UICC
global cancer control

IJC
International Journal of Cancer

High frequency of tumor-infiltrating FOXP3$^+$ regulatory T cells predicts improved survival in mismatch repair-proficient colorectal cancer patients

Daniel M. Frey[1], Raoul A. Droeser[1], Carsten T. Viehl[1], Inti Zlobec[2], Alessandro Lugli[2], Urs Zingg[1], Daniel Oertli[1], Christoph Kettelhack[1], Luigi Terracciano[2] and Luigi Tornillo[2]

[1] Department of Surgery, University Hospital of Basel, Basel, Switzerland
[2] Institute of Pathology, University Hospital of Basel, Basel, Switzerland

Regulatory T cells (T_{reg}) inhibit the generation of host-versus-tumor immunity *via* suppression of tumor-specific effector T-cell responses and development of immune tolerance to neoplastic cells. The transcription factor forkhead box P3 (FOXP3) is an intracellular key molecule for T_{reg} development and function and is considered to represent the most specific T_{reg} cell marker. The aim of this study was to analyze the frequency and prognostic impact of tumor-infiltrating FOXP3$^+$ T_{reg} in colorectal cancer (CRC) stratified by mismatch-repair (MMR) status. Using the tissue microarray technique, 1,420 tumor samples were immunohistochemically stained for FOXP3 and stratified into 1,197 MMR-proficient and 223 MMR-deficient CRCs. Additionally, the 1,197 MMR-proficient CRCs were randomized into 2 subgroups (Test Groups 1 and 2; $n = 613$ and 584, respectively). In both MMR-proficient CRC subgroups high frequency tumor-infiltrating FOXP3$^+$ T_{reg} was associated with early T stage ($p = 0.001$ and <0.001), tumor location ($p = 0.01$ and 0.045) and increased 5-year survival rate ($p = 0.004$ and <0.001), whereas in MMR-deficient CRCs an association between FOXP3$^+$ T_{reg} and absence of lymph node involvement ($p = 0.023$), absence of vascular invasion ($p = 0.023$) and improved 5-year survival rate ($p = 0.029$) could be detected. In a multivariable analysis including age, gender, T stage, N stage, tumor grade, vascular invasion, and tumor border configuration, a high FOXP3$^+$ T_{reg} frequency was an independent prognostic factor in both MMR-proficient CRC subsets ($p = 0.019$ and $p = 0.007$), but not in the MMR-deficient CRCs ($p = 0.13$). Therefore, high frequency of tumor-infiltrating FOXP3$^+$ T_{reg} is associated with early T stage and independently predicts improved disease-specific survival in MMR-proficient CRC patients.

Tumor Immunology

Tumor-infiltrating lymphocytes (TILs) are considered to be the primary host immune response against solid tumors. Recent results have shown a correlation between survival and density of TILs in colorectal cancer (CRC) patients.[1] Furthermore, there is accumulating evidence that the type of immune cells, rather than their sheer quantity, controls the efficiency of the host-versus-tumor immune response.[2] However, the role of TILs in predicting CRC prognosis remains a matter of ongoing debate. Controversy may arise from the uncertainty concerning the *in vivo* activity of TILs as well as from the lack of discriminating patients according to the underlying type of genomic instability (microsatellite stable *vs.* unstable). Approximately 10–15% of sporadic CRC and all cases with hereditary nonpolyposis colorectal cancer (HNPCC) are associated with high frequency of microsatellite instability as a result of inactivation or loss of expression of the mismatch repair (MMR) genes MLH1, MSH2 and MSH6 (so-called MMR-deficient tumors). Compared to the majority of sporadic CRC that are MMR-proficient tumors, MMR-deficient tumors are characterized *a priori* by a higher frequency of TILs and are associated with significantly improved prognosis.[3]

Increasing evidence suggests that regulatory T cells (T_{reg}) have the ability to inhibit the generation of host-versus-tumor immunity in the microenvironment of tumors *via* suppression of tumor-specific effector T-cell responses.[4,5] The transcription factor forkhead box P3 (FOXP3) is an intracellular key molecule for T_{reg} development and function and is considered to represent the most specific T_{reg} cell marker so far.[6–8] In the majority of solid tumors studied so far, high frequency of tumor-infiltrating FOXP3$^+$ T_{reg} predicted an impaired patient survival.[7–16] Paradoxically, increased frequency of T_{reg} was recently found to be associated with improved prognosis in lymphoma patients[17] and also in CRC

Key words: human colorectal cancer, tumor infiltrating lymphocytes, FOXP3, regulatory T cells, tissue microarray

Abbreviations: CRC: colorectal cancer; FOXP3: forkhead box P3; MMR: mismatch repair; ROC: receiver operating characteristic; TILs: tumor-infiltrating lymphocytes; TMA: tissue microarray; Treg: regulatory T cells

The authors declare that they have no financial competing interests.

Daniel M. Frey and Raoul A. Droeser contributed equally to the study.

DOI: 10.1002/ijc.24989

History: Received 15 May 2009; Accepted 24 Sep 2009; Online 23 Oct 2009

Correspondence to: Daniel M. Frey, Department of Surgery, University Hospital of Basel, Basel, Switzerland, Tel: +41-61-265-25-25, Fax: +41-61-265-72-50, E-mail: dfrey@uhbs.ch

patients.[18] However, the impact of tumor-infiltrating T_{reg} frequency and genetic tumor background (MMR-proficient *vs.* deficient) on clinical outcome of CRC patients has not been elucidated so far.

The objective of this study was to investigate the prognostic significance of tumor-infiltrating T_{reg} by analyzing immunohistochemical FOXP3 expression in tissue microarray (TMA) specimens obtained from a large series of CRC resections stratified into MMR-proficient and MMR-deficient samples and to correlate the findings with known clinicopathological features.

Material and Methods

Tissue microarray construction

A TMA of 1,420 unselected, nonconsecutive, primary CRCs was used. Construction of this TMA has been described previously.[19] Briefly, formalin-fixed, paraffin-embedded tissue blocks of CRC resections were obtained. Tissue cylinders with a diameter of 0.6 mm were punched from morphologically representative tissue areas of each donor tissue block and brought into 1 recipient paraffin block (30×25 mm^2), using our semiautomated tissue arrayer. Each punch was made from the center of the tumor such that each TMA spot consisted of at least 50% tumor cells.

Immunohistochemistry

Four-micron sections of TMA blocks were transferred to an adhesive-coated slide system (Instrumedics, Hackensack, NJ). Standard indirect immunoperoxidase procedures were used for immunohistochemistry (IHC; ABC-Elite, Vector Laboratories, Burlingame, CA). Briefly, the 1,420 punches were dewaxed and rehydrated in distilled water. Endogenous peroxidase activity was blocked using 0.5% H_2O_2. The sections were incubated with 10% normal goat serum (DakoCytomation, Carpinteria, CA) for 20 min and incubated with primary antibody at room temperature. The following primary antibodies were used: MLH1 (clone MLH-1, BD Biosciences, San Jose, CA), MSH2 (clone MSH-2, BD Biosciences), MSH6 (clone 44, Transduction Laboratories, San Jose, CA), FOXP3 (clone 236A/E7, Abcam, Cambridge, UK), CD3 (Clone F7.2.38, DakoCytomation, Switzerland), and CD8 (clone C8/144B, DakoCytomation, Switzerland). Subsequently, sections were incubated with peroxidase-labeled secondary antibody (DakoCytomation) for 30 min at room temperature. For visualization of the antigen, the sections were immersed in 3-amino-9-ethylcarbazole plus substrate-chromogen (DakoCytomation) for 30 min, and counterstained with Gill's hematoxylin.

Mismatch repair status

The 1,420 CRC were stratified according to DNA MMR status: (*i*) MMR-proficient tumors were defined as expressing MLH1, MSH2 and MSH6; (*ii*) MMR-deficient tumors lacking MLH1 or MSH2 and/or MSH6 at any age, or loss of MLH1 at less than 55 years.[20] Stratification revealed a TMA composition of 1197 MMR-proficient tumors and 223 MMR-deficient tumors (including presumed HNPCC tumors).

Clinicopathological features

The clinicopathological data included patient age at diagnosis, tumor diameter, tumor location (right-sided, left-sided or rectum), pT stage, pN stage, tumor grade, histologic subtype, vascular invasion, tumor border configuration, the presence of peritumoral lymphocytic inflammation at the invasive tumor front and disease-specific survival. Tumor border configuration and peritumoral lymphocytic inflammation was evaluated according to the method proposed by Jass *et al.*[21] using the original H&E slides of the resection specimens corresponding to each tissue microarray punch.

Evaluation of immunohistochemistry

The total number of TILs as well as the number of FOXP3$^+$, CD3$^+$, and CD8$^+$ cells contained within each punch, approximately the same as 1 high power (40×) field, was determined for each case. MLH1, MSH2 and MSH6, respectively, were scored as negative (0% staining) or positive (>0% staining).

Statistical analysis

To test the reproducibility of the associations of FOXP3 and clinico-pathological features, the larger cohort of 1,197 MMR-proficient CRCs was randomized into 2 subgroups of patients, containing ~50% of cases. The cut-off score with which to classify tumors as having a low or high FOXP3 cell count was obtained by performing receiver operating characteristic (ROC) curve analysis on Test Group 1 only.[22] With this method, the sensitivity and false positive rate for discriminating survivors and nonsurvivors of CRC was determined at each FOXP3 count, thereby generating a ROC curve. The (0, 1) criterion was used to select the score minimizing the trade-off between sensitivity and specificity and the reliability of the selected cut-off was tested by resampling of the data 500 times. The cut-off score was determined to be 17 cells/TMA punch, a value which corresponded as well to the median number of FOXP3 cells/punch. Chi-Square or Fisher's Exact test were used to determine the association of FOXP3 expression and clinicopathological features. Univariate survival analysis was carried out by the Kaplan–Meier method and log rank test. Subsequently, a Bonferroni correction was applied to the data to adjust for multiple comparisons. Therefore, only p-values ≤ 0.004 were considered significantly associated with FOXP3 expression. The assumption of proportional hazards was verified for FOXP3 by analyzing the correlation of Schoenfeld residuals and the ranks of individual failure times. Any missing clinico-pathological information was assumed to be missing at random. Subsequently FOXP3 was entered into multivariable Cox regression analysis and hazard ratios (HR) and 95% confidence intervals (CI) were used to determine the prognostic effect of FOXP3 on survival time. Spearman's rank correlation was used to

Table 1. Characteristics of patients with mismatch repair (MMR)-proficient (test group 1; $n = 613$ and test group 2; $n = 584$ groups) and MMR-deficient colorectal cancers ($n = 223$)

		MMR-proficient			
		Frequency *N* (%)			
Clinico-pathological features		Test Group 1	Test Group 2	*p*-value	MMR-deficient Frequency *N* (%)
Gender	Female	288 (49.6)	308 (50.5)	0.75	145 (65.0)
	Male	293 (50.4)	302 (49.5)		78 (65.0)
Tumor location	Left-sided	198 (34.4)	185 (30.7)	0.272	47 (21.1)
	Right-sided	159 (27.7)	184 (30.6)		145 (65.0)
	Rectum	218 (37.9)	233 (38.7)		31 (13.9)
pT stage	pT1	27 (4.7)	33 (5.6)	0.764	2 (0.9)
	pT2	92 (16.1)	97 (16.4)		14 (6.3)
	pT3	361 (63.1)	379 (63.9)		159 (71.6)
	pT4	92 (16.1)	84 (14.2)		47 (21.2)
pN stage	pN0	283 (51.1)	304 (51.7)	0.211	124 (56.1)
	pN1	136 (24.6)	172 (29.3)		50 (22.6)
	pN2	135 (24.4)	112 (19.1)		47 (21.3)
Tumor grade	G1–2	518 (88.7)	551 (89.9)	0.507	174 (78.0)
	G3	66 (11.3)	62 (10.1)		49 (22.0)
Histological subtype	Mucinous	42 (7.2)	47 (7.7)	0.754	30 (13.5)
	Other	542 (92.8)	566 (92.3)		193 (86.6)
Vascular invasion	Absent	405 (71.2)	429 (71.9)	0.797	168 (76.7)
	Present	164 (28.8)	168 (27.1)		51 (23.3)
Tumor border configuration	Pushing	211 (37.2)	219 (36.7)	0.887	83 (37.7)
	Infiltrating	357 (62.9)	377 (63.3)		137 (62.3)
Peritumoral lymphocytic inflammation	Absent	458 (80.5)	470 (78.7)	0.455	164 (74.6)
	Present	111 (19.5)	127 (21.3)		56 (25.5)
		Mean (range)			Mean (range)
Patient age at diagnosis	(years)	70.1 (36–96)	70.0 (30–96)	0.881	69.1 (37–93)
					59.5 (6–170)
Tumor diameter	(mm)	47.5 (5–130)	47.5 (4–150)	0.998	
		5-year survival rate (95%CI)			5-year survival rate (95%CI)
Survival rate	(95%CI)	53.8 (49–58)	53.3 (48–58)	0.706	71.2 (64–77)

analyze the correlation between $CD3^+$, $CD8^+$, and $FOXP3^+$ TILs. Analyses were carried out with SAS (V9.1, The SAS Institute, Cary, NC).

Results

Patients

Of the 1,420 patients 1,197 were classified as MMR-proficient; 613 and 584 patients were randomized into Test Groups 1 and 2, respectively (Table 1). No differences in clinico-pathological features were observed between these 2 subgroups, indicating appropriate randomization of cases. MMR-deficient tumors ($n = 223$) showed a preponderance for right-sided tumor location, poor differentiation, a more mucinous histologic subtype and a longer 5-year survival rate compared to MMR-proficient tumors underlining the representativity of this patient cohort.

Association of tumor-infiltrating $FOXP3^+$ T_{reg} frequency with clinicopathological features

MMR-proficient colorectal cancers. In Test Group 1 and 2, FOXP3 staining could be evaluated in 507 and 541 MMR-proficient tumors, respectively (Table 2). In the former, high expression of FOXP3 was significantly more frequent in rectal cancers than in right-sided tumors ($p = 0.01$). High expression of FOXP3 was also associated with earlier pT stages ($p = 0.001$) and, marginally, with the absence of vascular invasion ($p = 0.084$). High expression of FOXP3 in Test Group 2 was significantly more frequent in smaller tumors ($p = 0.003$), as well as in tumors with earlier pT

Table 2. Characteristics of patients with mismatch repair (MMR)-proficient (test group 1; $n = 507$ and test group 2; $n = 541$ groups) and MMR-deficient ($n = 204$) colorectal cancers

		MMR-proficient Test Group 1 Frequency *N* (%)			MMR-proficient Test Group 2 Frequency *N* (%)			MMR-deficient Frequency *N* (%)		
Clinico-pathological features		**FOXP3 Low**	**FOXP3 High**	***p*-value**	**FOXP3 Low**	**FOXP3 High**	***p*-value**	**FOXP3 Low**	**FOXP3 High**	***p*-value**
Gender	Female	124 (51.7)	134 (50.2)	0.739	133 (51.2)	142 (50.5)	0.885	77 (58.8)	53 (72.6)	0.049
	Male	116 (48.3)	133 (49.8)		127 (48.9)	139 (49.5)		54 (41.2)	20 (27.4)	
Patient age at diagnosis (years)	Mean (range)	70.0 (37–96)	69.2 (36–95)	0.354	68.6 (43–96)	70.4 (30–96)	0.066	68.7 (37–93)	69.9 (38–88)	0.497
Tumor diameter (mm)	Mean (range)	47.9 (5–130)	46.0 (5–120)	0.245	49.6 (5–150)	44.7 (5–100)	0.003	62.3 (6–170)	56.9 (6–170)	0.195
Tumor location	Left-sided	100 (42.4)	84 (31.6)	0.01	91 (35.9)	70 (24.9)	0.045	24 (18.3)	18 (24.7)	0.476
	Right-sided	68 (28.8)	62 (23.3)		80 (31.5)	80 (28.5)		89 (67.9)	46 (63.0)	
	Rectum	80 (33.9)	120 (45.1)		83 (32.7)	131 (46.6)		18 (13.7)	9 (12.3)	
pT stage	pT1–2	37 (15.6)	73 (27.8)	0.001	40 (15.9)	79 (28.6)	<0.001	9 (6.9)	7 (9.6)	0.499
	pT3–4	200 (84.4)	190 (72.2)		212 (84.1)	197 (71.4)		121 (93.1)	66 (90.4)	
pN stage	pN0	113 (49.3)	136 (53.1)	0.406	110 (43.7)	160 (58.8)	<0.001	67 (51.9)	50 (68.5)	0.023
	pN1–2	116 (50.7)	120 (46.9)		142 (56.4)	112 (41.2)		62 (48.1)	23 (31.5)	
Tumor grade	G1–2	212 (88.3)	244 (91.4)	0.254	232 (89.2)	262 (93.2)	0.098	102 (77.9)	58 (79.5)	0.791
	G3	28 (11.7)	23 (8.6)		28 (10.8)	19 (6.8)		29 (22.1)	15 (20.6)	
Histological subtype	Mucinous	16 (6.7)	15 (5.6)	0.624	22 (8.5)	11 (3.9)	0.027	17 (13.0)	9 (12.3)	0.894
	Other	224 (93.3)	252 (94.4)		238 (91.5)	270 (96.1)		114 (87.0)	64 (87.7)	
Vascular invasion	Absent	160 (67.8)	196 (74.8)	0.084	172 (98.0)	211 (75.9)	0.042	94 (72.9)	61 (85.9)	0.035
	Present	76 (32.2)	66 (25.2)		81 (32.0)	67 (24.1)		35 (27.1)	10 (14.1)	
Tumor border configuration	Infiltrating	148 (63.0)	168 (64.1)	0.791	166 (65.6)	174 (62.8)	0.503	84 (65.1)	41 (56.9)	0.252
	Pushing	87 (37.0)	94 (35.9)		87 (34.4)	103 (37.2)		45 (34.9)	31 (43.1)	
Peritumoral lymphocytic inflammation	Absent	193 (81.8)	210 (80.2)	0.645	209 (82.6)	205 (73.7)	0.014	100 (77.5)	47 (65.3)	0.061
	Present	43 (18.2)	52 (19.9)		44 (17.4)	73 (26.3)		29 (22.5)	25 (34.7)	
		Mean (range)			Mean (range)			Mean (range)		
Patient age at diagnosis	(years)	70.0 (37–96)	69.2 (36–95)	0.354	68.6 (43–96)	70.4 (30–96)	0.066	68.7 (37–93)	69.9 (38–88)	0.497
Tumor diameter	(mm)	47.9 (5–130)	46.0 (5–120)	0.245	49.6 (5–150)	44.7 (5–100)	0.003	62.3 (6–170)	56.9 (6–170)	0.195
		5-year survival rate (95%CI)			5-year survival rate (95%CI)			5-year survival rate (95%CI)		
5-year survival rate	(95%CI)	45.9 (39–53)	61.7 (55–68)	0.004	44.4 (38–51)	59.7 (51–64)	<0.001	66.6 (57–74)	82.7 (71–91)	0.029

Table 3. Prognostic effect of low and high FOXP3 expression after adjustment for age, gender, pT stage, pN stage, tumor grade, vascular invasion and tumor border configuration in colorectal cancer

	MMR-proficient Test Group 1		MMR-proficient Test Group 2		MMR-deficient	
Features	HR (95%CI)	*p*-value	HR (95%CI)	*p*-value	HR (95%CI)	*p*-value
High versus low FOXP3 expression	0.73 (0.6–0.9)	0.019	0.7 (0.6–0.9)	0.007	0.63 (0.3–1.2)	0.13
Increasing age	1.03 (1.01–1.04)	<0.001	1.04 (1.01–1.06)	<0.001	1.01 (0.99–1.0)	0.246
Female versus male gender	0.78 (0.6–1.0)	0.062	0.66 (0.5–0.9)	0.001	0.93 (0.5–1.6)	0.804
pT3–T4 versus pT1–2	2.42 (1.5–3.9)	<0.001	2.03 (1.3–3.1)	<0.001		–
pN0 versus pN1–2	2.55 (1.9–3.4)	<0.001	1.97 (1.5–2.6)	<0.001	2.76 (1.5–5.0)	<0.001
G3 versus G1–2	1.63 (1.1–2.4)	0.015	1.63 (1.1–2.4)	0.018	1.15 (0.6–2.2)	0.675
Presence versus absence of vascular invasion	1.85 (1.4–2.5)	<0.001	1.78 (1.4–2.4)	<0.001	1.51 (0.8–2.7)	0.171
Infiltrating versus pushing tumor border configuration	1.72 (1.2–2.4)	0.001	2.11 (1.5–3.0)	<0.001	1.5 (0.8–2.9)	0.221

stage ($p < 0.001$), and in lymph node negative cases ($p < 0.001$), and frequently found in rectal tumors ($p = 0.045$), in cancers with nonmucinous histology ($p = 0.027$) and in those with absence of vascular invasion ($p = 0.042$). Together, the results from Test Groups 1 and 2 underline the reproducible association of high FOXP3 expression with left-sided MMR-proficient CRCs and with earlier pT stage.

MMR-deficient colorectal cancer. FOXP3 expression could be evaluated in 204 cases. High FOXP3 expression in MMR-deficient cases was more commonly found in female patients ($p = 0.049$), in tumors with no lymph node involvement ($p = 0.023$), and in cases with an absence of vascular invasion ($p = 0.035$).

Association of tumor-infiltrating FOXP3$^+$ T_{reg} frequency with disease-specific survival

Univariate survival analysis. A significant effect of FOXP3 expression on disease-specific survival was observed for both MMR-proficient and -deficient CRCs. In MMR-proficient Test Group 1, the 5-year survival rate for patients with high *vs.* low expression of FOXP3 was 61.7% (95%CI: 55–68) and 45.9% (95%CI: 39–53), respectively (Fig. 1*a*; $p = 0.004$). Similarly in Test Group 2, survival time was significantly more favorable in patients with high FOXP3-expressing tumors compared to those with low expression (5-year survival rate 59.7% (95%CI: 51–64) and 44.4% (95%CI: 35–51), respectively) (Fig. 1*b*; $p < 0.001$). In MMR-deficient cases, high FOXP3 counts were again significantly linked to prolonged survival time compared to low expression of the marker (Fig. 1*c*; $p = 0.029$).

Multivariable analysis. To determine whether the beneficial prognostic effect of FOXP3 in both MMR-proficient and -deficient CRCs was maintained after adjustment for other well-established prognostic factors, multivariable analysis was performed including clinico-pathological features (age, gender, pT stage, pN stage, tumor grade, vascular invasion and tumor border configuration). In both MMR-proficient Test Group 1 and 2, high expression of FOXP3 was associated with an improved disease-specific survival time independently of these features (Test Group 1: *p*-value = 0.019; HR (95%CI) = 0.73 (0.6–0.9), Test Group 2: *p*-value = 0.007; HR (95%CI) = 0.7 (0.6–0.9)). In MMR-deficient cancers, high expression of FOXP3 did not maintain its favorable effect on outcome in multivariable setting (Table 3).

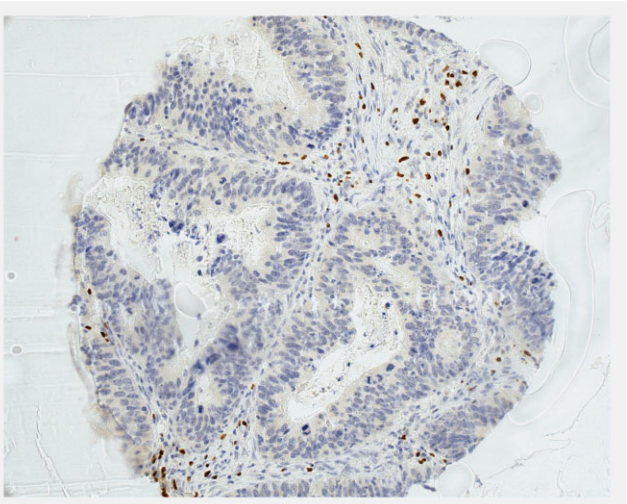

Figure 1. Nuclear FOXP3 staining in a mismatch repair (MMR)-proficient colorectal carcinoma (magnification ×40).

Correlation between CD3$^+$, CD8$^+$, and FOXP3$^+$ in MMR-proficient colorectal cancers

To evaluate the relationship between CD3$^+$, CD8$^+$, and FOXP3$^+$- positive cells in MMR-proficient CRCs, additional immunohistochemical staining were performed. The 1,108 and 1,071 cases of MMR-proficient tumors were evaluable for CD3$^+$ and CD8$^+$, respectively. A strong, positive

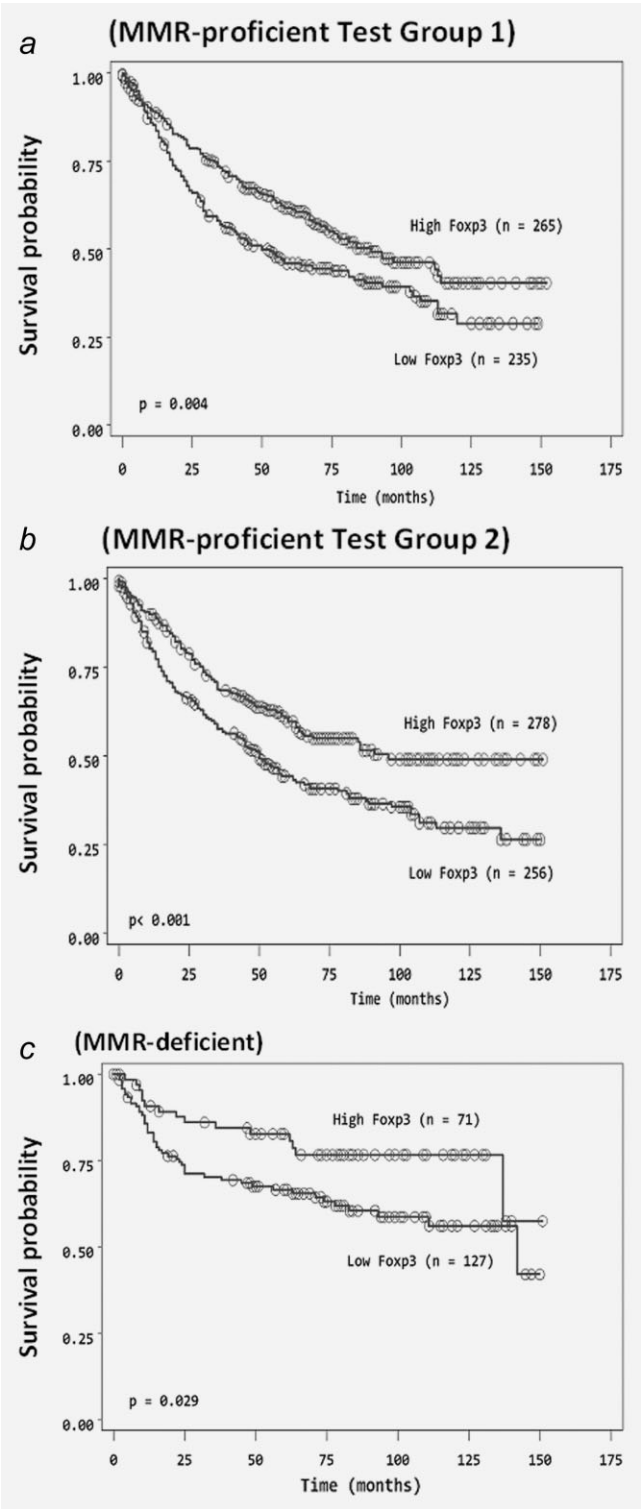

Figure 2. Survival time differences between low and high Foxp3 expression in mismatch repair (MMR)-proficient colorectal cancers in (*a*) Test Group 1 and (*b*) Test Group 2 and in MMR-deficient (*c*) colorectal cancers.

correlation was observed between the 3 TIL types. The correlation between CD3$^+$ and FOXP3 was $r = 0.31$ ($p < 0.001$) and $r = 0.49$ for CD8$^+$ and FOXP3$^+$ ($p < 0.001$).

Discussion

The aim of the present study was to analyze the prognostic impact of FOXP3$^+$ tumor-infiltrating T_{reg} in a large series of CRC samples stratified by MMR status using immunohistochemistry and TMA technology. Indeed, in univariate and multivariable analysis including age, gender, T stage, N stage, tumor grade, vascular invasion and tumor border configuration, a high FOXP3$^+$ T_{reg} frequency was associated with a significantly improved 5-year survival rate in MMR-proficient CRCs, but not in the MMR-deficient CRCs. By using 2 randomized cohorts of MMR-proficient cases in addition to ROC curve analysis, the effect of FOXP3 on outcome was found to be reproducible.

Tumor-infiltrating lymphocytes have been shown to inhibit tumor growth in a variety of solid tumors and a high frequency of TILs is associated with improved prognosis.[23] Accumulating evidence suggests that type, frequency and location of immune cells in CRC may have a higher prognostic value than the tumor-node-metastasis (TNM)-classification of the International Union against Cancer (UICC).[1,2,24] Tumor-infiltrating FOXP3$^+$ T_{reg} play a significant role in suppressing the host antitumor response and therefore might adversely affect prognosis. Accumulation of FOXP3$^+$ T_{reg} in the microenvironment of tumors has been demonstrated in ovarian,[7,9] lung,[10] breast,[8,11] pancreatic,[12] hepatocellular,[25–27] head and neck,[13] colorectal,[14,28] prostate[15] and anal carcinoma.[16] In most of these solid tumors, high frequency of tumor infiltrating FOXP3$^+$ T_{reg} was associated with poor patient survival. Moreover, increasing evidence indicates that tumor-specific T_{reg} exist that actively suppress antigen-specific antitumor immunity in tumor bearing patients.[29] In CRC, increased frequency of FOXP3$^+$ T_{reg} is found in peripheral blood,[14] in mesenteric lymph nodes,[30] as well as intratumorally.[28,31] Loddenkemper *et al.* analyzed a series of 40 CRC patients and found a significantly higher infiltration of FOXP3$^+$ T_{reg} in tumors of patients with limited disease than in tumors of patients with metastatic CRC.[28] Surprisingly, no association was found between total T_{reg} infiltration and patient survival neither with nor without stage correction. However, increased T_{reg} frequency has been found to be associated with improved prognosis in lymphoma patients.[17,32]

We used the transcription factor forkhead box P3 (FOXP3) to identify tumor-infiltrating T_{reg} which is an intracellular key control molecule for T_{reg} development and function and considered to represent the most specific T_{reg} cell marker so far.[6–8] Previous publications showed that the vast majority of T cells stained with the monoclonal antibody 236A/E7, which was also used in our study, were CD4$^+$CD25$^+$ T_{reg}.[33–35] We determined the cut-off score for the number of positive FOXP3 T_{reg} within the TIL population by ROC curve analysis. This method was used to select

the most clinically relevant cut-off score for positive FOXP3 expression which maximizes the correct number of patients who have died of CRC or are still alive.

In our previous publication on the same TMA we have shown that the significantly higher density of TILs in MMR-deficient versus MMR-proficient tumors correlates with a significantly better survival in MMR-deficient CRC patients. This was especially the case for the intraepithelial CD8 T cell count.[36] Our original hypothesis stated that a high frequency of tumor-infiltrating FOXP3$^+$ T_{reg} correlates with a poor prognosis for survival in CRC patients. Surprisingly, we found that high frequency of tumor-infiltrating FOXP3$^+$ T_{reg} correlates with considerable improved patient survival in MMR-proficient, but not MMR-deficient patients in univariate analysis. This effect was maintained following adjustment for multiple comparisons carried out to compensate for the exploratory nature of this analysis. In addition, multivariable analysis again highlighted the independent prognostic effect of FOXP3 expression in MMR-proficient CRC patients.

Our results are in line with a recently published study by Salama *et al.* who observed an association between a high frequency of tumor-infiltrating FOXP3$^+$ T_{reg} and improved survival in CRC patients.[18] In contrast to the previous data of Michel *et al.*, we found a significant higher amount of FOXP3$^+$ tumor-infiltrating T_{reg} in MMR-proficient CRC samples.[37] These results agree with a recently published study investigating the prevalence of FOXP3 and Interleukin 17 (IL 17) in MMR-proficient carcinomas. Le Gouvello *et al.* observed a higher expression of FOXP3, IL 17, IL1β, IL6 and transforming growth factor-β (TGF-β) in MMR-proficient CRC.[31] In our study, high density FOXP3$^+$ T_{reg} was significantly associated with early pT stage and location in both MMR-proficient subgroups, whereas in MMR-deficient tumors FOXP3 expression correlated with absence of lymph node involvement and vascular invasion.

MMR-deficiency is associated with longer disease-free and overall survival even in the case of deep local invasion of the primary tumor.[3,38,39] Tumors of the MMR-deficient phenotype are less likely to metastasize than those characterized by microsatellite stability. Furthermore, MMR-deficient tumors are related to an abundant neoplastic tissue infiltration of CD3$^+$ and CD8$^+$ T cells[40,41] that could result in recognition of frameshift-mutated neoantigens.[42]

The role of T_{reg} may differ according to the clinical stage and the genetic background of tumors (MMR-proficient *vs.* MMR-deficient; low *vs.* high antigenicity, respectively). It has been reported that the frequency of T_{reg} increases during tumor progression and that depletion of T_{reg} promotes tumor-specific immune responses which may be efficient to eradicate established tumors.[43–45] This may be the case for a sterile microenvironment and high tumor antigenicity, but most likely different for the gut. Considering this hypothesis and our results, high density of tumor-infiltrating T_{reg} seems to have the strongest prognostic value in the MMR-proficient CRC subgroup. The most important question concerning T_{reg} in solid tumors is whether these cells have a suppressive capacity. Recent findings suggest that human FOXP3$^+$ T_{reg} also possess an effector differentiation program resulting in IL-17 production.[46] Radhakrishnan *et al.* showed that these reprogrammed T_{reg} downregulated FOXP3 expression, secreted proinflammatory cytokines interferon-γ (IFN-γ), IL-17 and tumor necrosis factor-α (TNF-α), and mounted potent responses against self-antigens *in vivo*.[47] Surprisingly, these IL-17-producing T_{reg} did not cause a generalized autoimmunity, but rather a specific immune attack was fostered. The micro-environment of MMR-proficient CRC is rich in bacteria and cytokines and exerts a proinflammatory effect, thus favoring T_{reg} differentiation into IL-17$^+$ antigen-specific autoimmune effector cells. On the other hand, FOXP3$^+$ T_{reg} could also be beneficial in some cancers by downregulating an unresolved inflammatory response which could promote tumor progression.[48] However, the mechanisms leading to the observed correlation between T_{reg} infiltration and prognosis in MMR-proficent CRC and its clinical impact remain unclear and warrant further investigation. Functional studies of tumor-infiltrating FOXP3$^+$ T_{regs} are needed to clarify their role in antitumor response and to explain the relationship between T_{reg} infiltration and prognosis.

In conclusion, our study shows that a high frequency of tumor-infiltrating FOXP3$^+$ T_{reg} is associated with early pT stage and is an independent predictor of improved survival in MMR-proficient CRCs.

References

1. Pages F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R, Mlecnik B, Kirilovsky A, Nilsson M, Damotte D, Meatchi T, Bruneval P, *et al.* Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med* 2005;353:2654–66.
2. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, *et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006;313:1960–4.
3. Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol* 2005;23:609–18.
4. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005;6:345–52.
5. Wang HY, Wang RF. Regulatory T cells and cancer. *Curr Opin Immunol* 2007;19:217–23.
6. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299:1057–61.
7. Wolf D, Wolf AM, Rumpold H, Fiegl H, Zeimet AG, Muller-Holzner E, Deibl M, Gastl G, Gunsilius E, Marth C. The expression of the regulatory T cell-specific forkhead box transcription factor FoxP3 is associated with poor prognosis in ovarian cancer. *Clin Cancer Res* 2005;11:8326–31.
8. Bates GJ, Fox SB, Han C, Leek RD, Garcia JF, Harris AL, Banham AH. Quantification

of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *J Clin Oncol* 2006;24:5373–80.
9. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 2004;10:942–9.
10. Petersen RP, Campa MJ, Sperlazza J, Conlon D, Joshi MB, Harpole DH, Jr, Patz EF, Jr. Tumor infiltrating Foxp3+ regulatory T-cells are associated with recurrence in pathologic stage I NSCLC patients. *Cancer* 2006;107:2866–72.
11. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS, Linehan DC. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol* 2002;169:2756–61.
12. Hiraoka N, Onozato K, Kosuge T, Hirohashi S. Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. *Clin Cancer Res* 2006;12:5423–34.
13. Badoual C, Hans S, Rodriguez J, Peyrard S, Klein C, Agueznay Nel H, Mosseri V, Laccourreye O, Bruneval P, Fridman WH, Brasnu DF, Tartour E. Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers. *Clin Cancer Res* 2006;12:465–72.
14. Ling KL, Pratap SE, Bates GJ, Singh B, Mortensen NJ, George BD, Warren BF, Piris J, Roncador G, Fox SB, Banham AH, Cerundolo V. Increased frequency of regulatory T cells in peripheral blood and tumour infiltrating lymphocytes in colorectal cancer patients. *Cancer Immunol* 2007;7:7.
15. Miller AM, Lundberg K, Ozenci V, Banham AH, Hellstrom M, Egevad L, Pisa P. CD4+CD25high T cells are enriched in the tumor and peripheral blood of prostate cancer patients. *J Immunol* 2006;177:7398–405.
16. Grabenbauer GG, Lahmer G, Distel L, Niedobitek G. Tumor-infiltrating cytotoxic T cells but not regulatory T cells predict outcome in anal squamous cell carcinoma. *Clin Cancer Res* 2006;12:3355–60.
17. Carreras J, Lopez-Guillermo A, Fox BC, Colomo L, Martinez A, Roncador G, Montserrat E, Campo E, Banham AH. High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. *Blood* 2006;108:2957–64.
18. Salama P, Phillips M, Grieu F, Morris M, Zeps N, Joseph D, Platell C, Iacopetta B. Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. *J Clin Oncol* 2009;27:186–92.
19. Sauter G, Simon R, Hillan K. Tissue microarrays in drug discovery. *Nat Rev Drug Discov* 2003;2:962–72.
20. Hampel H, Stephens JA, Pukkala E, Sankila R, Aaltonen LA, Mecklin JP, de la Chapelle A. Cancer risk in hereditary nonpolyposis colorectal cancer syndrome: later age of onset. *Gastroenterology* 2005;129:415–21.
21. Jass JR, Atkin WS, Cuzick J, Bussey HJ, Morson BC, Northover JM, Todd IP. The grading of rectal cancer: historical perspectives and a multivariate analysis of 447 cases. *Histopathology* 1986;10:437–59.
22. Zlobec I, Steele R, Terracciano L, Jass JR, Lugli A. Selecting immunohistochemical cut-off scores for novel biomarkers of progression and survival in colorectal cancer. *J Clin Pathol* 2007;60:1112–6.
23. Ohtani H. Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human colorectal cancer. *Cancer Immunol* 2007;7:4.
24. Galon J, Fridman WH, Pages F. The adaptive immunologic microenvironment in colorectal cancer: a novel perspective. *Cancer Res* 2007;67:1883–6.
25. Gao Q, Qiu SJ, Fan J, Zhou J, Wang XY, Xiao YS, Xu Y, Li YW, Tang ZY. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *J Clin Oncol* 2007;25:2586–93.
26. Fu J, Xu D, Liu Z, Shi M, Zhao P, Fu B, Zhang Z, Yang H, Zhang H, Zhou C, Yao J, Jin L, *et al.* Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. *Gastroenterology* 2007;132:2328–39.
27. Yang XH, Yamagiwa S, Ichida T, Matsuda Y, Sugahara S, Watanabe H, Sato Y, Abo T, Horwitz DA, Aoyagi Y. Increase of CD4+ CD25+ regulatory T-cells in the liver of patients with hepatocellular carcinoma. *J Hepatol* 2006;45:254–62.
28. Loddenkemper C, Schernus M, Noutsias M, Stein H, Thiel E, Nagorsen D. *In situ* analysis of FOXP3+ regulatory T cells in human colorectal cancer. *J Transl Med* 2006;4:52.
29. Nishikawa H, Jager E, Ritter G, Old LJ, Gnjatic S. CD4+ CD25+ regulatory T cells control the induction of antigen-specific CD4+ helper T cell responses in cancer patients. *Blood* 2005;106:1008–11.
30. Clarke SL, Betts GJ, Plant A, Wright KL, El-Shanawany TM, Harrop R, Torkington J, Rees BI, Williams GT, Gallimore AM, Godkin AJ. CD4+CD25+FOXP3+ regulatory T cells suppress anti-tumor immune responses in patients with colorectal cancer. *PLoS ONE* 2006;1:e129.
31. Le Gouvello S, Bastuji-Garin S, Aloulou N, Mansour H, Chaumette MT, Berrehar F, Seikour A, Charachon A, Karoui M, Leroy K, Farcet JP, Sobhani I. High prevalence of Foxp3 and IL17 in MMR-proficient colorectal carcinomas. *Gut* 2008;57:772–9.
32. Alvaro T, Lejeune M, Salvado MT, Bosch R, Garcia JF, Jaen J, Banham AH, Roncador G, Montalban C, Piris MA. Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. *Clin Cancer Res* 2005;11:1467–73.
33. Roncador G, Brown PJ, Maestre L, Hue S, Martinez-Torrecuadrada JL, Ling KL, Pratap S, Toms C, Fox BC, Cerundolo V, Powrie F, Banham AH. Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol* 2005;35:1681–91.
34. Walker MR, Carson BD, Nepom GT, Ziegler SF, Buckner JH. De novo generation of antigen-specific CD4+CD25+ regulatory T cells from human CD4+CD25- cells. *Proc Natl Acad Sci USA* 2005;102:4103–8.
35. Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* 2007;37:129–38.
36. Baker K, Zlobec I, Tornillo L, Terracciano L, Jass JR, Lugli A. Differential significance of tumour infiltrating lymphocytes in sporadic mismatch repair deficient versus proficient colorectal cancers: a potential role for dysregulation of the transforming growth factor-beta pathway. *Eur J Cancer* 2007;43:624–31.
37. Michel S, Benner A, Tariverdian M, Wentzensen N, Hoefler P, Pommerencke T, Grabe N, von Knebel Doeberitz M, Kloor M. High density of FOXP3-positive T cells infiltrating colorectal cancers with microsatellite instability. *Br J Cancer* 2008;99:1867–73.
38. Gryfe R, Kim H, Hsieh ET, Aronson MD, Holowaty EJ, Bull SB, Redston M, Gallinger S. Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. *N Engl J Med* 2000;342:69–77.
39. Buckowitz A, Knaebel HP, Benner A, Blaker H, Gebert J, Kienle P, von Knebel Doeberitz M, Kloor M. Microsatellite instability in colorectal cancer is associated with local lymphocyte infiltration and low frequency of distant metastases. *Br J Cancer* 2005;92:1746–53.

40. Dolcetti R, Viel A, Doglioni C, Russo A, Guidoboni M, Capozzi E, Vecchiato N, Macri E, Fornasarig M, Boiocchi M. High prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in colorectal carcinomas with microsatellite instability. *Am J Pathol* 1999;154:1805–13.
41. Lynch HT, Smyrk TC, Watson P, Lanspa SJ, Lynch JF, Lynch PM, Cavalieri RJ, Boland CR. Genetics, natural history, tumor spectrum, and pathology of hereditary nonpolyposis colorectal cancer: an updated review. *Gastroenterology* 1993;104:1535–49.
42. Ishikawa T, Fujita T, Suzuki Y, Okabe S, Yuasa Y, Iwai T, Kawakami Y. Tumor-specific immunological recognition of frameshift-mutated peptides in colon cancer with microsatellite instability. *Cancer Res* 2003;63:5564–72.
43. Viehl CT, Moore TT, Liyanage UK, Frey DM, Ehlers JP, Eberlein TJ, Goedegebuure PS, Linehan DC. Depletion of CD4+CD25+ regulatory T cells promotes a tumor-specific immune response in pancreas cancer-bearing mice. *Ann Surg Oncol* 2006;13:1252–8.
44. Liyanage UK, Goedegebuure PS, Moore TT, Viehl CT, Moo-Young TA, Larson JW, Frey DM, Ehlers JP, Eberlein TJ, Linehan DC. Increased prevalence of regulatory T cells (Treg) is induced by pancreas adenocarcinoma. *J Immunother* 2006;29: 416–24.
45. Yu P, Lee Y, Liu W, Krausz T, Chong A, Schreiber H, Fu YX. Intratumor depletion of CD4+ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. *J Exp Med* 2005;201: 779–91.
46. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. *Blood* 2008;112:2340–52.
47. Radhakrishnan S, Cabrera R, Schenk EL, Nava-Parada P, Bell MP, Van Keulen VP, Marler RJ, Felts SJ, Pease LR. Reprogrammed FoxP3+ T regulatory cells become IL-17+ antigen-specific autoimmune effectors *in vitro* and *in vivo*. *J Immunol* 2008;181:3137–47.
48. Nam JS, Terabe M, Kang MJ, Chae H, Voong N, Yang YA, Laurence A, Michalowska A, Mamura M, Lonning S, Berzofsky JA, Wakefield LM. Transforming growth factor beta subverts the immune system into directly promoting tumor growth through interleukin-17. *Cancer Res* 2008;68:3915–23.