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FoxP3 demethylation is increased in human colorectal cancer and rat cholangiocarcinoma tissue

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ABSTRACT

Objectives: *FoxP3* expression is a marker for Tregs which are known to be involved in tumor immunity. We aimed to evaluate *FoxP3* promoter demethylation in human colorectal cancer (CRC) and rat intrahepatic cholangiocarcinoma (ICC).

Design and methods: Bisulfite-treated genomic DNA templates of shock frozen paired samples were studied from 13 anonymous CRC patients and from 10 male rats (n = 6 ICC induced by thioacetamide and n = 4 age-matched controls). Real-time PCR was carried out using a LightCycler 480 system. Human *FoxP3* and *CD3* promoter demethylations were estimated using previously described assays; and rat *FoxP3* promoter demethylation using a newly developed assay.

Results: A significant 3.5-fold increase of the demethylation in *FoxP3* promoter region was found in human CRC and rat ICC ($P < 0.05$). The average frequency of cells with *FoxP3* demethylation in patients suffering from CRC was 0.26% in normal tissue and 0.92% in tumor tissue (n = 11 paired samples). Although, no significant difference was found between the mean frequency of *CD3* demethylation in normal tissue (4.80%, n = 6) and in tumor tissue (4.14%, n = 6) from CRC patients, the ratio of demethylated *CD3*/*FoxP3* promoter areas was significantly lower in tumor specimens ($P < 0.05$). Using our novel assay, we found a significant increase in mean frequencies of cells with *FoxP3* demethylation in rats with ICC (7.42%, n = 6) in comparison to controls (2.14%, n = 4).

Conclusion: *FoxP3* seems to be an interesting biomarker for immune response to epithelial tumors. Functional consequences from the increase of Tregs remain to be demonstrated. Further studies with outcome data are necessary.

Introduction

There has been increasing interest in using *FoxP3* as a specific marker for regulatory T cells (Tregs) in order to elucidate its role not only in autoimmune diseases, but also in tumor immunity as well. *FoxP3*, an X chromosome-encoded forkhead transcription factor family member, is needed for the differentiation of Tregs and maintenance of its expression is essential for the suppressive function of $FoxP3^+$ Tregs [1–3]. Most studies evaluating regulatory T cells in colorectal cancer (CRC) and intrahepatic cholangiocarcinoma (ICC) have used immunostaining/immunohistochemical staining of *FoxP3* on sections obtained from formalin-fixed and paraffin-embedded conventional blocks or tissue microarray and/or fluorescent activated cell sorting (FACS) analysis of peripheral blood [4–11]. However, recent literature suggests that epigenetic mechanisms contribute to the stability of *FoxP3* expression, and that *FoxP3* promoter demethylation shows higher biological specificity for Tregs [2,12]. Since it is already known that depletion or inactivation of $FoxP3^+$ Tregs improves cellular antitumor immunity in human malignancies [1,2], we evaluated *FoxP3* promoter demethylation in paired, fresh frozen tumor samples from human patients with CRC using the assay described by Wieczorek et al. [12] and also in rats with ICC using a novel rat assay we developed for this purpose.

Materials and methods

Samples

Colorectal cancer (CRC)

After obtaining informed consent according to the ethical approval committee, fresh tumor and adjacent healthy tissue samples at a

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distance of at least 20 cm from the tumor location were collected from 13 colorectal cancer patients and then shock frozen. The material was shipped on dry ice for further analysis and stored at −20 °C/−80 °C for up to 8 years prior to the demethylation analyses reported here. The patient samples were anonymized according to ethical and legal standards after recording non-specific clinicopathological characteristics: gender and age, as well as cell specific clinicopathological characteristics: dedifferentiation grade (G), pTNM pathological stage and tumor localization as previously described [13,14]. The paired tumor and normal tissue samples studied were from 7 females with a mean age of 70 years (range 62–78 years) and 6 males with a mean age of 62 years (range 42–76 years). The dedifferentiation grade of the tumors was G1 (well) in 4, G2 (moderate) in 6, and G3 (poor) in 3 cases. Distant metastases were observed in 5 patients, and metastatic spread to regional lymph nodes in 6 patients. Both distal and regional lymph node metastases were seen in 4 patients who had large (>5 cm in diameter) tumors and/or local spread of the primary tumor (pT3/4N1/2pM1). The tumor was localized to the rectum in 5 patients and to the colon in 8.

Intrahepatic cholangiocarcinoma (ICC)

Ten male Sprague-Dawley rats were used in the study. The animal experiments were conducted according to the guidelines of the Committee on Animals of the University Medical Center Goettingen, Goettingen, Germany. Rat liver tissue from six rats was obtained after thioacetamide treatment (TAA) for 16 or 18 weeks to induce ICC as described previously [15]. The experimental group received TAA in their drinking water every day up to the time they were euthanized. By week 16, 80% of the TAA-treated rats had developed ICC and the experiment was stopped at week 18, when 100% of the TAA-treated rats had developed ICC. Liver tissue from four age-matched rats was used for controls. Animals of both experimental groups were killed under pentobarbital anesthesia. Liver tissues were rinsed and snap-frozen in liquid nitrogen. Samples were stored at −80 °C until further use.

FoxP3 and CD3 promoter demethylation assays

Genomic human and rat DNA were isolated using the DNAzol® reagent (Life Technologies, Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol. Before further applications the quality of isolated genomic DNA was checked on 0.8% agarose gels. Bisulfite reactions were done with 0.5 μg DNA using EZ DNA Methylation-Gold™ kit 200 according to the manufacturer's protocol (Zymo Research, HiSS Diagnostics GmbH, Freiburg, Germany).

Human *FoxP3* demethylation analysis was carried out as previously described by Wieczorek et al. [12]. This qPCR assay is capable of monitoring exclusively the patients' natural Treg status and excluding transiently *FoxP3*-expressing suppressive cells. We applied a straight correction by a factor of 2 in order to compensate for X-chromosome inactivation. Moreover, we did matched *CD3* promoter demethylation analysis of both tumor tissue and corresponding healthy tissue from the same patients according to the method of Sehouli et al. [16]. Finally, we developed a novel demethylation assay to study the *FoxP3* promoter region in rats.

Real-time PCR was carried out in a final reaction volume of 20 μL using a LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). Samples contained 2 μL of bisulfite-treated genomic DNA template or corresponding standard, 15 pmol of each primer and 5 pmol probe. Samples were analyzed in duplicate. Cycling conditions for human *FoxP3* and human *CD3* demethylation assays consisted of a 95 °C preheating step for 10 min and 50 cycles of 95 °C for 15 s followed by 1 min at 61 °C as described elsewhere [12,16]. Purified PCR product was quantified on a photometer (NanoDrop 2000c, Thermo Fisher Scientific Inc.) and appropriate dilutions thereof were used as standards. The novel demethylation assay for rat *FoxP3* was conducted using the following cycling conditions: 95 °C preheating step for 10 min and 50 cycles of 95 °C for 15 s followed by 1 min at 63 °C (methylated) or 55 °C for 30 s and 62 °C for 50 s (demethylated); purified PCR products were used as standards. Crossing points were computed by the second-derivative method using LightCycler 480 software. Chromosomal position and sequences of the applied amplification primers and hydrolysis probes for human and rat *FoxP3* assays as well as for the human *CD3* assay are presented in Table 1.

Software and statistical analyses

The Wilcoxon signed-rank test was applied for paired samples of CRC patients (both normal and tumor tissues). The significance of the differences between the control rat group and the rats with ICC was

Table 1
Chromosomal position and sequence of the applied amplification primers and hydrolysis probes used for human *FoxP3* (A), human *CD3* (B) as well as for our novel rat *FoxP3* promoter demethylation assay (C).

Oligonucleotide	Chromosomal position [a]	Length, bp	Sequence
A) Human *FoxP3* assay[b]			
CpG (methylation)-specific forward primer	X:49117219-46:1	28	GTTTTCGATTTGTTTAGATTTTTTCGTT
CpG (methylation)-specific reverse primer	X:49117283-307:1	25	CCTCTTCTCTTCCTCCGTAATATCG
CpG (methylation)-specific hydrolysis probe	X:49117256-73:1	18	ATGGCGGTCGGATGCGTC
TpG (demethylation)-specific forward primer	X:49117219-46:1	28	GTTTTTGATTTGTTTAGATTTTTTTGTT
TpG (demethylation)-specific reverse primer	X:49117283-307:1	25	CCTCTTCTCTTCCTCCATAATATCA
TpG (demethylation)-specific hydrolysis probe	X:49117256-78:1	23	ATGGTGGTTGGATGTGTTGGGTT
B) Human *CD3* assay [c]			
CpG (methylation)-specific forward primer	11:118213633-53:1	21	TTAAATATTGTTATATTTCGA
CpG (methylation)-specific reverse primer	11:118213686-707:1	22	AAATCTAACTACTACGACTTAC
CpG (methylation)-specific hydrolysis probe	11:118213670-87:1	18	TCGTCGGTTTTATAGCGT
TpG (demethylation)-specific forward primer	11:118213632-53:1	22	TTTAAATATTGTTATATTTTGA
TpG (demethylation)-specific reverse primer	11:118213686-709:1	24	AAAAATCTAACTACTACTACTTAC
TpG (demethylation)-specific hydrolysis probe	11:118213664-90:1	27	CTTACACTATAAAACCAACAACTTCTC
C) Novel Rat *FoxP3* assay			
CpG (methylation)-specific forward primer	X:16559893-915:1	23	ACGAGAACCCCCCCACCCTGCGA
CpG (methylation)-specific reverse primer	X:16560168-92:1	25	AGTCCTTACCTGCAGTGTGTCCGGC
CpG (methylation)-specific hydrolysis probe	X:16559968-60005:1	38	TCTGCGGCTTCCACACCGTGGTTTTTTCTTCTCGGTAT
TpG (demethylation)-specific forward primer	X:16559889-914:1	26	AAACTACGAGAACCCCCCCACCCTGC
TpG (demethylation)-specific reverse primer	X:16560171-97:1	27	CAAAAGTCCTTACCTGCAGTGTGTCCG
TpG (demethylation)-specific hydrolysis probe	X:16559969-60008:1	40	TCTGCGGCTTCCACACCGTGGTTTTTTCTTCTCGGTATAA

[a] According to http://www.ensembl.org, May 2013.
[b] According to Wieczorek et al. [12].
[c] According to Sehouli et al. [16].

calculated using the Mann–Whitney *U* test (IBM SPSS 20, Ehningen, Germany). $P < 0.05$ was considered significant and $P \leq 0.10$ a trend. Graphics were generated using GraphPad software (GraphPad Prism 4.0, San Diego, USA). Chromosomal positions of primers and probes were indicated by Ensembl (http://www.ensembl.org, May 2013). Data were presented as mean ±standard deviation.

Results

FoxP3 promoter demethylation in CRC patients

Using a quantitative real-time PCR-based technique reported by Wieczorek et al. [12], we observed that the mean frequency of cells with *FoxP3* demethylation in patients suffering from CRC was 0.26% (range 0.17–0.45%, n = 11) in normal tissue and 0.92% (range 0.35–2.54%, n = 11) in tumor tissue. *FoxP3* promoter demethylation was ca. 3.5-fold increased in tumor samples in comparison with corresponding normal tissue obtained in 11 patients ($P < 0.05$; Table 2A; Fig. 1). In the remaining two patients there was a difference in the opposite direction between their paired samples (*FoxP3* % = 0.60 vs. 0.44 and 0.29 vs. 0.10 respectively in normal and tumor tissues from these two patients). The reasons for the lack of differences in these two subjects are unknown but may be because of the presence of lesions with many dead cells containing limited intact DNA content. It has been reported elsewhere that the DNA of an untrimmed tumor block represents a varying mixture of tumor-induced stroma, epithelial cancer cells, and normal adjacent tissue [2]. Because the group differences remained statistically significant whether or not these two outliers were included ($P = 0.013$, n = 13) or excluded ($P = 0.003$, n = 11) from the *FoxP3* analyses we excluded results for these two outlier patients in our stratification and statistical analysis.

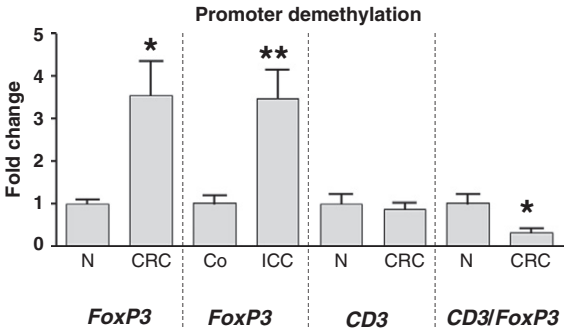

Fig. 1. Graphs show fold-changes in promoter demethylation of human *FoxP3* (n = 11 paired samples: normal (N) and tumor (CRC) tissue), *CD3* and the ratio *CD3*/*FoxP3* (n = 6 paired samples) in patients with sporadic colorectal cancer (CRC) as well as promoter demethylation of *FoxP3* in rats with intrahepatic cholangiocarcinoma (ICC, n = 6). Results were normalized to mean values of corresponding controls (Co, n = 4). Values with an asterisk were significantly different using the Wilcoxon signed-rank test (*$P < 0.05$) or using Mann–Whitney *U* test (**$P < 0.05$).

CD3 promoter demethylation in CRC patients

Using the quantitative real-time PCR-based technique described by Sehouli et al. [16], we found that the average frequency of *CD3* demethylated cells in CRC patients was 4.80% (range 1.75–8.45%, n = 6) in normal tissue and 4.14% (range 1.83–6.48%, n = 6) in tumor tissue ($P = 0.075$). Demethylation was not detectable in the seven remaining paired samples (these seven included the two outliers

Table 2
Percentage of (A) promoter demethylation of human *FoxP3*, (B) *CD3* and (C) ratio of *CD3*/*FoxP3* in CRC patients as well as (D) promoter demethylation of *FoxP3* in rats with ICC.

Variable	Description	n	Average ± standard deviation	Fold change	*P*-value
			Normal vs. tumor tissue	Tumor to normal tissue	
A) Demethylation of *FoxP3* promoter in patients with sporadic colorectal cancer					
Paired samples with both outliers		13	0.29 ± 0.13 vs. 0.82 ± 0.67, %	2.8	0.013[a]
Paired samples without both outliers		11	0.26 ± 0.09 vs. 0.92 ± 0.68, %	3.5	0.003[a]
Gender	Male	6	0.24 ± 0.09 vs. 0.66 ± 0.36, %	2.8	0.028[a]
	Female	5	0.29 ± 0.10 vs. 1.24 ± 0.88, %	4.3	0.043[a]
Histologic grade	G1/2	8	0.28 ± 0.10 vs. 0.76 ± 0.47, %	2.7	0.012[a]
	G3	3	0.22 ± 0.04 vs. 1.37 ± 1.07, %	6.2	0.109
Tumor size	pT2NxMx	3	0.30 ± 0.16 vs. 0.84 ± 0.70, %	2.8	0.109
	pT3/4NxMx	8	0.25 ± 0.06 vs. 0.96 ± 0.72, %	3.8	0.012[a]
Double metastases	pT3/4N1/2M1	4	0.23 ± 0.04 vs. 1.17 ± 0.96, %	5.1	0.068
	pTxN0M1, pTxN1/2M0 and pTxN0M0	7	0.28 ± 0.11 vs. 0.78 ± 0.50, %	2.8	0.018[a]
	pTxN0M0	5	0.28 ± 0.13 vs. 0.84 ± 0.57, %	3.0	0.042[a]
Lymph nodes	pTxN0Mx	6	0.27 ± 0.12 vs. 0.76 ± 0.55, %	2.8	0.027[a]
	pTxN1/2Mx	5	0.25 ± 0.06 vs. 1.13 ± 0.84, %	4.5	0.043[a]
Distant metastasis	pTxNxM0	6	0.29 ± 0.12 vs. 0.85 ± 0.51, %	2.9	0.027[a]
	pTxNxM1	5	0.23 ± 0.04 vs. 1.01 ± 0.91, %	4.4	0.043[a]
Age	<70 years	7	0.27 ± 0.11 vs. 1.02 ± 0.75, %	3.8	0.018[a]
	>70 years	4	0.24 ± 0.06 vs. 0.75 ± 0.60, %	3.1	0.068
Tumor location	Colon	7	0.28 ± 0.10 vs. 0.82 ± 0.79, %	2.9	0.018[a]
	Rectum	4	0.22 ± 0.07 vs. 1.11 ± 0.50, %	5.1	0.068
B) Demethylation of *CD3* promoter in patients with sporadic colorectal cancer					
Paired samples with detectable values		6	4.80 ± 2.58 vs. 4.14 ± 1.94, %	0.9	0.075
C) Ratio of demethethylated promoters of *CD3*/*FoxP3* (%) in patients with sporadic colorectal cancer					
Paired samples with detectable values		6	16.08 ± 8.57 vs. 4.90 ± 4.40, ratio	0.3	0.028[a]
D) Demethylation of *FoxP3* promoter in rats with intrahepatic cholangiocarcinoma					
Sprague-Dawley rats	Placebo and TAA treated rats	4 and 6	2.14 ± 0.84 vs. 7.42 ± 3.81, %	3.5	0.010[b]

Cancer staging according to the pTNM pathological classification: primary tumor (pT), regional lymph nodes (pN) and distant metastasis (pM); nonassessed category(-ies) for statistical evaluation (pTx/pNx/pMx).
[a] $P < 0.05$ Wilcoxon signed-rank test.
[b] $P < 0.05$ Mann–Whitney *U* test.

excluded from the *FoxP3* analysis) and these patients were consequently excluded from the statistical evaluation. Although there was no significant difference between *CD3* demethylation in tumor tissue and surrounding normal tissue (Table 2B), the ratio of demethylated *CD3*/*FoxP3* promoter areas was significantly lower in tumor specimens ($P < 0.05$; Table 2C; Fig. 1).

FoxP3 promoter demethylation in Sprague-Dawley rats with ICC

We established a novel assay for detection of *FoxP3* promoter demethylation in rat livers. Using this assay, we found that the mean frequency of cells with *FoxP3* demethylation was 2.14% (range 1.10–2.90%, n = 4) in age-matched control rats and 7.42% (range 3.98–12.69%, n = 6) in rats with TAA induced ICC. *FoxP3* promoter demethylation was ca. 3.5-fold increased in ICC tumor samples compared to results in the control rat tissues ($P < 0.05$; Table 2D; Fig. 1).

Discussion

Inflammation plays both positive and negative roles in tumor progression [5]. The distribution of tumor-infiltrating lymphocytes within a tumor is very non-homogenous and it is not known what causes lymphocytes to invade tumors or how these cells influence the tumor microenvironment [1,2,12,16–18]. In the current study we measured *FoxP3* demethylation in two epithelial tumor types: one very common form, sporadic CRC, and one relatively rare form, ICC. Both of these digestive tract neoplasias may evolve via a multistep progression to a malignant transformation [19]. The adaptive immune system is thought to play an important role in suppressing such tumor progression [18,20].

Quantitative measurements of Tregs using a demethylation method might be useful as an important diagnostic and/or prognostic variable [12]. Loddenkemper et al. [2] did immunostaining and *FoxP3* demethylation analysis in various malignancies, including colon cancer. They studied paraffin-embedded tumor tissues from 20 CRC patients and observed 4.1% (range 1.46–8.63%) *FoxP3* demethylation but they did not study any healthy control tissues. However, similar studies that did include healthy control tissue confirmed their results [16]. Similarly, Wieczorek et al. [12] found a higher percentage, 6.3% (range 1.9–12.3%), of *FoxP3* demethylation in 15 archival, formalin-fixed, paraffin-embedded samples of primary CRC and 1.5% (range 0.8–2.8%) in normal colon tissue (5 paired specimens from the same patients and 5 colon specimens from other patients). They studied only female patients with a mean age of 58 years and female controls with a mean age of 55 years and showed a 4.2-fold increase in the tumor samples. Using paired samples we confirmed the significant increase (mean of 4.3-fold) in females of a comparable age as well as a 2.8-fold change in males (Table 2A). These gender differences may be the result of increased tolerance of foreign antigens after pregnancy [12]. It is known that Treg infiltration is significantly greater in CRC than in healthy colon tissues [7]. It was suggested that significantly more intraepithelial *FoxP3*$^+$ cells are seen in tumor tissues from female patients suffering from CRC than in autologous normal mucosa [21]. Moreover, a significant increase of *FoxP3*$^+$ T lymphocytes was detected in tumor stroma relative to normal lamina propria. Another study using tissue microarrays with 216 colon carcinomas reported that the density of *FoxP3*$^+$ infiltration was similar in tumor stroma and epithelia, whereas the percentage of *CD8*$^+$ cells was higher in stroma. Authors of this study concluded that *FoxP3*$^+$ exerts a favorable influence on survival only in colon cancers with low *CD8*$^+$ infiltration [22].

Ling et al. [10] hypothesized that increased numbers of Tregs in the blood and in tumors of CRC patients may influence the immune response to cancer. Wieczorek et al. [12] also studied peripheral blood from both genders. The results for patients (mean age 64) with CRC (mean of 2.3%, range 0.6–19%, n = 27 including one outlier, or a mean of 1.6%, range 0.6–3.5%, n = 26 excluding that outlier) did not significantly differ from results in normal healthy control donors (average 1.4%, range 0.4–2.9%, n = 20, mean age 55).

The demethylation positive percentage using shock frozen tissues in our study seems to be lower than that using material from conventional blocks or tissue microarrays [2,12]. The formalin fixation and/or deparaffinization may be a possible explanation for the increased demethylation percentages seen in the literature. Time and temperature of storage could also be possible reasons. A major advantage of the use of formalin-fixed and paraffin-embedded blocks is the possibility to control pathologically the serial sections of the trimmed tumor tissue and thus precisely define the studied tissue cohort. A possible solution to this issue concerning the use of fresh frozen material would be the use of cryosection and laser capture microdissection.

Besides gender, additional clinicopathological variables must also be considered for analysis of *FoxP3* demethylation in CRC tissues. After stratification of the data according to histological grade there was more than twice the percentage of *FoxP3* demethylated cells in poorly differentiated tumors (6.2 fold) as that seen in well to moderately differentiated tumors (2.7 fold). This finding supports previously published results with 160 colon carcinomas and 25 normal mucosas showing an association between a significant increase in intraepithelial *FoxP3*$^+$ cells and poor tumor differentiation [21]. With increasing tumor diameter we found a significantly ($P = 0.012$) higher percentage of demethylated cells within the larger CRC tumors (<5 cm: 2.8 fold; >5 cm: 3.8 fold). Furthermore, a trend ($P \leq 0.10$) suggested that there was increased demethylation in tumor specimens if there was either a distant metastasis (4.4 fold, $P = 0.043$), lymph node metastasis (4.5 fold, $P = 0.043$), or the presence of both types of metastases (5.1 fold, $P = 0.068$). Our data confirmed the finding that a significant higher density of *FoxP3*$^+$ tumor infiltrating T lymphocytes was associated with fewer metastatic lymph nodes [21]. However, contrary to this immunofluorescence study, advanced age of patients did not dramatically influence the demethylation percentage in our cohort (<70 years: 3.8 fold, $P = 0.018$; >70 years: 3.1 fold, $P = 0.068$). We also observed more demethylated cells in rectal (5.1 fold, $P = 0.068$) than in colon cancer (2.9 fold, $P = 0.018$). *FoxP3* was previously studied immunohistochemically on a tissue microarray of 1420 untreated and unselected patients with sporadic CRC and results showed significant differences according to tumor locations [8], supporting results in our limited patient cohort ($P < 0.05$: right-sided versus rectal and rectal versus left-sided tumors; however $P > 0.05$ right vs. left-sided).

In our demethylation study using paired samples from patients with sporadic CRC we found a significantly ($P = 0.028$) lower *CD3*/*FoxP3* ratio in tumor than in normal tissue, but no significant decrease in *CD3* demethylation (Table 2B and C; Fig. 1). Interestingly, Sinicrope et al. [21] suggested that a low ratio of [intraepithelial *CD3*$^+$ tumor infiltrating lymphocytes/*FoxP3*$^+$ Tregs] and reduced numbers of the adaptive immune marker *CD3*$^+$ T cells were both associated with reduced patient survival time. Using tissue microarray and automated image analysis, Nosho et al. [18] quantified the densities of *CD3*$^+$ and *FoxP3*$^+$-cells within neoplastic epithelial areas in 768 colon and rectal carcinoma cases. Subsets were stratified according to various clinical, pathological and molecular features. Age, gender, body mass index, family history of CRC in any first degree relative, tumor location and grade did not significantly influence the densities of either tumor-infiltrating T-cell subsets. *CD3*$^+$ cell numbers did not differ; however *FoxP3*$^+$ cells showed significant differences according to disease stage; supporting our demethylation results. These authors reported that tumor-infiltrating *CD45RO*$^+$-cell density was significantly associated with improved prognosis in colorectal cancers, independent of clinical, pathologic and molecular features, including densities of *CD3*$^+$ and *FoxP3*$^+$ T-cell subsets [18]. Interestingly, in another study patients with low densities of *CD3*$^+$ cells and *CD45RO*$^+$ memory T cells in the center of their tumors and in the invasive margins had a very poor prognosis, similar to that of patients with concomitant distant metastasis [20]. Results of Nagorsen et al. [9] strongly supported the hypothesis

that tumor-infiltrating dendritic cells are a key factor at the interface between innate and adaptive immune responses to malignant disease. After Bonferroni corrections, they found that the *S100–FoxP3* pair significantly correlates not only with each other, but also with the *FoxP3–CD3* pair. Tumor infiltrating *S100*-positive dendritic cells showed positive correlations with both regulatory T cells and with survival in CRC patients.

Increased populations of *FoxP3*$^+$ *CD4*$^+$ cells were also detected in tumor stroma of hepatocellular carcinoma (HCC) suggesting a role for *FoxP3*$^+$*CD4*$^+$ cells in controlling the immune response to HCC during the progression of hepatocarcinogenesis [6]. Since the prevalence of *FoxP3*$^+$ Tregs was significantly higher in HCC than in non-tumor containing liver, the authors suggested that a high prevalence of Tregs seems to be an indicator of poor prognosis. Furthermore, while Treg infiltration showed no differences among different histological types of tumor, there were differences between primary and metastatic hepatic tumors.

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary hepatic malignancy after hepatocellular carcinoma [5]. It is known that ICC arises from the intrahepatic bile ducts. *FoxP3*$^+$ lymphocyte numbers were found to be increased predominantly in the intratumor area of ICC using tissue microarrays [5]. We were able to demonstrate a mean 3.5-fold change in *FoxP3* demethylation in rat ICC tumors using a new assay developed to study rat tissues. These results suggest that this in vivo model of ICC is comparable to human ICC and that our novel demethylation assay is a feasible alternative to immunostaining methods.

Additionally, activated macrophages (referred to as the M2-phenotype) are involved in promoting tumor growth, remodeling tissues, promoting angiogenesis, and suppressing adaptive immunity in various human tumors. M2-phenotype macrophages induce the infiltration and differentiation of regulatory T cells, and regulatory T cells induce macrophage differentiation toward the M2 phenotype; changes mediated by chemokines [4,23]. In a recent study of patients with ICC it was reported that the number of regulatory T cells (*FoxP3*$^+$ *CD4*$^+$ cells) correlated with the number of *CD163*$^+$ M2-phenotype macrophages [4]. Hasita et al. [4] provided suggestive evidence that tumor-associated macrophages contribute to cancer progression via Stat3 activation and that this activation affects the tumor microenvironment and promotes tumor development. These authors also suggested that evaluation of M2-phenotype tumor-associated macrophages could be used to predict the clinical prognosis of ICC patients. Moreover, they also suggested that Stat3 inhibitors might be effective in preventing tumor progression in ICC patients by regulating the tumor microenvironment.

Conclusion

A summary of both the recent literature and the data presented here suggests that *FoxP3* is an interesting biomarker for the prognosis of these two epithelial tumors, CRC and ICC. However, elucidation is still needed. The novel rat *FoxP3* demethylation assay presented here will be useful to investigate numerous questions concerning the multistep progression to malignant transformation in different rat models.

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