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COX-2 expression and the prevalence of regulatory T cells in tumor and non-tumor sites of hepatocellular carcinoma

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Abstract

Aim: The expression of cyclooxygenase-2 (COX-2) plays a role in the differentiation and guidance of regulatory T cells (FOXP3$^+$ Tregs), and this mechanism has also been studied extensively in hepatocellular carcinoma (HCC). In this study, we investigated the expression of COX-2 and prevalence of FOXP3$^+$ Tregs in tumor and non-tumor sites to elucidate their association with the clinicopathological features of HCC and disease outcome.

Method: This study involved 44 patients with HCC who had undergone hepatectomy without any preoperative treatment. Paraffin-embedded nodules (n=44) were sectioned for immunostaining with COX-2 and FOXP3 monoclonal antibodies, and the degree of COX-2 expression and prevalence of FOXP3$^+$ Tregs were measured.

Results: COX-2 expression in the non-tumor sites showed a positive correlation with the number of FOXP3$^+$ Tregs ($p<0.001$). In addition, in the non-tumor sites, the high FOXP3$^+$ Tregs prevalence group was significantly associated with TNM stages ($p=0.003$) and AFP ($p=0.027$). The expression of COX-2 in the non-tumor sites was also significantly associated with disease-free survival ($p=0.005$).

Conclusion: The present findings suggest the association of COX-2 expression in the non-tumor sites with disease-free survival and thus the recurrence of HCC. In addition, COX-2 expression and the prevalence of FOXP3$^+$ Tregs have been positively correlated in the non-tumor sites, indicating that their interaction influences the outcome of HCC. To prevent the recurrence of HCC, it may be necessary to inhibit the expression of COX-2.

Key words: hepatocellular carcinoma, COX-2, FOXP3, immunostaining

Introduction

Hepatocellular carcinoma (HCC) is a frequently encountered malignant tumor and accounts for 90% of primary liver cancer cases. Despite the availability of various treatments including hepatectomy, radiofrequency thermal ablation (RFA), microwave coagulation therapy (MCT), transcatheter arterial chemoembolization (TACE), and the use of novel molecularly targeted agents (such as sorafenib), HCC remains a disease with poor prognosis and is in need of better treatment methods.

There have been many reports on the association between immunoreactive characteristics of HCC and disease prognosis, and the most representative example of this is regulatory T cells (Tregs). Tregs, a subset of T cells that function specifically in immune suppression, account for 5-10% of CD4$^+$ T cells and induce immunological tolerance.[1,2] However, Tregs also suppress tumor immunity and play a role in the development and progression of tumors.[3] A transcription factor forkhead and winged helix family of transcription factor P3 (FOXP3) is specifically induced in Tregs and is currently

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used as a marker to assess the prevalence of Tregs.[3–5] There are two known subtypes of Tregs : naturally occurring Treg (T_R^{nat}), which is produced in the thymus, and adaptive Treg (T_R^{adapt}), which is derived from naïve $CD4^+$ T cells in periphery. Differentiation into T_R^{adapt} occures mainly in a cell-to-cell contact dependent manner in the vicinity of tumors.[6] Analyzing tumor-infiltrating lymphocytes (TILs), many studies have reported the association between the number of Tregs and outcome of HCC.[7–10] Tregs, which are often associated with poor prognosis, are abundant in tumors and are thus identified within TILs. These Tregs are considered to be differentiated in the periphery.

Cyclooxygenase-2 (COX-2) is the major enzyme in the arachidonic acid cascade and is involved in the synthesis of prostaglandins from arachidonic acid. It is overexpressed during inflammation, cell growth, differentiation, and tumorigenesis.[11,12] In addition, COX-2 is upregulated in different types of cancers, and poor prognosis is the common characteristic of cancers overexpressing COX-2. In HCC, COX-2 is overexpressed not only in tumor site, but also in non-tumor site, and its expression in both sites is reportedly to be associated with disease outcome.[12–16]

The relationship between the differentiation and activation of T_R^{adapt} and the arachidonic acid cascade has been shown in recent years. Mahic et al. reported that the differentiation and activation of T_R^{adapt} depends on the synthesis of prostaglandin E_2 (PGE_2) following the expression of COX-2.[17,18] If this is the case, both tumor and non-tumor sites can promote the differentiation and activation of T_R^{adapt}. It is therefore of interest to investigate which population of T_R^{adapt} is dominantly associated with poor prognosis of HCC.

We investigated the expression of COX-2 and prevalence of $FOXP3^+$ Tregs in tumor and non-tumor sites using immunohistochemical analyse to evaluate the possible correlation between the two parameters. We conducted clinicopathological analyse to determine whether they influence the outcome of HCC.

Method

Patients and Specimens

This was a retrospective study of 44 patients with HCC (44 paraffin-embedded hepatic nodules) who had undergone hepatectomy at Kinki University Hospital between July 2005 and December 2007. We selected cases without any preoperative treatments such as RFA or TACE.

Because all subjects had been under observation at our hospital, we had access to their complete clinical records, which included the date of recurrence and death. The last observation was performed on May 31, 2011, and the period of observation was 2.9-86.7 months (mean, 35.1 months).

Clinicopathological features were age, sex, hepatitis virus infection (HBsAg-positive, HCV-Ab-positive, and both negative [Non-B/Non-C]), the Child-Pugh classification, the TNM classification, degrees of differentiation (WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated), alpha-fetoprotein (AFP) level, portal vein invasion (VP), intrahepatic metastasis (IM), and tumor size (Table 1). None of the patients with hepatitis virus infection were positive for both HBsAg and HCV-Ab. Because all patients had Child-Pugh class A cirrhosis, this factor was excluded from other analyses. In accordance with the 7th edition of the American Joint Committee on Cancer/the International Union Against Cancer (AJCC/UICC) TNM Classification System, cases were classified into Stage I (n=18), Stage II (n=10), Stage IIIa (n=11), Stage IIIb (n=4), and StageIVa (n=1).

Immunohistochemical Staining of COX-2 and FOXP3 (Fig. 1)

Thin sections (4 μm in thickness) were made from paraffin blocks of hepatic nodules and placed on glass slides for immunostaining. Paraffin was removed from sections using xylene

Table 1 Clinicopathologic features of patients.

Variable	Value
Age, years (median, range)	66.5, 47-82
Gender (male/female)	36/8
Virus infection [HBV/HCV/Non-BNonC]	17/21/6
Child-Pugh classification (A/B/C)	44/0/0
TNM stage (I/II/III/IV)	18/10/15/1
Histologic grade (WD HCC/MD HCC/PD HCC)	10/27/7
AFP, ng/ml (median, range)	19, 1-44001
VP (presence/absence)	19/25
IM (presence/absence)	15/29
Tumor size, mm (median, range)	41.4, 11–162

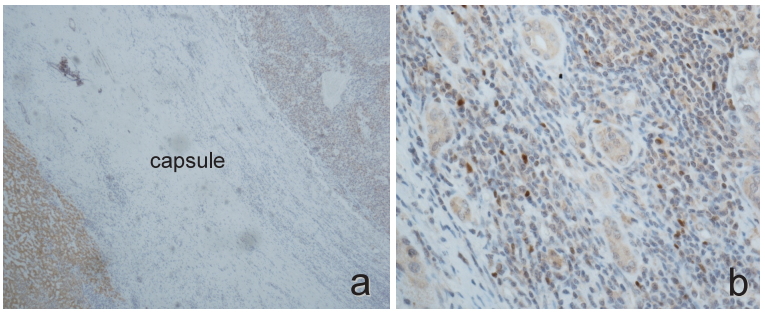

Fig. 1 A representative result of immunostaining analysis using a monoclonal antibody against COX-2 (a) and FOXP3 (b). In this particular case, the COX-2 antibody reacted to the non-tumor site (a, on the left of capsule) more strongly than to tumor site (a, on the right of capsule) (40× magnification). In non-tumor site in the vicinity of cancer, FOXP3$^+$ Tregs-positive cells have a clearly stained nucleus (b).

and alcohol. Antigen retrieval for COX-2 and FOXP3 was performed by autoclaving at 121 °C for 20 min with citrate buffer (pH 6) and Tris-EDTA buffer (pH 9) [1 M Tris-HCl (pH 7.5) : 0.5 M EDTA (pH 8) : H_2O = 1 : 0.2 : 100], respectively. To block endogenous peroxidase, a 30-min incubation with 0.3% H_2O_2 in methanol (30% H_2O_2 : methanol = 1.5 : 150) was performed for COX-2, and a 10-min incubation with 3% H_2O_2 in distilled water (30% H_2O_2 : distilled water = 15 : 135) for FOXP3. For the primary antibody reaction, mouse monoclonal IgG antibody against COX-2 (1 : 100 dilution ; DAKO Inc., Tokyo, Japan) and mouse monoclonal IgG antibody against FOXP3 (1 : 25 dilution ; Abcam Co., Tokyo, Japan) were used to incubate sections at 4 °C for 24 h. The secondary antibody reaction was performed at room temperature for 60 min using Envision+system-HRP Labelled Polymer (DAKO), and peroxidase products was developed using phosphate buffer saline (PBS) : diaminobenzidine (DAB) : H_2O_2 = 150 ml : 60 mg : 45 μl. Sections were washed 3 times for 5 min each between steps with PBS for COX-2 and Tris buffer saline (TBS) for FOXP3. Optimum staining conditions were determined using positive and negative controls.

Immunostaining evaluation methods

COX-2 immunoreactivity in tumor and non-tumor sites was expressed as a fraction of cells positive for COX-2. After immunostaining, images of entire sections were captured, and the area with or without tumors was measured in ImageJ software (National Institutes of Health, MD). Then, the total area of COX-2 positive cells in both tumor and non-tumor sites was calculated to determine the fraction (%) of positive cells. Scores of 0 to 4 were used to grade the expression rates (0, negative ; 1, 1-25% ; 2, 26-50% ; 3, 51-75% ; and 4, 76-100%), and scores of 3 and higher were considered to be COX-2-positive.[19,20] FOXP3$^+$ Tregs were counted in tumor sites as well as non-tumor sites within two high-power fields of the tumor margin. The total number of FOXP3$^+$ Tregs observed in 10 high-power fields was used in analysis. When a tumor was surrounded by a capsule, we counted FOXP3$^+$ Tregs-positive cells that were present outside the capsule but within two high-power fields of the capsule. Using the median value of FOXP3$^+$ Tregs-positive cell numbers, we divided samples into high and low prevalence groups. Evaluation of immunostaining was performed by two pathologists who were otherwise not involved in the present study.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software, and the correlation between the two groups was analyzed using the $\chi 2$ test, Student's t test, Mann-Whitney U test, and the Kruskal-Wallis test. The Kaplan-Meier method and log-rank test were performed to estimate survival rate and differences between survival curves, respectively. Multivariate and univariate survival analyse were performed using Cox's regression model to identify the association between clinicopathological features, results of immunostaining, and disease prognosis. For analysis, the median value was used to divide the category of age, AFP, and tumor size into two groups for comparison. Differences were considered significant at $p < 0.05$.

Results

The relationship between COX-2 expression and the number of FOXP3$^+$ Tregs

Based on the degree of COX-2 expression, samples were divided into COX-2 positive and negative groups, and we counted the number of FOXP3$^+$ Tregs in each group. In tumor sites, the total number of FOXP3$^+$ Tregs in 10 high-power fields was 8.5±17.5 (median±SD), with 3.0±6.8 in the COX-2-positive group and 9.5±21.9 in the negative group. No correlation was found between the number of FOXP3$^+$ Tregs and the expression of COX-2 (Kruskal-Wallis test, P=0.226) (Fig. 2a). In non-tumor sites, the total number of FOXP3$^+$ Tregs in 10 high-power fields was 11.5±20.6, with 18.0±23.1 in the COX-2-positive group and 6.0±11.4 in the negative group, showing that the number of FOXP3$^+$ Tregs was significantly increased in the COX-2-positive group (Kruskal-Wallis test, $p<0.001$) (Fig. 2b).

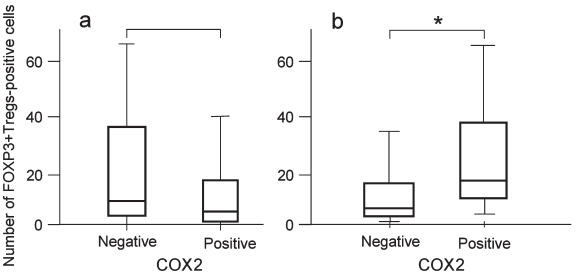

Fig. 2 The relationship between COX-2 expression and the number of FOXP3$^+$ Tregs in tumor (a) and non-tumor (b) sites. A horizontal line in each box represents the median value. While the number of FOXP3$^+$ Tregs had no correlation with COX-2 expression in tumor sites (a ; Kruskal-Wallis test, p=0.226), the two factors were significantly correlated in non-tumor sites (b ; Kruskal-Wallis test, *$p<0.001$).

Table 2a Correlation between clinicopathologic findings and the prevalence of COX-2 in tumor sites.

	Prevalence of COX-2		
Variable	Positive	Negative	*P* value
Age, years (mean±SD)	65±8.3	70±7.0	0.451†
Gender (male/female)	24/2	12/6	**0.030‡**
Viral infection HBV(+)/HCV(+)/nonBC	13/9/4	4/12/2	0.102‡
TNM stage (I/II/III/IV)	11/6/9/0	7/4/6/1	0.555‡
Histologic grade (WD/MD/PD)	7/17/2	3/10/5	0.187‡
AFP, ng/ml (median, range)	55, 1-44001	9, 2-17120	0.138§
VP (presence/absence)	11/15	8/10	0.888‡
IM (presence/absence)	9/17	5/13	0.632‡
Tumor size, mm (median, range)	40, 10-162	47, 16-140	0.960§

† Student's t test ; ‡ $\chi 2$ test ; § Mann-Whitney U test

Table 2b Correlation between clinicopathologic findings and the prevalence of COX-2 in non-tumor sites.

	Prevalence of COX-2		
Variable	Positive	Negative	*P* value
Age, years (mean±SD)	66±7.2	67±8.5	0.293†
Gender (male/female)	15/3	21/5	0.828‡
Viral infection HBV(+)/HCV(+)/nonBC	6/6/6	11/15/0	**0.006‡**
TNM stage (I/II/III/IV)	5/4/9/0	13/6/6/1	0.314‡
Histologic grade (WD/MD/PD)	5/10/3	5/17/4	0.771‡
AFP, ng/ml (median, range)	266, 2-28576	19, 1-44001	0.441§
VP (presence/absence)	9/9	10/16	0.447‡
IM (presence/absence)	8/10	7/19	0.402‡
Tumor size, mm (median, range)	49, 10-162	39, 16-140	0.453§

† Student's t test ; ‡ $\chi 2$ test ; § Mann-Whitney U test

Association between COX-2 expression, FOXP3$^+$ Tregs, and clinicopathologic features

COX-2 expression

In tumor sites (Table 2a), the COX-2-positive group had a significantly higher number of male patients (P=0.003). In non-tumor sites (Table 2b), the expression of COX-2 was significantly higher in Non-B/Non-C patients (P=0.006). Other findings were not significant.

FOXP3$^+$ Tregs

There were no significant correlations in tumor sites (Table 3a). In non-tumor sites (Table 3b), the high prevalence group was significantly associated with higher TNM stages (p=0.003) and high levels of AFP (p=0.027).

Association between COX-2 expression, FOXP3$^+$ Tregs, and disease prognosis

The overall 5-year survival rate was 48.8%, and the 3-year disease-free survival rate was 35.0%. The log-rank test was performed to analyze the 5-year survival rate and 3-year disease-free survival rate in COX-2-positive and negative groups in tumor and non-tumor sites, and high and low FOXP3 prevalence groups, to determine the differences between each two groups (Table 4). In non-tumor sites, there was a significant difference in the 3-year disease-free survival rate between COX-2-positive and negative groups (p=0.005) (Fig. 3). In non-tumor sites, the high FOXP3 prevalence group which showed a correlation with COX-2 expression, tended to be associated with shorter 3-year disease-free survival rates (p=0.080) (Fig.4).

In addition, using 11 variables including clinicopathologic features, COX-2 expression, and the prevalence of FOXP3$^+$ Tregs, we perfor-

Table 3a Correlation between clinicopathologic findings and the prevalence of Tregs in tumor sites.

	Prevalence of FOXP3+ Tregs		
Variable	High	Low	*P* value
Age, years (mean±SD)	68.0±6.0	64.6±6.2	0.887†
Gender (male/female)	17/5	19/3	0.446‡
Viral infection HBV(+)/HCV(+)/nonBC	9/10/2	8/11/3	0.879‡
TNM stage (I/II/III/IV)	6/7/8/1	12/3/7/0	0.388‡
Histologic grade (WD/MD/PD)	6/14/2	4/13/5	0.232‡
AFP, ng/ml (median, range)	10, 1-15280	71, 2-44001	0.091§
VP (presence/absence)	8/14	11/11	0.373‡
IM (presence/absence)	5/17	9/13	0.344‡
Tumor size, mm (median, range)	43, 17-140	40, 10-162	0.362§

† Student's t test ; ‡ $\chi 2$ test ; § Mann-Whitney U test

Table 3b Correlation between clinicopathologic findings and the prevalence of Tregs in non-tumor sites.

	Prevalence of FOXP3+ Tregs		
Variable	High	Low	*P* value
Age, years (mean±SD)	67±7.3	65±8.6	0.288†
Gender (male/female)	17/5	19/3	0.391‡
Viral infection HBV(+)/HCV(+)/nonBC	8/11/3	9/10/3	0.982‡
TNM stage (I/II/III/IV)	4/4/13/1	14/6/2/0	**0.003**‡
Histologic grade (WD/MD/PD)	3/14/5	7/13/2	0.320‡
AFP, ng/ml (median, range)	471, 2-28576	10, 1-44001	**0.027**§
VP (presence/absence)	11/11	8/14	0.455‡
IM (presence/absence)	10/12	5/17	0.159‡
Tumor size, mm (median, range)	45, 10-162	39, 16-140	0.715§

† Student's t test ; ‡ $\chi 2$ test ; § Mann-Whitney U test

Table 4 Survival rate comparison between the experimental groups in tumor and non-tumor sites.

		Overall 5-year survival rates (%)	*P*-value*	Disease-free 3-year survival rates (%)	*P*-value*
Tumor site	Prevalence of COX-2 (Positive/Negative)	51.9/45.7	0.576	41.6/25.0	0.543
	Prevalence of FOXP3$^+$ Tregs (High/Low)	36.9/61.6	0.126	33.3/42.1	0.570
Non-tumor site	Prevalence of COX-2 (Positive/Negative)	40.5/54.9	0.502	12.5/50.0	**0.005**
	Prevalence of FOXP3$^+$ Tregs (High/Low)	38.2/60.5	0.243	21.0/47.6	0.080

*log-rank test

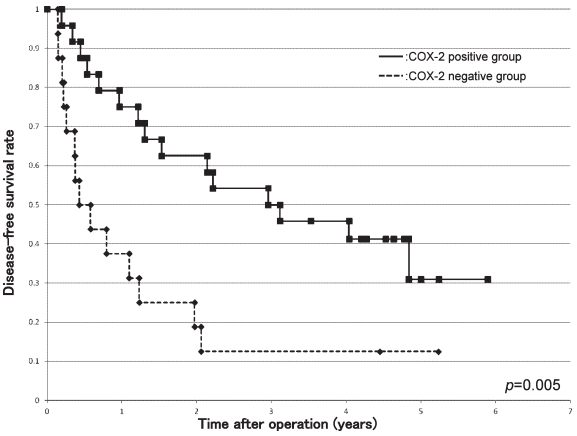

Fig. 3 In non-tumor sites, disease-free survival periods were significantly different between COX-2-positive (solid lines) and COX-2-negative (dotted lines) groups (log-rank test, p=0.005).

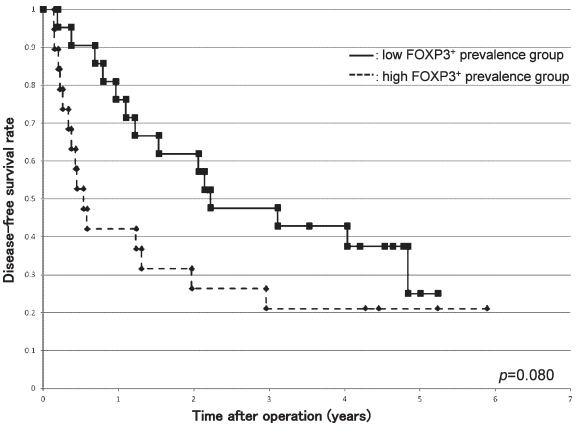

Fig. 4 In non-tumor sites, the high FOXP3$^+$ prevalence group (dotted lines) tended to have shorter disease-free survival periods that those of the low prevalence group (solid lines) (log-rank test, p=0.080).

med COX regression analysis of the overall survival and disease-free survival periods. During the overall survival period, the level of AFP was the significant factor in the tumor site (Table 5), while no significant factor was revealed in the non-tumor site (data not shown). During the disease-free survival period, no significant factor was associated with the tumor site (data not shown), while the expression of COX-2 was the significant factor in the non-tumor site (Table 6).

Discussion

COX-2 is upregulated in many types of cancers, and there are many reports on the association of COX-2 with the prognosis of HCC.[11–16] In the present study, overexpression of COX-2 in the non-tumor site was associated with a shorter disease-free survival period. Pathologically well-differentiated cancers reportedly express higher levels of COX-2, and COX-2 expression is also higher in HCV-related HCC than in HBV-related HCC.[12,21–23] It has been, however, difficult to obtain a consensus on the involvement of COX-2 in outcomes of cancer cases, largely because of the tumor-dependent expression of COX-2. The expression of COX-2 in non-tumor sites, which generally increases in proportion to

Table 5 Univariate and multivariate analyses of prognostic factors associated with overall survival in tumor sites.

Variable	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% confidence interval)	*P* value	Hazard ratio (95% confidence interval)	*P* value
Age (≥66.5y/<66.5y)	1.493(0.575-3.877)	0.410	3.894(0.737-20.583)	0.110
Gender (male/female)	1.246(0.410-3.789)	0.698	0.566(0.082-3.897)	0.563
Viral infection (presence/absence)	0.632(0.206-1.934)	0.421	0.367(0.057-2.360)	0.367
TNM Stage (I/II/III/IV)	2.114(0.836-5.347)	0.114	15.885(0.883-285.624)	0.061
AFP, ng/ml (≥19/<19)	3.221(1.143-9.076)	**0.027**	13.198(2.754-63.259)	**0.001**
Histologic grade (WD/MD, PD)	1.890(0.546-6.539)	0.315	2.459(0.217-27.797)	0.467
VP (presence/absence)	2.244(0.869-5.800)	0.095	0.719(0.067-7.752)	0.786
IM (presence/absence)	1.702(0.660-4.393)	0.271	0.204(0.020-2.079)	0.179
Tumor size (≥41/<41)	0.840(0.332-2.126)	0.713	0.304(0.053-1.764)	0.184
Prevalence of COX-2 (negative/positive)	0.767(0.302-1.948)	0.576	0.279(0.044-1.769)	0.176
Prevalence of FOXP3$^+$ Tregs (low/high)	2.119(0.793-5.662)	0.134	2.035(0.472-8.774)	0.341

the degree of chronic inflammation due to hepatitis virus infection, is higher and more stable than that of tumor sites.[24,25] The correlation between the differentiation and activation of T_R^{adapt} and the expression of COX-2 may explain the positive correlation between COX-2 expression and the high prevalence of FOXP3$^+$ Tregs in the non-tumor sites (Fig. 2b). Although there were 6 cases of Non-B/Non-C HCC in the present study, COX-2 was overexpressed in all non-tumor sites (Table 2b), suggesting the involvement of COX-2 in carcinogenesis of metabolic liver diseases, such as non-alcoholic fatty liver disease (NAFLD). It is difficult to explain the absence of a correlation between the overall survival period and COX-2 expression or the prevalence of FOXP3$^+$ Tregs in the present study. This may be because patients had received additional treatment such as TACE and RFA due to the recurrence of HCC, and the effects of these treatments may have generated a certain degree of bias.

In this study, we defined a non-tumor site as the area within two fields of tumor margins at 400× magnification. Currently, there is no clear definition of such an area in counting the number of Tregs. According to our literature search, however, many studies define such an area to be within 10 mm of tumor margins.[26–29] Here, we found that the number of FOXP3$^+$ Tregs was markedly reduced in the area beyond the two fields of tumor margins at 400× magnification. In the present 44 cases, the number of FOXP3$^+$ Tregs (the total number in 10 fields) beyond two fields of tumor margins was 1.5±2.1 (median± SD). Because this number was thought to generate a large bias in statistical analysis, we used an area within two fields of tumor margin as a non-tumor area. The expansion of Tregs in the periphery requires the involvement of tumor-associated antigens (TAAs), tumor growth factor-beta (TGF-β), and interleukin-10 (IL-10)

Table 6 Univariate and multivariate analyses of prognosis factors associated with disease-free survival in non-tumor sites

Variable	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% confidence interval)	*P* value	Hazard ratio (95% confidence interval)	*P* value
Age (≥66.5y/<66.5y)	1.763(0.836-3.719)	0.136	1.017(0.339-3.054)	1.017
Gender (male/female)	1.672(0.672-4.156)	0.269	0.055(0.001-2.219)	0.124
Viral infection (presence/absence)	0.273(0.219-1.535)	0.273	1.368(0.216-8.664)	0.739
TNM Stage (I/II/III/IV)	1.886(0.888-4.007)	0.099	1.738(0.542-5.579)	0.353
AFP, ng/ml (≥19/<19)	0.911(0.438-1.897)	0.804	0.632(0.203-1.967)	0.428
Histologic grade (WD/MD, PD)	1.533(0.621-3.789)	0.354	3.099(0.876-10.958)	0.079
VP (presence/absence)	1.402(0.674-2.916)	0.366	0.911(0.348-2.384)	0.849
IM (presence/absence)	1.624(0.751-3.513)	0.218	0.165(0.008-3.336)	0.240
Tumor size (≥41/<41)	1.525(0.722-3.219)	0.269	2.904(0.733-11.503)	0.129
Prevalence of COX-2 (negative/positive)	2.829(1.341-5.968)	**0.006**	8.991(1.538-52.572)	**0.015**
Prevalence of FOXP3$^+$ Tregs (low/high)	1.897(0.899-4.002)	0.093	0.631(0.151-2.636)	0.528

secreted by macrophages in the vicinity of tumor. The influence of these molecules on T cells is reduced with increasing distance from a tumor.[30] Using the present definition, we analyzed the prevalence of FOXP3$^+$ Tregs in non-tumor sites and observed a positive correlation with the expression of COX-2, but not with the disease-free survival period (Fig. 4). However, the TNM stages and levels of AFP were higher in the high prevalence group than in the low prevalence group (Table 3b), suggesting the involvement of FOXP3$^+$ Tregs in the progression of HCC.

Using the concept of TILs, many studies have corroborated the association between tumor-infiltrating Tregs and the prognosis of tumors. However, the present study did not find a correlation between the prevalence of FOXP3$^+$ Tregs in tumor sites and disease outcome. The reasons for this might have been the small number of cases in this study and a potentially close structural relationship between the two sites. Because COX-2 plays a role in angiogenesis,[11,12,31,32] T_R^{adapt} differentiated in non-tumor site may infiltrate tumor site through newly developed blood vessels. This may also explain the high prevalence of FOXP3$^+$ Tregs within the two fields from tumor margin at 400× magnification. The reason for the relatively small number of cases in this study was because we excluded cases with a preoperative TACE and RFA treatment, which are often performed prior to HCC surgery at our hospital. Cases with preoperative treatment were excluded because such treatment often alters intra- and extra-tumoral microenvironments. We excluded HCC cases with preoperative treatments or cases with other types of cancers, such as metastatic liver cancer that originated from colorectal cancer.

The present study suggests that inhibition of COX-2 expression may be useful to prevent HCC recurrence. Non-steroidal anti-inflammatory analgesics are representative examples of COX-2

inhibition. Clinical studies of colorectal cancers and adenomas reported that a selective COX-2 inhibitor induced apoptosis of colorectal cancer cells and inhibited the recurrence of colorectal adenomas,[33–37] indicating that similar results may be obtained with HCC. In addition, although eicosapentaenoic acid (EPA) reportedly inhibits the expression of COX-2, it may also suppress the recurrence of HCC through nutritional benefits.[38]

In summary, the present study shows that the expression of COX-2 in non-tumor site is associated with the period of disease-free survival and also suggests that COX-2 induces Tregs in the vicinity of tumor. The degree of COX-2 expression in non-tumors can be used to immunohistochemically identify a group of HCC patients with a high risk of recurrence, and thus, it is a useful indicator for postoperative supplemental therapy in clinical practice.

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Conflict of interest statement

The authors declare no conflict of interest.

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