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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].