	p
Age, years (mean ± SD)	66.0 ± 7.638	67.3 ± 7.442	0.5936
Gender, male/female	16/3	14/6	0.2991
Radiological subclass, main duct type/branch duct type	11/8	5/15	0.0414*
Tumor size, mm (median/range)	41.947 ± 20.942	23.2 ± 12.891	0.0066*
Tumor location, Ph included/Ph excluded	13/6	14/6	0.9150
Mucus nodule (EUS finding), mm (median/range)	18.211 ± 11.253	6.725 ± 11.128	0.0015*
Maximum dilated branch (EUS finding), mm (median/range)	43.632 ± 19.842	30.250 ± 10.020	0.0592
Main pancreatic duct (EUS finding), mm (median/range)	13.894 ± 7.333	6.600 ± 4.223	0.0004*
Pathological grade, IPMA/IPMB/IPMC/I-IPMC	0/1/6/12	11/8/1/0	<0.0001*
TNM stage (UICC), stage 0/stage 1/stage 2/stage 3/stage 4a/stage 4b	6/3/5/1/3/0	1/0/0/0/0/0	0.9708
CEA, ng/ml	10.067 ± 17.473	3.305 ± 2.569	0.0954
CA19-9, ng/ml	597.333 ± 2,346.817	259.503 ± 16.109	0.2761
Number of CD8+ iELs (median/range)	3.843 ± 3.005	26.750 ± 21.570	<0.0001*
Number of CD8+ T cells infiltrating tumor (median/range)	52.474 ± 28.385	92.700 ± 40.459	0.0012*

* Significant.

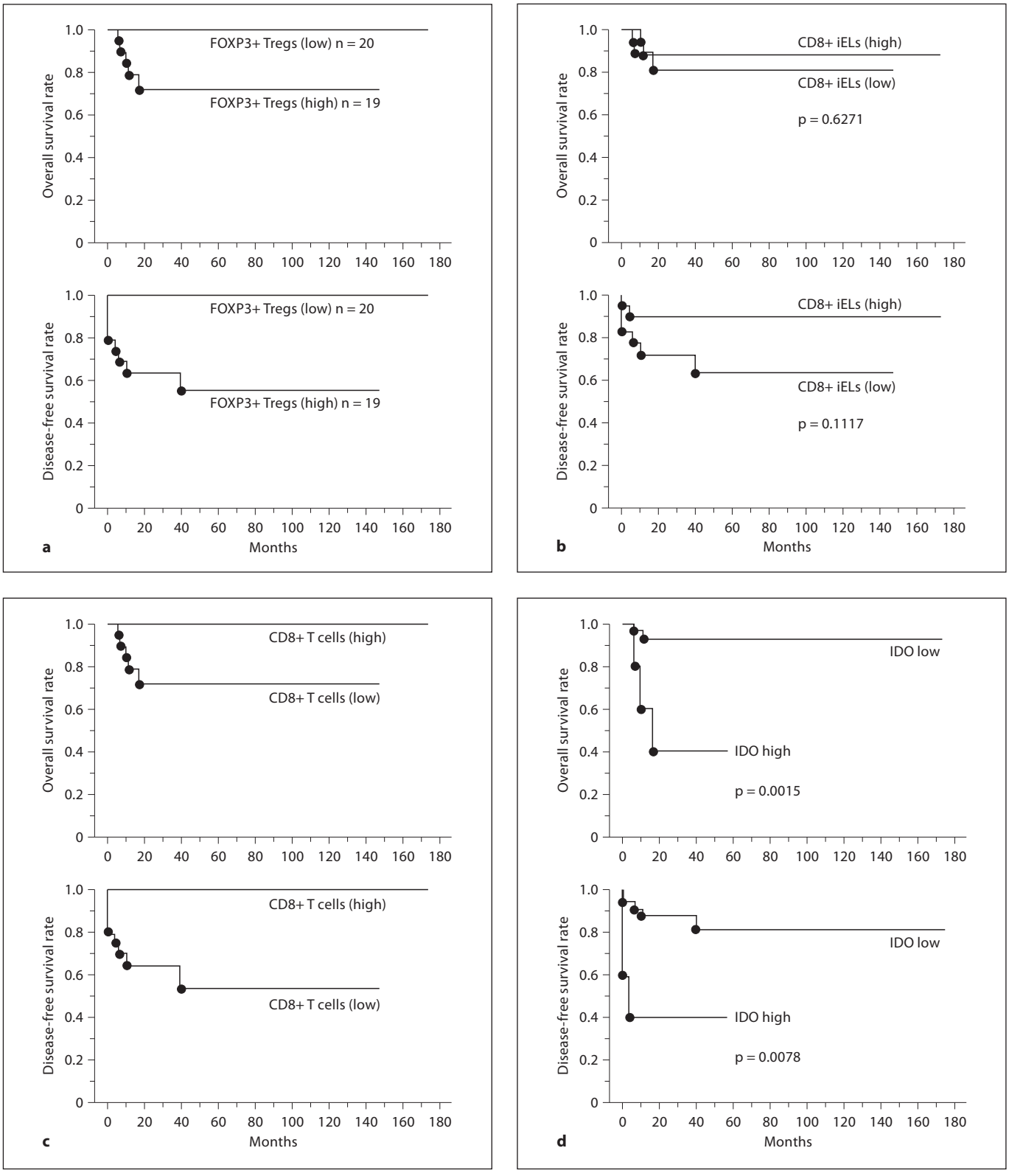

Fig. 3. Kaplan-Meier survival curve of IPMNs. Overall survival and disease-free survival were worse in the high Tregs group (**a**), low CD8+iEL group (**b**), and low CD8+ T cells group (**c**) than in the other groups. Overall survival and disease-free survival were significantly worse in the IDO protein expression group (**d**) than in the nonexpression group.

Table 3. Correlation between clinicopathologic findings and tumoral IDO expression

	IDO high	IDO low	p
Age, years (mean ± SD)	68.800 ± 7.694	66.353 ± 7.499	0.5011
Gender, male/female	5/0	25/9	0.9752
Radiological subclass, main duct type/branch duct type	2/3	16/23	0.9602
Tumor size, mm (median/range)	51.400 ± 20.971	29.529 ± 17.950	0.0258*
Tumor location, Ph included/Ph excluded	5/0	22/12	0.9710
Mucus nodule (EUS finding), mm (median/range)	21.000 ± 14.748	11.044 ± 11.826	0.1376
Maximum dilated branch (EUS finding), mm (median/range)	53.800 ± 20.303	34.265 ± 15.002	0.0478*
Main pancreatic duct (EUS finding), mm (median/range)	10.600 ± 5.320	10.088 ± 7.200	0.5549
Pathological grade, IPMA/IPMB/IPMC/I-IPMC	0/0/1/4	11/9/6/8	0.9747
TNM stage (UICC), stage 0/stage 1/stage 2/stage 3/stage 4a/stage 4b	1/0/2/0/2/0	6/3/3/1/1/0	0.0427*
CEA, ng/ml	17.880 ± 25.057	4.785 ± 8.826	0.0265*
CA19-9, ng/ml	2,043 ± 4,448.143	27.091 ± 27.841	0.0075*
Number of FOXP3+ Tregs infiltrating tumor (median/range)	92.00 ± 70.299	39.588 ± 31.974	0.0066*
Number of CD8+ iELs (median/range)	10.000 ± 9.274	16.412 ± 20.295	0.4946
Number of CD8+ T cells infiltrating tumor (median/range)	49.400 ± 12.422	76.588 ± 41.754	0.1606

* Significant.

present study, we investigated the relationship between host immunoevasion mechanism and multistage carcinogenesis of IPMNs of the pancreas, especially focusing on FOXP3+ Tregs and IDO expression in the tumor cells. First, the number of FOXP3+ Tregs infiltrating in the tumor microenvironment was increased according to the multistage carcinogenesis of IPMNs. Even in the stroma of IPMA, some lymphocytes are FOXP3+ Tregs. These finding suggest that tumor evasion from host antitumor immunity already begins in these premalignat lesions in the pancreas, and, according to the progression of carcinogenesis, FOXP3+ Tregs were gradually increased. The increase of FOXP3+ Tregs might mean a more suppressive microenvironment of antitumor immunity, suggesting that FOXP3+ Tregs might play the role of promoting the carcinogenesis of IPMNs. The prevalence of tumor-infiltrating Tregs increased in a stepwise manner, whereas the prevalence of CD8+ T cells and CD8+ iELs decreased during the progression of carcinogenesis of IPMNs. The decreased number of CD8+ T cells and CD8+ iELs could also be important for tumor progression through the immunosuppressive microenvironment of the IPMNs. According to past reports, FOXP3+ Tregs were increased in a variety of tumors and a few studies have also investigated whether the increase of FOXP3+ Tregs is associated with poor prognosis in ovarian, pancreas and hepatocellular carcinoma [7, 15, 16]. But no prognostic influence of Tregs was found in anal squamous cell carcinomas [32] and an increase in Tregs was associated with a good prognosis in Hodgkin's lymphoma [33] and the conclusion about its correlation with prognosis was contradictory. In this study, the absolute number of Tregs was significantly correlated with overall survival and disease-free survival. On the other hand, CD8+ T cells and CD8+ iELs are associated with good prognosis in some cancers [7, 34, 35]. In this study, the absolute number of CD8+ T cells in the cancer stroma was also correlated with good prognosis. A positive prognostic effect of infiltrating CD8+ T cells has been reported in various solid cancers but a negative prognostic effect of CD8+ T cell infiltration has been observed in EB virus-associated nasopharyngeal carcinoma and human papilloma virus-associated anal carcinomas [32, 36].

In a recent study, FOXP3+ Tregs proliferated extensively in response to dendric cells, processing protein antigens in the steady state or after maturation in vivo in the lymph nodes [37]. The IDO+ dendric cell (DC) efficiently suppressed all T cell responses to a particular antigen, despite the fact that the same antigen was presented by many other IDO– antigen-presenting cells. IDO+ DCs might promote Tregs development in the tumor-draining lymph node [38]. But little is known about that effect of other IDO+ cells on FOXP3+ Tregs in the tumor microenvironment. In our study, the cases of IDO expression in the tumor were positively correlated with the number of Tregs in the cancer stroma. IDO expression in the tu-

mor might influence the recruitment and/or maintenance and/or prolongation of FOXP3+ Tregs. Also, the cases of IDO expression in the tumor decreased the number of CD8+ T cells in the cancer stroma. According to past reports, high IDO expression had a significantly reduced number of stoma CD3+ or CD8+ cells in the cancer and the activation of IDO is critically involved in the regulation of immune responses to establish the immune tolerance of pregnant mice on their fetuses, or to induce T cell unresponsiveness [39]. Cell proliferation of alloreactive T cells is thereby arrested in the G1 phase of the cell cycle via local tryptophan deprivation and the accumulation of toxic, proapoptotic catabolites [40]. In this study, IDO expression in the cancer had an influence on increasing the number of FOXP3+ Tregs and decreasing the number of CD8+ T cells in the tumor microenvironment. In our study, IDO was only expressed in the invasive or noninvasive carcinoma, but adenomas or borderline tumors were not expressing IDO in the tumor. Three cases of tumoral IDO expression were tumor death and 2 cases of tumoral IDO expression were recurrence. High-level expression of IDO was one of the most reliable makers for the prognosis of IPMNs. According to past reports, IDO expression in the tumor was one of the reliable prognosis factors in colorectal, endometrial and ovarian cancers [26, 29, 41].

In conclusion, FOXP3+ Tregs play a role in controlling the immune surveillance against IPMNs of the pancreas from the premalignant stage to established carcinoma. Only established cancers acquire a high level of IDO protein in the tumor of IPMNs. High-level IDO expression in the tumor might influence FOXP3+ Tregs and CD8+ T cells and maintaining an immunosuppressive microenvironment. The number of FOXP3+ Tregs and the high level expression of tumoral IDO were both reliable markers to predict poor prognosis and recurrence of IPMNs.

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