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Original Article

High intratumoral FOXP3$^+$ T regulatory cell (Tregs) density is an independent good prognosticator in nodal negative colorectal cancer

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Abstract: Immunologic profiling of colorectal cancer (CRC) may help to predict the tumors metastatic potential and patients with an aggressive tumor, although not yet metastasized at time of surgery might benefit from adjuvant therapy. In this study we evaluated the prognostic significance of FOXP3$^+$ T regulatory cells (Tregs), CD3$^+$ and CD8$^+$ lymphocyte densities and conventional histopathologic features in nodal negative (n = 820, UICC stage II) CRC. Immunohistochemical studies showed that high expression of FOXP3$^+$ Tregs is significantly linked to a better clinical outcome (P = 0.0001). In multivariate analysis including tumor stage, tumor grade, type of tumor invasion margin (pushing vs. infiltrating type), lymphovascular invasion (absent vs. present), CD3$^+$, CD8$^+$ and FOXP3$^+$ Tregs expression, only low tumor stage, absence of lymphovascular invasion and high Foxp3 Tregs density showed prognostic significance (P = 0.0132, P = 0.0022 AND P = 0.0234, respectively). Our findings argue towards a clinical utility of FOXP3$^+$ Tregs immunostaining as an independent good prognostic biomarker in stage II colorectal cancers. FOXP3$^+$ Tregs immunoscoring, assessment of tumor stage and lymphovascular invasion may help to define stage II cancers with a potentially aggressive behavior and CRC patients who might benefit from adjuvant therapy. A two-scale immunosore related to the median count of FOXP3$^+$ Tregs proved to be easy and quick to perform.

Keywords: Colorectal cancer, immunoscore, FOXP3$^+$ Tregs, prognosis

Introduction

Despite recent advances in therapy, colorectal cancer (CRC) remains the second leading cause of death from cancer in Western countries [1]. In advanced metastatic disease, surgery alone is not curative and therefore adjuvant chemotherapy is needed. Patients with nodal negative CRC generally show a favorable clinical course. However, approximately one third of stage II CRC recurs or show progressive disease, suggesting failure to detect occult disease [2]. Antitumoral immune response is an important factor for prognosis in CRC with high numbers of tumor-infiltrating lymphocytes (TIL) being associated with improved patient survival [3, 4]. Therefore, immunologic profiling of the CRC might predict the tumor's metastatic potential and patients with a potentially aggressive tumor, although not yet metastasized at time of surgery might benefit from adjuvant therapy. Cytotoxic T lymphocytes (CTLs; CD8$^+$) have the ability to kill target cells upon being exposed to a tumor cell antigen/HLA1 complex for which their T-cell receptor is specific [5]. The transcription factor forkhead box P3 (FOXP3$^+$) is an intracellular key molecule for regulatory T cell (Treg) development and function and is considered the most specific Treg cell marker [6, 7]. Tregs have been suggested to down regulate the antitumor immune response by reducing the activation of conventional T cells [8]. However, conflicting reports on the prognostic impact of Tregs in different human tumor types exist. Whereas Tregs have been associated with adverse outcomes in breast and hepatocellular

carcinomas [9, 10], studies on other tumor types found FOXP3+ T lymphocytes to be associated with a favorable prognosis, including CRC and gastric cancer [11-14]. Of note, most of these studies have been performed under different conditions. From the clinical standpoint of view, a practicable, easy and quick to perform immune scoring method should be introduced comparable to accepted scoring systems for therapeutically important molecular targets in other human cancers. For example, in breast cancer, guidelines for the evaluation of the hormone receptor status under highly standardized conditions exist [15]. To further elucidate the importance of immune response in CRC, we performed an immunohistochemical (IHC) study on a cohort of nodal negative CRC patients examining the expression of FOXP3+ Tregs, CD3+ and CD8+ T cells in respect to overall survival (OS) and to conventional clinicopathologic data as histologic tumor type, tumor grade, tumor stage, lymphovascular invasion, tumor margin infiltration type and tumor localization. Our data show that high FOXP3+ expression in nodal negative CRC is associated with improved survival in stage II CRC.

Materials and methods

Patients and tissue microarray (TMA) construction

Two different TMAs with a total of 1800 CRC samples were included in this study. The first TMA was manufactured from resection specimens of 1420 CRC patients at the Institute of Pathology of the University Hospital of Basel. Raw survival data were obtained from the responsible physicians for all of the 1420 patients. The median follow up time was 46 months (range 1-152 months). The second TMA included samples from 380 CRC patients, whose tumor resection specimens were examined at the Institute of Pathology of the University Medical Center Hamburg-Eppendorf. For this TMA too, overall survival data were available for all of the 380 patients with a median follow up period of 36 months (range 1-179 months). TMA construction was as described [16]. In brief, hematoxylin and eosin-stained sections were made from each block to define representative tumor regions. One tissue cylinder with a diameter of 0.6 mm was then punched from the tumor on the "donor" tissue block using a home-made semi-automated precision instrument and brought into empty recipient paraffin blocks. Four µm sections of the resulting TMA blocks were transferred to an adhesive coated slide system (Instrumedics Inc., Hackensack, New Jersey). Patient information and clinical data such as age, sex, localization and type of tumor, pTNM-stage and carcinoma grade were retrospectively retrieved from clinical and pathological databases (**Table 1**). All tumors were re-classified by two pathologists (PL, AM). Follow-up data were obtained from local cancer register boards or via attending physicians. For statistical analyses, tumor localizations were grouped as follows: right-sided cancer (cecum, ascendens), cancer of the transverse colon, left-sided colon cancer (descending and sigmoid colon, rectum). The utilization of tissues and clinical data was according to the Hamburger Krankenhaus Gesetz (§12 HmbKHG) and approved by our local Ethical Committee.

Immunohistochemistry

After deparaffinization, the tissue sections were pretreated for 25 minutes in a Sharp™ microwave oven (approximately 98°C) for heat-induced epitope retrieval at pH 6.0 (TRIS-EDTA-buffer). Standard indirect immunoperoxidase procedures were used for visualization of bound antibody (Envision system, DAKO, Glostrup, Denmark). Diaminobenzidine was used as the chromogen. Primary antibodies were used according to manufacturer's instructions (CD8, DakoCytomation, clone C8/144B ready to use; CD3, Dako Cytomation FLEX polyclonal rabbit anti-human CD3, ready to use; FOXP3; clone 206D, Biolegend, San Diego, CA, dilution 1:50).

Measurement of T-cell density

After staining for the T-cell markers, TMA slides were analyzed under a microscope (Zeiss, Axio Scope) by one observer (TH). Numbers of stained T cells were counted in each tissue core (0.6 mm diameter), representing approximately one 40 × high power field (HPF). Evaluation of the T-cell marker density was carried out blinded to clinicopathologic information. Only complete tissue cores with at least 20% viable tumor tissue were included in the analysis. No attempt was made to evaluate the various tissue compartments separately (eg,

Table 1. Clinicopathological parameter, CD3+, CD8+ and FOXP3+ Treg expression in stage II CRC

	CD3+			CD8+			FOXP3+		
	low	high	*p*-value	low	high	*p*-value	low	high	*p*-value
Frequency n (%)									
Gender			.0749			.7413			.3194
Female	338 (49.1)	76 (57.6)		377 (50.7)	37 (48.7)		394 (50.1)	20 (58.8)	
Male	350 (50.9)	56 (42.4)		367 (49.3)	39 (51.3)		392 (49.9)	14 (41.2)	
Age			.0841			.6181			.5138
≤ 49	31 (4.5)	8 (6.0)		34 (4.6)	5 (6.6)		37 (4.7)	2 (5.9)	
50-75	402 (58.4)	88 (66.7)		443 (59.5)	47 (61.8)		467 (59.4)	23 (67.6)	
≥ 76	255 (37.1)	36 (27.3)		267 (35.9)	24 (31.6)		282 (35.9)	9 (26.5)	
Localisation			.0015			.0034			.6396
Right colon	169 (32.6)	42 (35.9)		187 (31.5)	24 (55.8)		202 (33.5)	9 (27.3)	
Left colon	149 (28.7)	16 (13.7)		160 (27.0)	5 (11.6)		157 (26.0)	8 (24.2)	
Rectum	201 (38.7)	59 (50.4)		246 (41.5)	14 (32.6)		244 (40.5)	16 (48.5)	
Grade			.5725			.8357			.9407
G1	15 (2.2)	2 (1.5)		16 (2.1)	1 (1.3)		16 (2.0)	1 (2.9)	
G2	653 (94.9)	124 (93.9)		704 94.6)	73 (96.0)		745 (94.8)	32 (94.2)	
G3	20 (2.9)	6 (4.6)		24 (3.2)	2 (2.6)		25 (3.2)	1 (2.9)	
Stage			.0569			.0349			.0549
pT1	48 (7.0)	15 (11.4)		56 (7.5)	7 (9.2)		59 (7.5)	4 (11.8)	
pT2	157 (22.8)	39 (29.5)		169 (22.7)	27 (35.5)		186 (23.7)	10 (29.4)	
pT3	414 (60.2)	70 (53.0)		445 (59.8)	39 (51.3)		464 (59.0)	20 (58.8)	
pT4	69 (10.0)	8 (6.1)		74 (10.0)	3 (4.0)		77 (9.8)	0 (0.0)	
Infiltration margin			.3739			.7947			.5133
Pushing type	192	45		227 (58.7)	10 (55.6)		230 (58.8)	7 (50.0)	
Infiltrative type	130	38		160 (41.3)	8 (44.4)		161 (41.2)	7 (50.0)	
Histology type			.3311			.3954			.1693
Non-mucinous	668 (97.1)	130 (98.5)		723 (97.2)	75 (98.7)		764 (97.2)	34 (100)	
Mucinous	20 (2.9)	2 (1.5)		21 (2.8)	1 (1.3)		22 (2.8)	0 (0.0)	
Peritumoral lymphocyts			.0134			< .0001			.2461
Absent	240 (74.3)	50 (60.2)		285 (73.4)	5 (27.8)		282 (71.9)	8 (57.1)	
Present	83 (25.7)	33 (39.8)		103 (26.6)	13 (72.2)		110 (28.1)	6 (42.9)	
Lymphovascular invasion			.7613			.3545			.3976
Absent	276 (85.4)	72 (86.7)		334 (86.1)	14 (77.8)		335 (85.5)	13 (92.9)	
Present	47 (14.6)	11 (13.2)		54 (13.9)	4 (22.2)		57 (14.5)	1 (7.1)	

T-cell densities were grouped in relation to the count distribution quantities according to the median into “low” (n < median) and “high” (n ≥ median). Abbreviations: CRC, colorectal cancer.

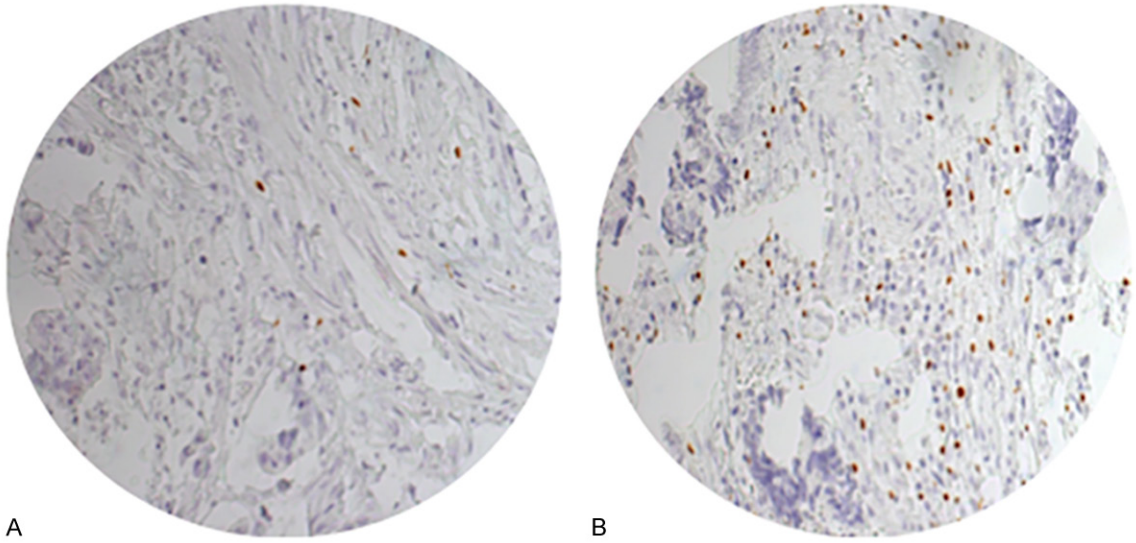

Figure 1. Representative microphotographs of immunohistochemical staining for intratumoral FOXP3+ cells in stage II CRC, magnification 100 ×. FOXP3+ staining was always nuclear. A case with "low density" of intratumoral FOXP3+ stained cells is shown in (A), whereas a case with "high density" of FOXP3+ stained cells is shown in (B).

stroma, epithelium). Results of cell densities were exported into an Excel file, and individual cores were matched to corresponding clinicopathologic data. T-cell densities were grouped in relation to the count distribution quantities according to the median into "low" (n < median) and "high" (n ≥ median, **Table 1**; **Figures 1** and **3**).

Statistical analysis

Statistical calculations were performed with JMP® 10.0.2 software (2012 SAS Institute Inc., NC, USA). Contingency tables and the chi square test were performed to search for associations between tested immunologic parameters and tumor phenotype. Survival curves were calculated according to Kaplan-Meier. The Log-Rank test was applied to detect significant survival differences between groups. Cox proportional hazards regression analysis was performed to test the statistical independence and significance between pathological, immunological and clinical variables.

Results

CD3+, CD8+ and FOXP3+ Tregs immunohistochemistry

A total of 238 from 1800 cancer tissue samples (13.2%) were non-informative due to either absence or less than 20% of unequivocal cancer tissue. All of the remaining 1562 CRC were examined for CD3+, CD8+ and FOXP3+ Tregs immunostaining. Representative examples of FOXP3+ Tregs cell staining in colorectal cancers are shown in **Figure 1.**

The staining results of CD3+, CD8+ and FOXP3+ Tregs densities and their relationship to clinical parameters in 820 nodal negative CRC (stage II) are shown in **Table 1**.

High intratumoral CD8+ and CD3+ densities were significantly associated with presence of peritumoral lymphocytes ($P = 0.0134$ and $P < 0.0001$, respectively) and with right-sided tumor localization ($P = 0.0015$, $P = 0.0034$, **Table 1**). No correlation between FOXP3+ expression and patient's gender, age group, tumor grade, tumor stage, tumor localization, histologic tumor type, tumor margin infiltration type, peritumoral lymphocytic infiltration or occurrence of lymphovascular invasion could be observed (**Table 1**).

Survival analysis of stage II CRC

In nodal negative CRC (stage II cancers, n = 820), absence of lymphovascular invasion ($P = 0.0001$, **Figure 2A**), low tumor stage ($P = 0.0004$, **Figure 2B**), low tumor grade ($P = 0.0124$), pushing type of the invasive tumor front ($P = 0.0245$) and high density of intratumoral FOXP3+ Tregs cells ($P = 0.0355$, **Figure**

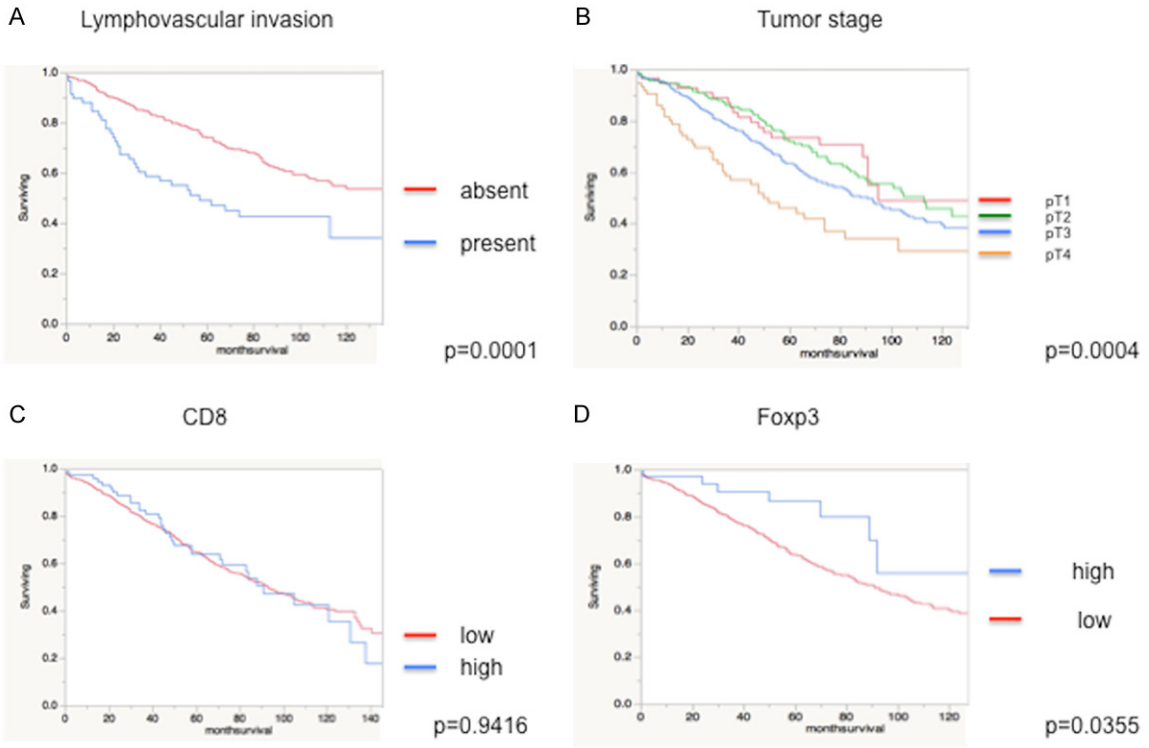

Figure 2. Prognostic relevance of lymphovascular invasion (A), tumor stage (B), intratumoral CD8+ cells (C) and intratumoral FOXP3+ positive cells (D) in stage II CRC.

2D) were significantly related to better patient survival, whereas all other tested parameter including tumor localization, intratumoral CD3+ and CD8+ T cell density showed no prognostic significance (data not shown).

Multivariate analysis of stage II CRC

In a multivariate analysis, including tumor stage, tumor grade, tumor invasive margin type, lymphovascular invasion, CD3+, CD8+ and FOXP3+ Tregs expression, only low tumor stage (P = 0.0132), absence of lymphovascular invasion (P = 0.0022) and high FOXP3+ Tregs density (P = 0.0234) showed significance (**Table 2**).

Discussion

Considering mounting evidence of the prognostic importance of immune cell infiltration in CRC and other gastrointestinal malignancies [14, 17-19], Galon et al have recently suggested an immunoscore as a component of cancer classification, based on a quantitative automated IHC method, that grades CD3+ and CD8+ infiltration at the invasive margin and tumor center [20].

Here we show that a semiquantitative analysis of immune cell infiltration on one core needle biopsy from the tumor-center using IHC could provide a rapid, inexpensive and powerful prognostic scoring method to identify subgroups of nodal negative CRC that show an aggressive behavior and might benefit from adjuvant therapy.

In this IHC study we evaluated the significance of intratumoral CD3+, CD8+ and FOXP3+ cells in archival tumor tissue samples from 820 nodal negative (UICC stage II) CRC patients. Our findings show that high intratumoral FOXP3+ (Treg) expression, low tumor stage and absence of lymphovascular tumor invasion are strong independent favorable prognostic markers in stage II CRC.

Our findings are comparable to results of previous studies. For example, in an IHC study on 967 stage II and stage III colorectal cancers, Salama et al also found a link between high density of FOXP3+ Tregs in tumor tissue and improved survival [12].

In another study on 87 CRC patients with stage II tumor high intraepithelial FOXP3+ showed to

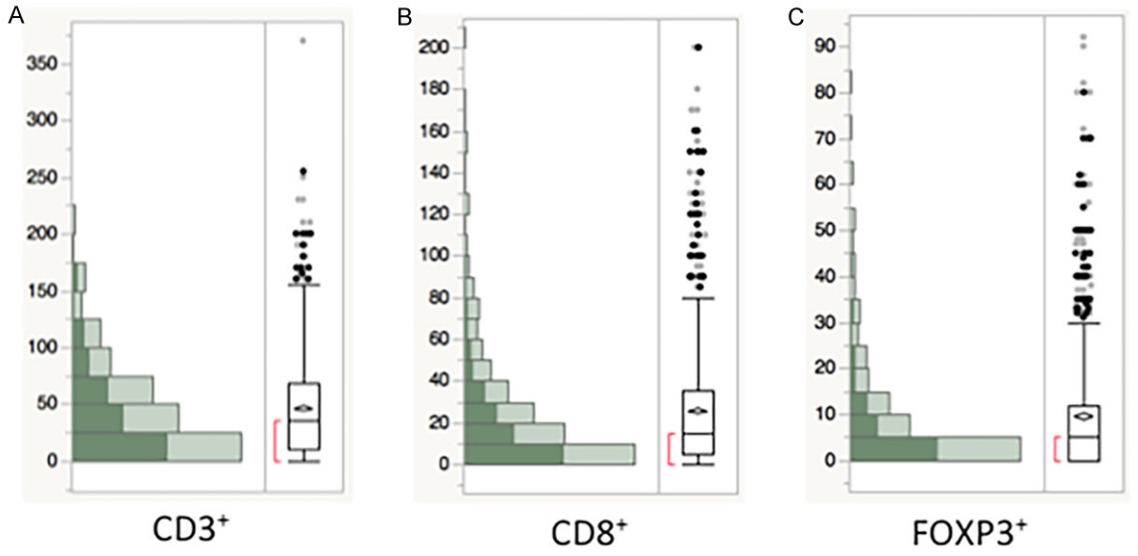

Figure 3. Schematic representation showing distribution and frequency of intratumoral CD3+, CD8+ and FOXP3+ stained cells in CRC (n = 1562). The number of stained cells was counted under a light microscope. A. Number CD3+ cells ranged from 0 to 200 (mean: 47). B. Number of CD8+ cells ranged from 0 to 370 (mean: 26). C. Number of FOXP3+ stained cells ranged from 0 to 92 (mean: 10). Each median served as cut off point for classification of intratumoral cells into "low"-(n < median) and "high" density-(n ≥ median).

Table 2. Multivariate analysis of FOXP3+ expression in stage II colorectal cancer

n analyzable	Tumor stage	Tumor grade	Invasive margin type	Lymphovascular invasion	CD3+	CD8+	FOXP3+
820	0.0132	0.1204	0.3672	0.0022	0.7482	0.1600	0.0234

be an independent factor for disease free survival [21].

Frey et al examined 1197 MMR-proficient and 223 MMR-deficient CRC and found that a high FOXP3+ Treg frequency is an independent prognostic factor in MMR-proficient CRC, but not in MMR-deficient CRCs. High densities of FOXP3+ Tregs were associated with early T stage and independently predicted improved disease-specific survival in MMR-proficient CRC patients [22].

The question remains, whether the immune cell count obtained from a single core needle biopsy adequately represents the whole tumor. Ling et al have recently thoroughly studied the prognostic importance of immune cells at different intratumoral subsites of CRC by a semi-quantitative IHC analysis using a light microscope and found no prognostic discrepancy between infiltration of FOXP3+ cells in the tumor invasive front and the tumor-center, a high infiltration rate in both subsites being significantly associated with a better prognosis [13]. Similar approaches using core biopsies from the tumor-center are routinely being applied in pathological practice to identify subgroups of cancers for therapy, for example to evaluate the hormone receptor status of breast cancer [15]. Recently, a study using TMAs of hepatic metastases from well-differentiated neuroendocrine tumors demonstrated good correlation between Ki67 labeling index (Ki67LI) in one to three random core biopsies and whole sections of G1 tumors in nearly 100% and in about 50% of G2 tumors. Thus, the authors concluded that a single needle core biopsy randomly taken from within a tumor or metastasis usually provides adequate proliferation assessment, despite the presence of intratumoral heterogeneity [23].

Based on the concordance of our results with previous data, we assume that our analysis of immune cells using TMAs constructed from one 0.6-mm core per patient yields representative results comparable to random core needle biopsies.

The strong association between classical prognostic features such as tumor stage, tumor grade, nodal status and prognosis in our patent set provide indirect proof for the validity of our clinical data [24]. Semi-quantitative IHC analysis has certainly the disadvantage of inter-observer variability. However, considering the heterogeneity of diagnostic tumor-tissue samples, which might also include tumor necrosis and normal tissue, the light microscope analysis method, has on the other hand the benefit of more accurately identifying true tumor tissue and excluding normal tissue and necrotic areas [25].

Studies on different subsets of T lymphocytes suggest that tumor infiltrating $CD8^+$ cells (TILs) have the strongest prognostic impact [13, 26]. However, quantification of intratumoral lymphocytes including TILs can be difficult [27]. Our semiquantitative IHC approach counting T-cells regardless their intratumoral localization and using the median to stratify the $FOXP3^+$ cell count into two groups ("low" and "high") has proved to be easy and quick to perform and to be statistically relevant (**Figures 2D** and **3**). It is well known that right and left-sided CRC show distinct differences in epidemiology, pathogenesis, genetic and epigenetic alterations, molecular pathways and prognosis (reviewed in [28]). MSI cancers are more likely right-sided and more frequently show peritumoral lymphocytes (PTL) as compared to microsatellite stable cancers [29-31]. In our study, we have also found higher intratumoral $CD8^+$ and $CD3^+$ densities in right-sided compared to left-sided CRC. However, $FOXP3^+$ cell density was independent of tumor localization in our study (**Table 1**). Therefore, we conclude that the prognostic impact of $FOXP3^+$ cell infiltration is independent of the MSI status of CRC. This is in line with reports of Ling et al, who evaluated lymphocyte infiltration in molecular subgroups of CRC defined by MSI screening and CIMP status and found that even though MSI tumors are more likely infiltrated, the prognostic importance of lymphocyte infiltration is independent of these molecular characteristics [13].

In summary, our data show that high $FOXP3^+$ Tregs density, low tumor stage and absence of lymphovascular invasion are independent good prognosticators in nodal negative (UICC stage II) CRC. In conclusion, our findings strongly argue towards a clinical utility of $FOXP3^+$ immunostaining as a prognostic biomarker in stage II CRC. Immunoscoring may help to define patients with nodal negative but aggressive disease who could potentially benefit from adjuvant therapy.

We suggest a two-scale immunoscoring-system based on the median T cell count that proved to be easy and quick to perform.

Disclosure of conflict of interest

None.

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