Research Article

Cancer Immunology Research

Combined Low Densities of FoxP3$^+$ and CD3$^+$ Tumor-Infiltrating Lymphocytes Identify Stage II Colorectal Cancer at High Risk of Progression

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Abstract

The densities of CD3$^+$ and CD8$^+$ tumor-infiltrating lymphocytes (TILs), combined with tumor–node–metastasis (TNM) staging, have prognostic value for patients with nonmetastatic colorectal cancer. We compared the prognostic value of CD3$^+$ and FoxP3$^+$ TILs at the invasive front, TNM classifiers, and microsatellite (MS) status in a trial set of patients with stage II and III colorectal cancer ($n = 413$), by recursive partitioning with a classification and regression tree (CART). Significant prognostic factors and interactions were reassessed by logistic regression and Cox proportional-hazards modeling in the trial and a validation set ($n = 215$) of patients with stage II colorectal cancer. In the trial set, CART indicated that TIL numbers were of value only in predicting recurrence risk for stage II cancers, where low densities of FoxP3$^+$ TILs ranked first and low densities of CD3$^+$ TILs further stratifying risk. Multivariate analysis showed that TILs interacted with tumor stage (FoxP3$^+$, $P = 0.06$; CD3$^+$, $P = 0.02$) and MS instability (MSI; FoxP3$^+$; $P = 0.02$). In stage II MS-stable cancers, concomitant low densities of both FoxP3$^+$ and CD3$^+$ TILs identified patients with the highest progression risk in the trial [HR 7.24; 95% confidence interval (CI), 3.41–15.4; $P < 0.001$] and the validation (HR 15.16; 95% CI, 3.43–66.9; $P < 0.001$) sets. FoxP3$^+$ and CD3$^+$ TIL load in colorectal cancer was more informative than other prognostic factors before the cancer progressed to lymph nodes. This prognostic information about TILs, including FoxP3$^+$ cells, suggests that randomized controlled trials might be refined to include interactions between TNM status, molecular classifiers, and post-surgical treatments.

Introduction

Translational studies have clarified the role of the local immune response in controlling the progression of colorectal cancer. High intra- and peri-tumoral densities of CD3$^+$ T cells, CD8$^+$ cytotoxic lymphocytes, and memory CD45RO$^+$ cells predict a better post-surgical outcome (1, 2). Densities of CD3$^+$ and CD8$^+$ cells at these locations defined an immunoscore assessing the extent of the antitumor response in early colorectal cancer that was more informative than the analysis of a single T-cell population at a given location (3). With a retrospective analysis of colorectal cancer cohorts (4), an international consortium presented Immunoscore as a reliable predictor of cancer recurrence in patients with nonmetastatic colorectal cancer and an improvement upon the prognostic power of conventional tumor–node–metastasis (TNM; T, size and spread of primary cancer; N, number of metastatic lympho nodes; M, metastases spread to distant organs) staging (5).

Although not included in Immunoscore, high densities of FoxP3$^+$ tumor-infiltrating lymphocytes (TILs) predict a favorable

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colorectal cancer outcome (6–13). The positive prognostic value of FoxP3$^+$ TILs is counter-intuitive, given that the expression of the Foxp3$^+$ transcription factor is typical of T-regulatory cells (Tregs), an immunosuppressive population associated with poor prognosis in other cancers (14).

Before the FoxP3$^+$ TILs can function as an immune biomarker predictive of colorectal cancer outcome, their prognostic value is needed to be analyzed. First, densities of FoxP3$^+$ TILs might be influenced by the tumor microsatellite (MS) status (6, 8, 15). Indeed, FoxP3$^+$ TILs had no prognostic value for colorectal cancer with MSI in a study stratified by tumor mismatch-repair status (7). Second, the prognostic value of FoxP3$^+$ TILs is unclear in the context of nodal involvement, which may reflect tumor immune evasion (16). In patients with stage II/III colorectal cancer, FoxP3$^+$ TILs presented as a stage-independent prognostic factor, although stages II and III were not separately analyzed (6). Finally, the interaction of FoxP3$^+$ with other T cells is unclear. Densities of FoxP3$^+$ TILs predicted survival of patients with early colorectal cancer independently of, and more accurately than, densities of CD8$^+$ and CD45RO$^+$ TILs in one study (6), but were less informative than densities of CD45RO$^+$ TILs in another study (8). FoxP3$^+$ TILs were also reported to significantly interact with the prognostic value of CD8$^+$or CD3$^+$ TILs (9, 11, 12). The aim of this study was to see whether densities of FoxP3$^+$ cells at the invasive tumor front can add to the prognostic significance of CD3$^+$ TILs in a patient series of pT3/pT4 colorectal cancer stratified by nodal involvement and MS status.

Materials and Methods

Study population

The trial set included formalin-fixed, paraffin-embedded (FFPE) tumor specimens from 413 consecutive patients with stage II/III pT3-T4 colorectal cancer who had received radical surgery at the Humanitas Clinical and Research Center (Milan, Italy), from 1997 to 2006.

The external validation set included tissues of stage II colorectal cancer from 215 consecutive patients who had undergone surgery from 2010 to 2015 at the St. Orsola-Malpighi Hospital (Bologna, Italy; $n = 74$), or from 2008 to 2014 at the Grande Ospedale Metropolitano Niguarda (Milan, Italy; $n = 141$).

The absence of metastasis at diagnosis was assessed definitively in all patients by combining histopathologic findings, surgical records, and perioperative imaging (abdominal CT and chest radiography in all patients). The observation period started immediately after surgery. To monitor postsurgical tumor recurrences, thoraco-abdominal CT, abdominal ultrasonography, and chest radiography were done according to common protocols for surveillance. Patients with rectal cancer treated with neo-adjuvant radiotherapy were not included in the study. The 5-fluorouracil chemotherapy was administered on clinical grounds and not in the context of prospective trials. MS status was systematically assessed by analysis of mononucleotide repeats.

MSI assignment was based on the analysis of repeats in mononucleotides *BAT26* and *BAT25*. DNA was purified from paraffin sections of formalin-fixed tissue with neoplastic cell content above 50%. After DNA extraction by proteinase-K digestion, *BAT26* and *BAT25* loci were amplified by fluoresceinated primers, and PCR products analyzed by capillary electrophoresis (ABI PRISM 310 DNA Sequence; PE Applied Biosystems). Finally, the MSI phenotype tumours were investigated for MMR protein defects by IHC (17, 18).

Ethics approval and consent to participate

The study was conducted in accordance with the Helsinki Declaration on ethical principles for medical research involving human subjects. Samples from both the internal and external set were obtained complying with protocols approved by the local Ethical Committee and Institutional Review Board at Humanitas Clinical and Research Center (approval no. 1052 and further acknowledgment of 14 May 2013). All patients provided their informed consent to the referring physician or to other clinicians involved in the study at each participating center.

IHC

FFPE and 2-μm thin sections of tumor were deparaffinized and exposed to an antigen-retrieval system (1 mmol/L ethylenediamine tetraacetic acid, pH 8, for 30 minutes at 98°C). Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes at room temperature; nonspecific staining was reduced with Background Sniper (Biocare Medical) for 15 minutes at room temperature. Then, slides were incubated for 60 minutes at room temperature in moist chamber with the specific antibodies for CD3 (1:50, clone F7238; Dako) and FoxP3 (dilution 1:100, clone 236/E7, Abcam), or mouse IgG (Dako) for negative controls. To reveal bound antibodies, the slides were exposed to MACH 4 Universal HRP Polymer system (Biocare Medical) following manufacturer's instructions, followed by incubation with DAB-chromogen X50 (Dako) as chromogen. Finally, nuclei were lightly counterstained with a freshly made hematoxylin solution (Harris Hematoxylin, DiaPath).

Image analysis

From whole-tissue slides, we obtained digital images of three randomly chosen, nonadjacent, nonoverlapping areas at the tumor invasive front. The selected areas had to include 50% of tumor gland and 50% of bordering stroma. The observer who selected the areas of interest was blinded to tumor MS status and to any patient clinical data. The Image-Pro Premier 9.2 (Media Cybernetics) analysis software calculates on the total digital captured area the percentage of TIL immune-reactive area (IRA%) on the basis of red, green, and blue color segmentation. For each specimen, the average IRA% from the three different areas was calculated and used for subsequent statistical analysis.

Statistical analysis

We weighed the relationship between IRA% of CD3$^+$ and FoxP3$^+$ TILs by linear regression, and assessed their association with patient demographics, clinicopathologic features at diagnosis by Wilcoxon/Mann–Whitney test.

For evaluating CD3$^+$ and FoxP3$^+$ TIL densities as prognostic factors, we first used recursive partitioning analysis to identify foretelling variables. These variables included patient demographics and clinicopathologic tumor features, assessed by the classification and regression tree (CART; Salford Systems) software, which also identifies cutoff values for continuous variables. CART, commonly used in data mining, was used to build a model predicting the likelihood of recurrence (dependent variable) testing several independent variables (i.e., demographics, clinical, pathologic, molecular features, and TIL densities; refs. 19, 20). The algorithm weighs each independent variable in predicting the dependent one, moving along a tree-like decision structure. In the decision tree, each node hierarchically identifies the independent variable with the best predictive value. From each node, branches emerge, as long as predictive variables can be identified. Afterward, the prognostic values of CD3$^+$ and FoxP3$^+$ TIL densities were retested by logistic regression, and the variables significantly associated with disease relapse at univariate analysis were entered in a multivariate model including possible interactions. Independent predictors of disease-free survival (DFS) were tested in a Cox proportional hazards (21) model to evaluate progression. DFS was calculated from diagnosis until March 1, 2013, which was the date of data censoring for the trial set, and December 31, 2017, for the validation set. To analyze the survival of patients with colorectal cancer grouped according to TIL density, we plotted Kaplan–Meier curves and obtained the relative log-rank tests. All statistical analyses except for recursive partitioning were managed by STATA (version 13.1). Two-sided P values of <0.05 were considered statistically significant.

Results

Densities of FoxP3$^+$ and CD3$^+$ TILs by patient demographics and tumor features

Variable densities of CD3$^+$ TILs were detectable at the invasive front of 97.3% of tissues from the trial set (402 of 413), and FoxP3$^+$ TILs were detected in 82.3% (340 of 413) of tissues. Linear regression analysis showed no significant correlation ($P = 0.26$) between the density of FoxP3$^+$ TILs (median, 0.37%; 2nd–3rd quartile, 0.13–0.68) and the density of CD3$^+$ TILs (median, 2.42%; 2nd–3rd quartile, 0.92–5.78).

Densities of FoxP3$^+$and CD3$^+$ TILs by patient demographics and tumor molecular/pathologic features are detailed in Supplementary Table S1. MSI correlated with a higher density of CD3$^+$ TILs ($P < 0.001$) and with lower densities of FoxP3$^+$ cells ($P < 0.001$). No FoxP3$^+$ immunoreactivity was detectable in 21 of 66 (31.8%) of these tumors.

Data mining for the prognostic impact of TILs in stage II/III colorectal cancer

CART analysis (Fig. 1) identified TNM staging as the highest hierarchical node (OR, 2.97; 95% CI, 1.85–4.77; $P < 0.001$) in the prognostic tree, stage III accounting for 70 of 102 (68.6%) postoperative recurrences. Densities of FoxP3$^+$ TILs or CD3$^+$ TILs had no predictive value in stage III colorectal cancer, in which only nodal status had further predictive value (N2 vs. N1; OR, 3.56; 95% CI, 1.93–6.58; $P < 0.001$). In contrast, both FoxP3$^+$ and CD3$^+$ TILs had a place in the prognostic tree of stage II colorectal cancer. Low densities of FoxP3$^+$ cells ranked first in this decisional branching (OR 5.20; 95% CI, 2.26–11.9; $P < 0.001$), identifying 23 of 32 (71.9%) recurrences. Secondary branching of low-FoxP3$^+$ tumors by CD3$^+$ TILs further improved the identification of recurrences (OR, 4.46; 95% CI, 1.58–12.6; $P < 0.001$). The combination of low densities for both Foxp3$^+$ and CD3$^+$ TILs predicted 16 of 32 (50.0%) recurrences with 20 of 179 (11.2%) false positives, and thus 83% accuracy.

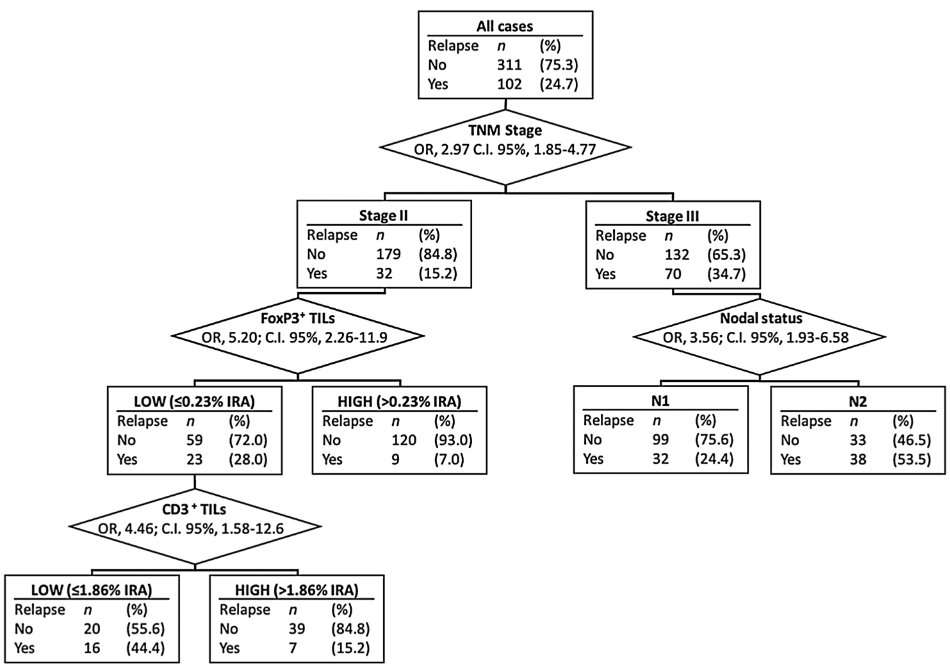

Figure 1.

Hierarchical recursive analysis of factors predicting postsurgical recurrences in 413 patients with stage II/III colorectal cancer. CART modeled (Salford Systems) by entering patient demographics, tumor pathologic features (including MS status), as well as densities of FoxP3$^+$ and CD3$^+$ TILs. TNM staging (stage III vs. stage II) ranked as the hierarchically highest prognostic node ($P < 0.001$, χ^2 test). Within stage III, only nodal status (N2 vs. N1) was a further discriminating factor ($P < 0.001$). In contrast, the software-identified cutoff values of TIL densities efficiently predicting recurrences in stage II colorectal cancer. In this subset, low densities of FoxP3$^+$ TILs ranked first in the decisional branching ($P < 0.001$), whereas low densities of CD3$^+$ TILs further discriminated the outcome of colorectal cancer with low density of FoxP3$^+$ TILs ($P < 0.001$). MS status was not recognized as a discriminating predictor.

Analytic assessment of prognostic variables and of their interactions

At logistic regression analysis (Supplementary Table S2), postoperative recurrences were significantly associated with lower (below median) densities of FoxP3$^+$ (OR, 2.24; 95% CI, 1.41–3.56; $P < 0.001$) and CD3$^+$ TILs (OR, 1.59; 95% CI, 1.01–2.50; $P = 0.04$). Other variables significantly associated with recurrences included stage III ($P < 0.001$), pT4 local invasion (0.03), and angio-invasion ($P = 0.002$). A protective effect was observed for MSI ($P = 0.05$). At multivariate analysis, densities of both FoxP3$^+$ and CD3$^+$ TILs interacted with tumor stage ($P = 0.06$ and $P = 0.02$, respectively), densities of FoxP3$^+$ cells significantly interacting also with the MS status of stage II colorectal cancer (0.03). A stratified analysis showed that below-median densities of FoxP3$^+$ and CD3$^+$ TILs were associated with disease progression in patients with stage II MS-stable (MSS) colorectal cancer, but not in patients with MSI or stage III cancer (Table 1).

ROC curve analysis confirmed that densities of TILs can predict postsurgical progression in patients with stage II MSS colorectal cancer (Fig. 2), AUC being 0.77 for FoxP3$^+$ cells and 0.71 for CD3$^+$ cells. The estimation of the cutoffs returned values of IRA% matching those adopted by the CART. ROC analysis also confirmed the absence of any predictive value of TILs in stage II MSI cancers (AUC: 0.45 for FoxP3$^+$; 0.53 for CD3$^+$ TILs), and in stage III tumors (AUC: 0.55 and 0.53, respectively).

At Cox proportional hazards model (ref. 21; Table 2), low densities of FoxP3$^+$ and CD3$^+$ TILs, as defined by ROC cutoffs, were both independent predictors of poor DFS in stage II MSS colorectal cancer (FoxP3$^+$: HR, 5.61; 95% CI, 2.38–13.2; $P < 0.001$; and CD3$^+$: HR, 5.76; 95% CI, 2.16–15.35; $P < 0.001$). Deep local invasion (pT4) was the only additional predictor of recurrence (HR, 3.88; 95% CI, 1.29–11.7; $P = 0.02$). Tumors with high densities of both FoxP3$^+$ and CD3$^+$ TILs had no recurrence. The outcome of cancers with a discordant pattern of TILs (high/low or low/high densities) was better than that of tumors with low densities for both FoxP3$^+$ and CD3$^+$ TILs. As a result, coexisting low densities of both TIL markers provided the strongest predictor of poor outcome (HR, 7.24; 95% CI, 3.41–15.4; $P < 0.001$ vs. all other combinations). The recurrence rate of cancers with low densities for both FoxP3$^+$ and CD3$^+$ TILs (50%) exceeded the sum of the recurrence rate of tumors with discordant densities (15%), as observed in additive models of biological interaction.

Kaplan–Meier curves (Fig. 3) recapitulated the statistical analysis by showing that stage II and MSS colorectal cancer (Fig. 3A) harboring both low-density FoxP3$^+$ and low-density CD3$^+$ TILs had a 5-year DFS lower than 60%, which was worse than that of tumors with high densities for both cell types (100% 5-year DFS; $P < 0.001$) or discordant densities (85% 5-year DFS, $P < 0.001$). Conversely, TIL densities did not predict DFS of patients with stage III MSS colorectal cancer (Fig. 3B), or that of the subjects with MSI cancer (Fig. 3C).

Data validation in the external set of stage II MSS colorectal cancer

In the external validation set of stage II MSS colorectal cancer, demographics and tumor pathologic features showed no significant association with densities of FoxP3$^+$ and CD3$^+$ cells (Supplementary Table S3). ROC curve analysis showed that AUC of

Table 1. Tumor densities of FoxP3$^+$ and CD3$^+$ TILs as predictors of colorectal cancer postsurgical recurrence, stratified by tumor stage and MS status[a]

		Stage II						Stage III ($n = 202$)		
		MSS ($n = 170$)			MSI ($n = 41$)					
		Rate of recurrence (%)	OR (95%CI)	P	Rate of recurrence (%)	OR (95%CI)	P	Rate of recurrence (%)	OR (95%CI)	P
FoxP3$^+$ TILs	Above median	4/86 (4.7)	1.00 ref.		2/21 (9.5)	1.00 ref.		29/101 (27.7)	1.00 ref.	
	Below median	24/84 (28.6)	8.75 (2.74–28.0)	<0.001	2/20 (10.0)	1.36 (0.15–12.4)	0.79	41/101 (40.6)	1.60 (0.86–2.96)	0.14
CD3$^+$ TILs	Above median	5/85 (5.9)	1.00 ref.		2/21 (9.5)	1.00 ref.		35/101 (34.7)	1.00 ref.	
	Below median	23/85 (27.1)	6.78 (2.26–20.4)	0.001	2/20 (10.0)	0.78 (0.08–7.25)	0.83	35/101 (34.7)	0.88 (0.47–1.63)	0.68
Local Invasion	pT3	24/160 (15.0)	1.00 ref.		2/33 (6.1)	1.00 ref.		NS		
	pT4	4/10 (40.0)	4.89 (0.94–24.4)	0.05	2/8 (25.0)	5.71 (0.59–55.2)	0.13			
Nodal status	N1	NA						32/131 (24.4)	1.00 ref	
	N2							38/71 (53.5)	3.48 (1.87–6.46)	<0.001

[a]Logistic regression, multivariate analysis.

Abbreviations: NA, not applicable; NS, variable not qualified ($P > 0.10$) for being entered into multivariate analysis of stage III colorectal cancer.

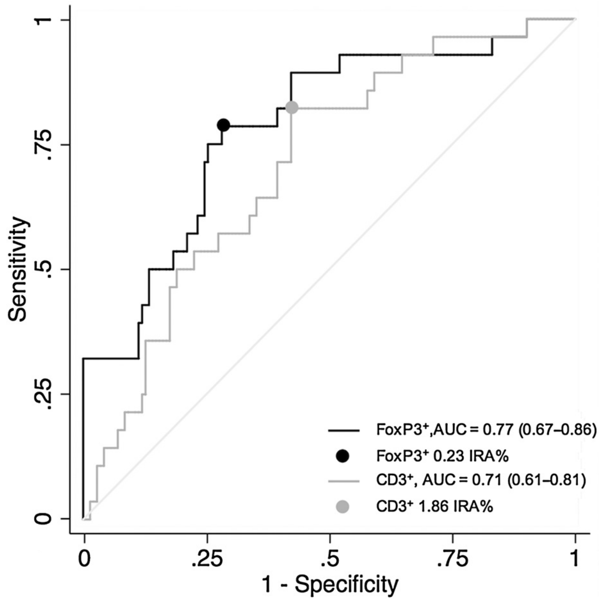

Figure 2.
ROC curves for densities of FoxP3$^+$ and CD3$^+$ TILs as predictors of postsurgical cancer recurrence in patients with stage II MSS colorectal cancer. FoxP3$^+$ TILs: AUC, 0.77 (bootstrap standard error, 0.05; 95% CI, 0.67–0.86); at cutoff 0.23 IRA%, sensitivity, 0.79; specificity, 0.71. CD3$^+$ TILs: AUC, 0.71 (bootstrap standard error, 0.05; 95% CI, 0.61–0.81); at cutoff 1.86 IRA%, sensitivity, 0.82; specificity, 0.58.

FoxP3$^+$ and CD3$^+$ TILs (0.78 and 0.71, respectively) were superimposable to those computed from corresponding tumors of the trial set (Supplementary Fig. S1). Cox multivariate analysis employing trial set cutoffs confirmed that both FoxP3$^+$ (HR 5.15; 95% CI, 1.94–13.7; $P = 0.001$) and CD3$^+$ (HR 2.78; 95% CI, 1.23–6.28; $P = 0.001$) TILs are independent predictors of recurrence (Supplementary Table S4). Again, the combination of low-density FoxP3$^+$ and low-density CD3$^+$ TILs predicted the worst outcome (HR 5.31; 95% CI, 2.45–11.5; $P < 0.001$), as confirmed by Kaplan–Meier curves (Fig. 3D).

The prognostic value of TILs was independent of adjuvant therapy. Kaplan–Meier curves of 327 patients with stage II MSS colorectal cancer (a cohort arrived at by merging the trial and validation sets) showed that combined information about density of FoxP3$^+$ and CD3$^+$ TILs predicted DFS in treated ($n = 119$, 36.4%) and untreated patients (Supplementary Fig. S2).

Table 2. Low densities[a] of FoxP3$^+$ and CD3$^+$ TILs, and their combination, to predict DFS in stage II MSS colorectal cancer

		Rate of recurrence (%)		HR (95%CI)[b]	P
FoxP3$^+$ TILs	High	7/109	(6.4)	1.00, ref.	
	Low	21/61	(34.4)	5.61 (2.38–13.2)	<0.001
CD3$^+$ TILs	High	5/87	(5.8)	1.00, ref.	
	Low	23/83	(27.7)	5.76 (2.16–15.3)	<0.001
FoxP3$^+$/CD3$^+$ TILs	High/high	0/58	(0.0)	1.00, ref.	
	High/low	7/51	(13.7)		
	Low/high	5/29	(17.2)		
	Low/low	16/32	(50.0)	7.24 (3.41–15.4)	<0.001

[a]Defined by optimal cutoffs at ROC curves (Fig. 2): 0.23% IRA for Fox P3$^+$ cells, 1.86% IRA for CD3$^+$ cells.

Discussion

Studies have shown that the extent of the local immune response to colorectal cancer is a determinant of the patient outcome (1–3, 5, 16, 22). This study demonstrates that postoperative recurrences are better predicted by densities of FoxP3$^+$ TILs at the invasive front of stage II MSS colorectal cancer than by CD3$^+$ TILs. By introducing the concept of a synergistic interaction of FoxP3$^+$ and CD3$^+$ TILs in determining the protective effect of the local immune response, this work supports the inclusion of the density of FoxP3$^+$ cells as a prognostic variable.

Following a meta-analysis supporting the prognostic value of FoxP3$^+$ TILs (23), our study demonstrates the prognostic value of FoxP3$^+$ TILs at the tumor front of stage II colorectal cancer through the analysis of whole-tissue sections and by statistical analysis. The statistical analysis included recursive partitioning, which weighs the impact of a candidate marker by allowing for interactions with other prognostic variables (24). The results were confirmed by conventional multivariate and interaction models. The lack of correlation between the densities of FoxP3$^+$ and CD3$^+$ TILs along with their different association with MSI does not suggest that infiltration by FoxP3$^+$ cells is a simple homeostatic response to an effective T-cell recognition. Rather, our data suggest individual recruitment of FoxP3$^+$ and CD3$^+$ TILs, which then synergistically interact in protecting against cancer progression (25).

Even though high density of FoxP3$^+$ TILs correlates with improved outcomes in patients with stage II colorectal cancer, FoxP3$^+$ TILs are also associated with immunosuppressive functions. Indeed, FoxP3 is expressed by activated effector T cells (26) and by T cells that, once sorted from colorectal cancer, inhibit IFNγ production and T-cell proliferation (27). The latter are *bona fide* immunosuppressive Tregs. Colonic microflora can also divert T-cell killing activity away from cancer cells (28). Therefore, it has been proposed that FoxP3$^+$ Tregs trimmed by the colonic milieu may attenuate the Th17-mediated proinflammatory and tumor-enhancing response induced by bacterial exposure (10, 15), thus freeing other TILs to target antigens expressed by cancer cells. Consistent with this view, Tregs induced tumor regression in a mouse model of intestinal polyposis (29). In addition to their regulatory functions modulating the immune response to the colonic commensal microflora, FoxP3$^+$ cells could activate effector and memory TIL abilities rather than suppressor functions (30). Effector and memory TILs are otherwise associated with Tregs shaped by microenvironmental stimuli, and require a complex mix of phenotypic features for their proper identification (31).

Various factors slow the introduction of immune-based prognostic markers into the clinical routine. Consensus on which T-cell subsets and locations best serve for prognosis in colorectal cancer is lacking. Indeed, a meta-analysis confirmed the prognostic value of the immune infiltrate but did not validate the individual impact of T-cell subtypes and sites (32).

Densities of CD3$^+$ and CD8$^+$ cells within the tumor and at the invasive margin were proposed as informative for colorectal cancer prognostication (3) and adopted by the Immunoscore consortium (4, 5). Our results endorse the inclusion of FoxP3$^+$ cell into the panel of markers aimed at predicting recurrences of stage II colorectal cancer.

The most controversial issue is the prognostic value of TILs densities across TNM stages. In this study, TILs stratified patient

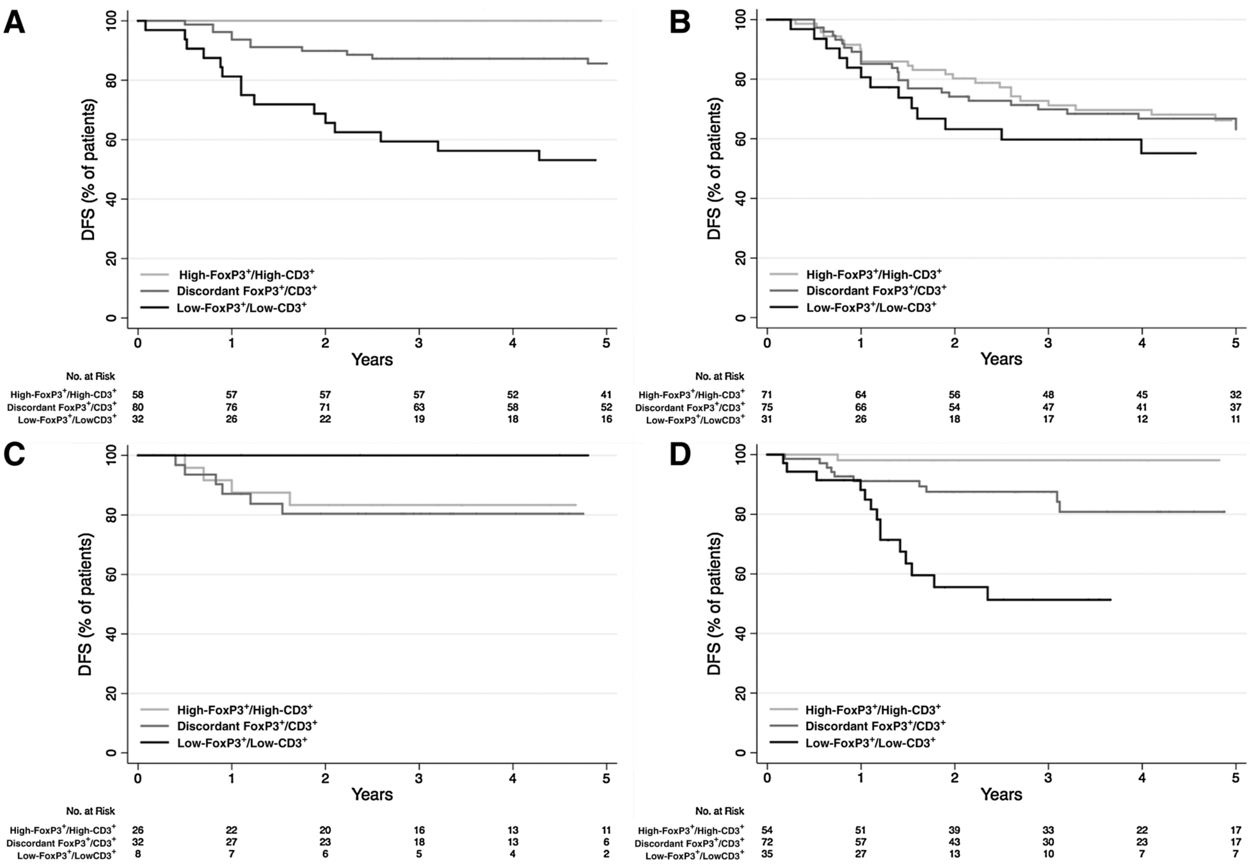

Figure 3.

Kaplan–Meier curves for the duration of DFS in patients with stage II/III colorectal cancer. **A,** MSS stage II trial set cancers stratified by combined analysis of $FoxP3^+$ and $CD3^+$ TIL densities (low vs. high, by cutoff at ROC curves). The outcome for patients with MSS CRCs harboring low densities of $FoxP3^+$ cells and low densities of $CD3^+$ cells was significantly worse than that of patients with cancers with high densities of both cell types ($P < 0.001$) or discordant TIL densities (low-high or high-low; $P = 0.001$). **B,** MSS stage III cancers stratified by combined analysis of $FoxP3^+$ and $CD3^+$ TIL densities. The outcome for patients with MSS CRCs harboring low densities of both $FoxP3^+$ and $CD3^+$ TILs was similar to that of patients with high densities of both cell types ($P = 0.26$) or discordant TIL densities ($P = 0.42$). **C,** MSI stage II/III trial set cancers, stratified by combined analysis of $FoxP3^+$ and $CD3^+$ TIL densities. The outcome for patients with MSI CRCs harboring low densities of both $FoxP3^+$ and $CD3^+$ TILs was like that of patients with high densities of both cells types ($P = 0.26$) or discordant TIL densities ($P = 0.22$). **D,** MSS stage II validation set cancers stratified by combined analysis of $FoxP3^+$ and $CD3^+$ TIL densities. The outcome for patients with MSS CRCs harboring low densities of both $FoxP3^+$ and $CD3^+$ TILs was significantly worse than that of cancers with high densities of both cell types ($P < 0.001$) or discordant TIL densities ($P = 0.002$).

survival across stage II and III, but neither $CD3^+$ nor $FoxP3^+$ TILs had prognostic value in stage III colorectal cancer. This stands in conflict with studies reporting that $CD3^+$ and $FoxP3^+$ TILs have prognostic value independent of colorectal cancer stage (2, 7, 8, 33) and to some extent in conflict with the Immunoscore results (5), in which stage III CRCs were under-represented, and increasing nodal involvement modified the patient outcome independently of the Immunoscore with multivariate analysis. In a scenario in which the interaction between nodal invasion and prognostic value of TILs is overlooked, it would be assumed that the magnitude of the local immune reaction controls tumor progression across stages with unmodified efficiency. Our results contradict this notion and support the integration of immunometric data with the TNM system. Furthermore, other immune cells, such as tumor-associated macrophages, tertiary lymphoid tissue (34), and neutrophils, modify cancer cell behavior (35) and may predict the outcome for patients with stage III colorectal cancer (36).

At present, neither discordant biological theories nor the lack of clarity on the hierarchical positioning of the immune markers versus the TNM system should delay the implementation of studies needed to complete the validation of the best immunoscore for stage II colorectal cancer. Neither $FoxP3^+$ nor $CD3^+$ cells have a prognostic impact in MSI colorectal cancer, so that the exclusion of these cancers strengthens the predictive power of TILs. TIL analysis in stage II colorectal cancer would be prognostically efficient even in the absence of MSI screening, due to the low recurrence rate of mismatch-repair deficient cancers, explained by the persistent renewal of neo-antigens to improve immune surveillance (37). At any event, universal screening for MSI is currently an advocated standard (38, 39), so that TNM classification would be best empowered by the parallel inclusion of TILs and MS status assessment.

Features that pose the greatest risk of excluding patients with stage II colorectal cancer from such assessment include inadequate sampling of lymph nodes, poorly differentiated histology, pT4 invasion, and perforation (40). None of these confers a risk of recurrence superior to that reported for immune markers by large reference studies. Accordingly, we found that the prognostic impact of low-FoxP3$^+$-CD3$^+$ TILs in stage II MSS colorectal cancer exceeded the weight of pT4. This reinforces the concept that only the introduction of a prognostic immunoscore can optimize the benefits of adjuvant therapy in stage II colorectal cancer. Although the limited number of patients with stage II cancers treated with adjuvant therapy does not allow for conclusive answers, our results suggest that the immune response is more relevant than chemotherapy in determining the outcome of stage II colorectal cancer. Thus, therapeutic efforts and innovative approaches should focus on patients with low TIL loads. Such approaches might include the use of ILs (IFNγ) and/or antibodies (anti-CTL4 or anti-PD-1) to boost the immune response of patients with MSS colorectal cancer. Patients with MSI colorectal cancer might benefit not only from anti-PD1 therapies, but also from vaccination strategies based on frameshifted peptides.

Our results from the implementation of Immunoscore and including information about FoxP3$^+$ TILs suggest the value of further testing within randomized controlled trials, such as those of the TOSCA trial (41) and of the IDEA collaboration (42).

Disclosure of Potential Conflicts of Interest

S. Siena is a consultant/advisory board member for Amgen, Bayer, Seattle, Bristol-Myers Squibb, Checkmab, Celgene, Daiichi-Sankyo, Incyte, Merck, Novartis, and Roche. A. Mantovani is a consultant/advisory board member for BiovelocITA, Novartis, Merck, Compugen, Roche, and Astra Zeneca. No potential conflicts of interest were disclosed by other authors.

Disclaimer

The funding sources did not have access to the raw data and had no role in study design; data collection, analysis, or interpretation; or writing of the report. The corresponding author had full access to all the data and final responsibility for the decision to submit the article for publication.

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