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Occurrence and significance of tumor-associated neutrophils in patients with colorectal cancer

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Inflammatory cells are an essential component of the tumor microenvironment. Neutrophils have emerged as important players in the orchestration and effector phase of innate and adaptive immunity. The significance of tumor-associated neutrophils (TAN) in colorectal cancer (CRC) has been the subject of conflicting reports and the present study was designed to set up a reliable methodology to assess TAN infiltration in CRC and to evaluate their clinical significance. CD66b and myeloperoxidase (MPO) were assessed as candidate neutrophil markers in CRC using immunohistochemistry. CD66b was found to be a reliable marker to identify TAN in CRC tissues, whereas MPO also identified a subset of $CD68^+$ macrophages. CRC patients ($n = 271$) (Stages I–IV) were investigated retrospectively by computer-assisted imaging on whole tumor sections. TAN density dramatically decreases in Stage IV patients as compared to Stage I–III. At Cox analysis, higher TAN density was associated with better prognosis. Importantly, multivariate analysis showed that prognostic significance of TAN can be influenced by clinical stage and 5-fluorouracil(5-FU)-based chemotherapy. On separate analysis of Stage III patients ($n = 178$), TAN density had a dual clinical significance depending on the use of 5-FU-based chemotherapy. Unexpectedly, higher TAN density was associated with better response to 5-FU-based chemotherapy. Thus, TAN are an important component of the immune cell infiltrate in CRC and assessment of TAN infiltration may help identify patients likely to benefit from 5-FU-based chemotherapy. These results call for a reassessment of the role of neutrophils in cancer using rigorous quantitative methodology.

Introduction

CRC is a major public health problem, representing the third most commonly diagnosed cancer and the fourth cause of cancer death worldwide.[1] Prediction of outcome in patients with CRC remains difficult because of the complexity, dynamicity and heterogeneity of this pathology, which displays distinct

Key words: colorectal cancer, predictive markers, prognosis, neutrophils, innate immunity

Abbreviations: BRAF: v-raf murine sarcoma viral oncogene homolog B1; CRC: colorectal cancer; DFS: disease free survival; DSS: disease specific survival; IM: invasive tumor margin; IRA: immunoreactive area; IT: intratumoral compartment; KRAS: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; MAPK: mitogen-activated protein kinases; MMR: mismatch repair system; MPO: Myeloperoxidase; MSI: microsatellite instability; MSS: microsatellite stability; TAM: tumor-associated macrophages; TAN: tumor-associated neutrophils; TNM: tumor-node-metastasis; 5-FU: 5-fluorouracil

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What's new?

While inflammatory cells are common in the tumor microenvironment, the associations of specific cell types with tumor development and disease progression are unclear. This is true particularly in the case of tumor-associated neutrophils (TAN), for which previous reports have identified conflicting roles in tumor progression. The present study links TAN density in colorectal cancer (CRC) tissues with patient outcome, indicating that TANs are an important component of tumor-infiltrating inflammatory cell populations in this disease. The prognostic relevance of TANs in CRC was influenced by disease stage and 5-fluorouracil-based chemotherapy, with higher TAN density associated with better therapeutic response.

clinical and molecular features.[2] Today, prognosis using the worldwide used tumor-node-metastasis (TNM) system, which is based on the anatomical extent of the tumor at diagnosis, has been questioned and alternative prognostic and predictive markers are needed to guide the selection of the most appropriate therapy for individual patients.[3,4]

Inflammation, including soluble and cellular effectors, affects all stages of tumor development and infiltration of inflammatory cells and increased expression of pro-inflammatory mediators have been reported in CRC.[5–7] For instance, transformed epithelial cells produce and secrete inflammatory mediators such as chemokines (*e.g.*, CCL3 and CXCL1) and pro-inflammatory cytokines (*e.g.*, IL-1β, TNF-α) sustaining the recruitment and activation of leukocytes.[8] In human CRC, recent efforts have described that the immune landscape displayed a spatiotemporal heterogeneity and might have clinical significance for prognosis. For instance, the quantification of the adaptive immune response has been proposed as prognostic factor and high densities of tumor-infiltrating T lymphocytes were associated with better survival.[4,9–12]

Among tumor-infiltrating innate immune cells, macrophages were the best characterized cells and were implicated in tumor initiation and progression.[13] A general view is that tumor-associated macrophages (TAM) resemble alternatively activated (M2) macrophages, promoting immunosuppression, tumor angiogenesis and progression and are generally associated with poor prognosis.[13,14]

Neutrophils play a primary role in the acute phase of inflammation and in resistance against invading pathogens. Recent evidence is consistent with the view that neutrophils are integrated in the orchestration of innate and adaptive immune responses.[15–18] Neutrophils are a component of the inflammatory microenvironment of tumors.[14,19,20] Recent evidence indicates that neutrophils are endowed with considerable plasticity depending on environmental cues.[21–24] In mouse models, tumor-associated neutrophils (TAN) have been reported to have a N2 phenotype[22] reminiscent of M2 or M2-like polarized macrophages.[13,25] Neutrophils have been identified in diverse human tumors including head and neck squamous cell carcinoma,[26] hepatocellular carcinoma,[27] renal cell carcinoma[28] and lung bronchoalveolar carcinoma.[29] In general, neutrophil infiltration has been associated with worse prognosis.[26–29] In CRC, the presence and significance of TAN have been the object of conflicting reports.[2,30–32] These discrepant results may reflect different methodological approaches.

The present study was designed to define a reliable methodological approach to quantify TAN infiltration in CRC tissues and to assess its clinical significance. Unexpectedly, higher TAN density was associated with better prognosis in Stage I–IV CRC. Importantly, an interaction was observed between clinical stage, TAN infiltration and 5-fluorouracil(5-FU)-based chemotherapy treatment and, in Stage III patients, TAN density had a dual clinical significance depending on the use of 5-FU therapy. In Stage III patients, higher TAN density was associated with higher chance of responding to 5-FU-based chemotherapy. Thus, TAN density may help identify patients more likely to respond to 5-FU-based chemotherapy. These unexpected results call for a rigorous reassessment of the role of TAN in cancer.

Material and Methods

Patients and study design

Tissue specimens from 271 patients with Stages I–IV CRC who consecutively underwent radical surgical resection at the Humanitas Clinical and Research Center (Rozzano, Milan, Italy) from January 1997 to November 2006 were retrieved from previous series.[10,12] A clinical retrospective database containing demographics, clinical and histopathologic data was assembled from the institutional intranet by investigators who were blinded to the results of molecular and immunological phenotyping of the cancers. A single pathologist, who was also unaware of the molecular phenotypes, reviewed tissue specimens. Patients who received neoadjuvant radiotherapy were excluded from the study because of the possible interference between this therapy and the assessment of the local immune response. Histopathological findings, surgical records (including intraoperative liver ultrasonography), and perioperative imaging (abdominal CT and chest radiography) were used to determine the presence of metastasis in all patients. The patients' follow-up started immediately after surgery and the mean follow-up of the cohort was 4.78 years (SD = 2.78) for disease specific survival (DSS) and 4.38 years (SD = 2.92) for disease free survival (DFS). The detection of tumor recurrence or death was computed from diagnosis until data were censored on May 30, 2010. Thoracic and abdominal CT, abdominal ultrasonography and chest radiography were used in the post-surgical phase, to monitor the insurgence of tumor recurrence. 5-FU-based chemotherapy treatment was administered to patients by a nonrandom assignment according to adjuvant protocols in use at the time of surgery. The study was approved

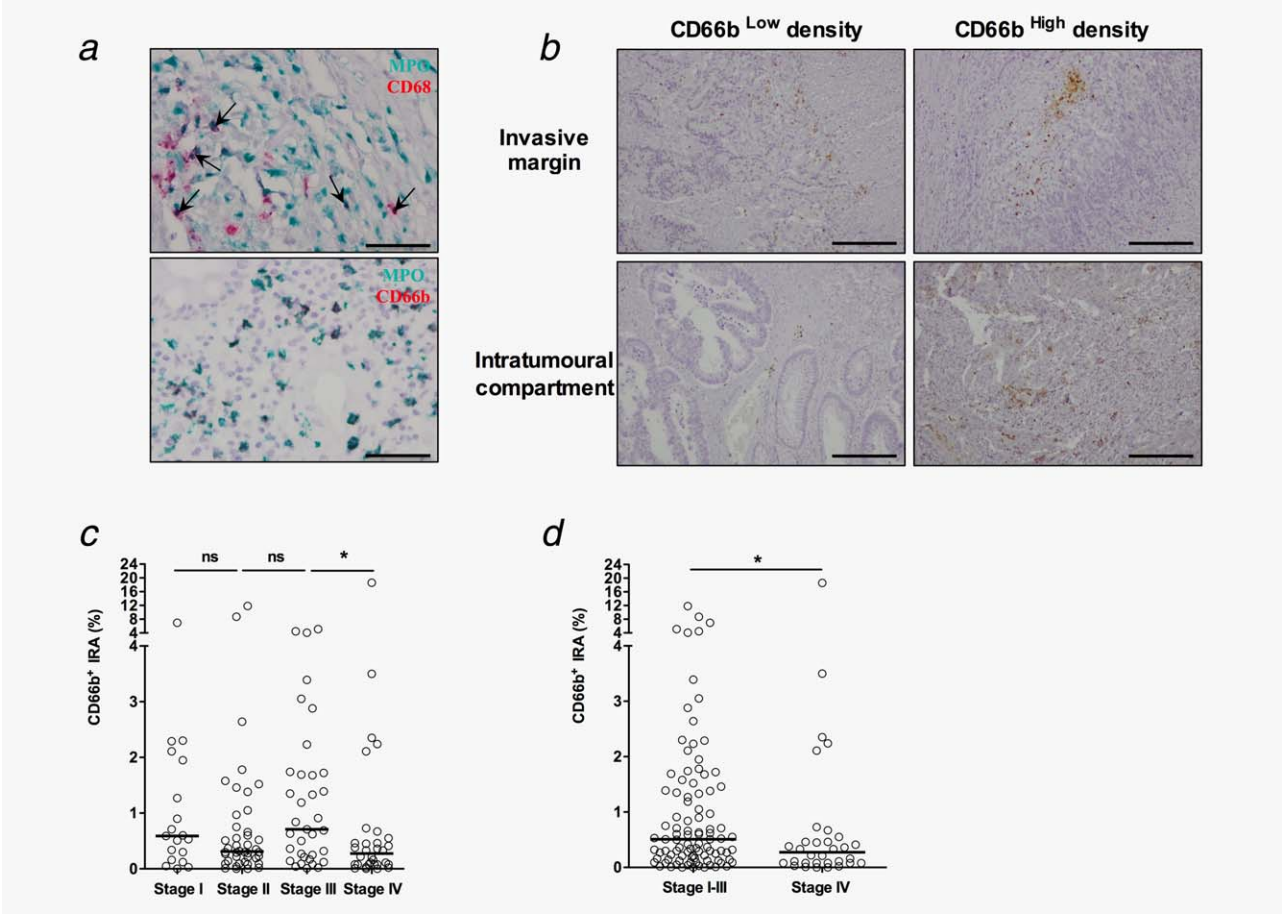

Figure 1. Analysis of neutrophil infiltration in CRC tissue sections. (*a*) Immunostaining analysis for CD68 (red) and MPO (green) (upper panel) and CD66b (red) and MPO (green) (lower panel) in CRC tissue sections. Double positive cells for MPO and CD68 are indicated by arrows (magnification: 40×). Bar: 50 μm, (*b*) Histological analysis of CRC samples stained with monoclonal anti-CD66b antibody. Density of neutrophils was analyzed in the invasive margin of the tumor (upper panel) and in the intratumoral compartment (lower panel) (magnification: 10×). Bar: 200 μm, (*c, d*) $CD66b^+$ immunoreactive area found in whole tumor sections of patients with Stage I ($n = 19$), Stage II ($n = 43$), Stage III ($n = 35$) and Stage IV ($n = 31$) CRC. The median value is shown. *$p \leq 0.05$; two-tailed Mann–Whitney *U* test.

by the Ethical Committee of the Humanitas Clinical and Research Center, and written informed consent was obtained by the referring physician at the time of surgery.

Immunohistochemistry

Formalin-fixed and paraffin-embedded 2 μm thin tissue sections were deparaffinised and treated with an antigen unmasking solution (DIVA Decloaker, Biocare Medical, CA). Endogenous peroxidases were blocked with a 3% solution of H_2O_2 for 20 min at room temperature (RT). Then, primary mouse monoclonal anti-CD66b antibody (clone G10F5; BD Pharmingen, CA) or control antibody were applied for 1 hr at RT followed by a secondary antibody (HRP rabbit/mouse; MACH 4 Biocare) for 30 min at RT. 3,3′-Diaminobenzidine tetrahydrochloride (Biocare) was used as chromogen and slides were subsequently counterstained with haematoxylin solution.

In some experiments, rabbit polyclonal anti-MPO antibody (Dako) was used with mouse monoclonal anti-CD68 (clone PG-M1; Dako) or mouse monoclonal anti-CD66b antibody followed by incubation with goat antirabbit poly-horseradish peroxidase (HRP) and goat antimouse poly-alkaline phosphatase (AP). AP reaction was obtained using red chromogen (Warp red Chromogen Kit; Biocare Medical) and HRP reaction was obtained using a green chromogen (Vina Green Chromogen Kit, Biocare Medical).

Computer-assisted image analysis

$CD66b^+$ immunoreactive area (IRA) was measured by using a computer-assisted image analysis system[10] in three contiguous but not overlapping fields at the invasive tumor margin (IM) (~50% of the entire microscopic field was cancerous tissue) and in three not overlapping fields microscopic areas in the intratumoral compartment (IT). The image analysis software automatically selected the $CD66b^+$ immunoreactive area on the basis of RGB color segmentation and assessed the percentage for each field. The mean of values obtained in three distinct regions was used for data analysis. The same light intensity level was applied for all images acquired. All

Table 1. Correlation between patients' demographics and clinicopathologic features and CD66b+ IRA (IM and IT)

		$CD66b^+$ IRA IM		$CD66b^+$ IRA IT	
	N	**Median value (IQR)**	p^1	**Median value (IQR)**	p^1
Age at diagnosis (years)					
≤65	66	0.27 (0.07–0.52)		0.11 (0.04–0.42)	
>65	62	0.15 (0.06–0.58)	0.71	0.11 (0.02–0.43)	0.79
Sex					
Male	73	0.17 (0.07–0.54)		0.13 (0.04–0.33)	
Female	55	0.16 (0.03–0.58)	0.48	0.08 (0.01–0.43)	0.84
Microsatellite status					
MSI	8	0.19 (0.06–0.56)		0.21 (0.06–0.48)	
MSS	120	0.16 (0.12–1.20)	0.46	0.11 (0.02–0.36)	0.39
Anatomical site					
Colon	99	0.17 (0.06–0.58)		0.10 (0.02–0.36)	
Rectum	29	0.20 (0.08–0.54)	0.55	0.20 (0.04–0.42)	0.23
TNM Stage					
Stage I	19	0.39 (0.07–0.65)		0.08 (0.04–0.42)	
Stage II	43	0.16 (0.08–0.36)	0.14	0.13 (0.01–0.36)	0.59
Stage III	35	0.39 (0.08–1.31)	0.55	0.24 (0.05–0.46)	0.40
Stage IV	31	0.07 (0.03–0.35)	**0.04**	0.08 (0.01–0.33)	0.62
Grade					
G1–G2	110	0.17 (0.06–0.53)		0.10 (0.02–0.37)	
G3	18	0.25 (0.07–1.19)	0.67	0.20 (0.01–0.33)	0.79
Tumor cell type					
Adenocarcinoma	119	0.20 (0.06–0.58)		0.13 (0.02–0.37)	
Variants	9	0.11 (0.06–0.38)	0.64	0.08 (0.03–0.25)	0.98
Vascular invasion					
No	94	0.29 (0.07–0.79)		0.13 (0.02–0.46)	
Yes	34	0.09 (0.03–0.35)	**0.02**	0.11 (0.02–0.27)	0.72

[1]Linear regression analysis: % $CD66b^+$ IRA was entered as a dependent, continuous variable, continuous variable. $p \leq 0.05$.
Abbreviations: IRA, Immunoreactive area; IM, invasive margin; IT, intratumoral compartment; MSI, microsatellite instability; MSS, microsatellite stability; TNM, tumor-node-metastasis; IQR, interquartile range.

images were digitized at 10× objective magnification. The expert pathologist who selected the areas of interest was blinded to tumor microsatellite status and to any patients' clinical data.

Microsatellite status and KRAS and BRAF mutations analysis

Microsatellite status [*i.e.*, microsatellite stability (MSS) and microsatellite instability (MSI) caused by a defective mismatch repair system (MMR)] was determined preliminarily for all cancers by testing instability at mononucleotide repeats, as previously described.[10,33] *KRAS* and *BRAF* mutation status was assessed as previously described.[34] Briefly, *KRAS* mutations (codon 12, 13 and 61) were assessed in genomic DNA extracted from paraffin-embedded sections by direct sequencing. The oligonucleotide primer sequences used for amplifying the *KRAS* codon 12 and 13 were 5′-TTATTATAAGGCCTGCTGAAAATG-3′ (forward) and 5′-CCTCTATT GTTGGATCATATTCGT-3′ (reverse); for amplifying the *KRAS* codon 61 were 5′-GGAAGCAAGTAGTAATTG ATGGAG-3′ (forward) and 5′-TTTATGGCAAATACACAAAG AAAG-3′ (reverse). $BRAF^{c.1799T>A}$ mutation was determined on DNA extracted from paraffin-embedded sections by Real-Time PCR using a TaqMan SNP Genotyping Assay (Applied Biosystem), as previously described.[34]

Statistical analysis

The existence of an association between $CD66b^+$ IRA and patients' characteristics or tumor features was assessed by linear regression analysis. Patients' outcomes were dichotomized by survival (death *vs.* alive) for DSS and relapse (local failure/distant metastasis *vs.* no local failure/distant metastasis) for DFS, respectively. The $CD66b^+$ IRA cut-off score was selected based on median (Table 2) or upper quartile values (Table 3). The univariate and multivariate Cox proportional hazards models were used

Table 2. Univariate and multivariate analysis for DSS and DFS in patients with colorectal cancer

		DFS				DSS			
		Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	N	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
Age at diagnosis (years)[1]	128	1.00 (0.97–1.02)	0.69			0.98 (0.96–1.00)	0.12		
Sex									
Male	73	1.00 Ref.				1.00 Ref.			
Female	55	0.80 (0.46–1.38)	0.42			0.72 (0.39–1.33)	0.29		
Microsatellite status									
MSS	120	1.00 Ref.				1.00 Ref.			
MSI	8	N.A.	**0.01**	NA	NA	N.A.	**0.03**	NA	NA
Anatomical location									
Colon	99	1.00 Ref.				1.00 Ref.			
Rectum	29	0.99 (0.53–1.85)	0.98			0.84 (0.40–1.75)	0.64		
TNM stage									
I	19	1.00 Ref. 2.^{112} (0.46-9.78)		1.00 Ref. 1.89^{2} (0.40-8.83)		1.00 Ref. 2		1.00 Ref.[1]	
II	43		0.34		0.42				
III	35	5.80 (1.34–25.1)	**0.02**	5.45 (1.24–24.0)	**0.02**	4.24 (1.47–12.2)	**0.008**	3.94 (1.33–11.7)	**0.01**
IV	31	18.7 (4.40–79.2)	**<0.001**	12.4 (2.83–54.4)	**<0.001**	30.1 (11.4–79.4)	**<0.001**	20.4 (7.45–55.6)	**<0.001**
Tumor grade									
G1–G2	110	1.00 Ref.				1.00 Ref.			
G3	18	1.34 (0.63–2.84)	0.44			1.91 (0.89–4.12)	0.10		
Tumor cell type									
Adenocarcinoma	119	1.00 Ref.				1.00 Ref.			
Variants	9	1.79 (0.71–4.49)	0.22			1.50 (0.54–4.19)	0.44		
Vascular invasion									
No	94	1.00 Ref.		1.00 Ref.		1.00 Ref.		1.00 Ref.	
Yes	34	3.76 (2.19–6.45)	**<0.001**	2.00 (1.12-3.57)	**0.02**	5.26 (2.89–9.58)	**<0.001**	2.35 (1.23–4.50)	**0.01**
CD66b^{+} IRA IM									
Low≤0.1717	64	1.00 Ref.				1.00 Ref.			
High>0.1717	64	0.51 (0.30–0.89)	**0.02**		^	0.35 (0.18–0.69)	**0.002**		
CD66b^{+} IRA IT									
Low ≤ 0.11	65	1.00 Ref.				1.00 Ref.			
High > 0.11	63	0.55 (0.32–0.95)	**0.02**		^{2}	0.45 (0.24–0.85)	**0.01**		

Table 2. Univariate and multivariate analysis for DSS and DFS in patients with colorectal cancer (Continued)

		DFS				DSS			
		Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	N	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	*p*
5-FU[4]									
No	73	1.00 Ref.				1.00 Ref.			
Yes	55	1.42 (0.83–2.41)	0.20		[2,3,4]	1.36 (0.75–2.45)	0.31		

Age entered as a continuous variable. $p \leq 0.05$.
[1]Reference population, CRC patients at clinical Stages I and II. All patients with Stage I CRC *survived during the follow*-up period.
[2]Interaction between 5-FU, TNM Stage and CD66b$^+$ IRA IT density, $p = 0.15$.
5-FU-chemotherapy in patients with Stage III–IV MSS CRC, HR 0.50 (0.27–0.92), $p = 0.025$.
[3]Interaction between 5-FU, TNM Stage and CD66b$^+$ IRA IM density, $p = 0.76$.
[4]Cox proportional hazard-rate model in Stage II–IV MSS CRC; interaction between 5-FU therapy and CD66b+ IRA IT density, $p = 0.04$. Interaction between 5-FU therapy and CD66b+ IRA IM density, $p = 0.63$.
Abbreviations: IRA, Immunoreactive area; IM, invasive margin; IT, intratumoral compartment; TNM, tumor-node-metastasis; HR, hazard ration; CI, *confidence interval*.

to evaluate the role of CD66b$^+$ IRA as well as other clinicopathological features in predicting patients' outcome. All variables with *p*-values ≤ 0.2 were entered in multivariate model. In multivariate analysis, interactions were also assessed, and further subgroup analysis was considered for *p*-values ≤ 0.2. Kaplan–Meier curves of DSS and DFS were plotted and the log-rank test was used to compare the curves of patient subgroups. Mann–Whitney *U* test, Log-rank Test and Spearman's correlation were used as specified. For each test, only two-sided and $p \leq 0.05$ was considered significant. Analyses were done using Epi Info version 3.4.3, StatsDirect Statistical software (version 2.5) and GraphPad Prism software (Version 4.1).

Results

Reliable assessment of TAN and evolution during CRC progression

Currently, there is no consensus concerning the best way to stain and identify neutrophils in cancer tissues, by an antibody-based approach. Therefore, we conducted a first set of experiments to characterize a specific and reliable marker to stain neutrophils in CRC tissues, focusing our attention on myeloperoxidase (MPO) and CD66b, two molecules previously proposed as neutrophil markers in CRC tissues.[31,32] Double staining in immunohistochemistry for CD66b plus MPO and CD68 plus MPO revealed that all CD66b$^+$ cells were MPO$^+$, corresponding to neutrophils. We also observed CD66b$^-$ MPO$^+$ cells and CD68$^+$ MPO$^+$ cells. These cells are freshly recruited monocytes and immature macrophages. Thus, MPO cannot be considered specific for TAN in CRC (Fig. 1*a*). Therefore, we evaluated the pattern and density of TAN by using CD66b as neutrophil marker and a computer-aided imaging analysis system. For each tumor, the IRA value for CD66b was blindly analyzed in whole tissue sections, encompassing both the IT and the IM (Fig. 1*b*). Interestingly, the density of TAN remained unchanged from Stages I–III but was dramatically decreased from Stages I–III to Stage IV (Fig. 1*c* and 1*d*), suggesting that neutrophil infiltration is a dynamic process evolving during the course of tumor progression.

Neutrophil infiltration, clinicopathological features and CRC outcome

Patient demographics and clinicopathological features are detailed in Table 1. Upon linear regression analysis, increasing densities of neutrophils at the IM were inversely correlated with the presence of distant metastasis at diagnosis and with the presence of vascular invasion (Table 1).

Median values of CD66b$^+$ IRA at the IM and within neoplastic glands were used to divide tumors into high and low TAN infiltration. Values were entered into a Cox proportional hazard model to evaluate their potential impact on CRC outcome alongside the clinicopathological features. Coherently, at univariate analysis, high TNM stage, vascular invasion, and microsatellite-stable tumor status were significantly associated with worse outcome, either DFS or DSS (Table 2). In the model,

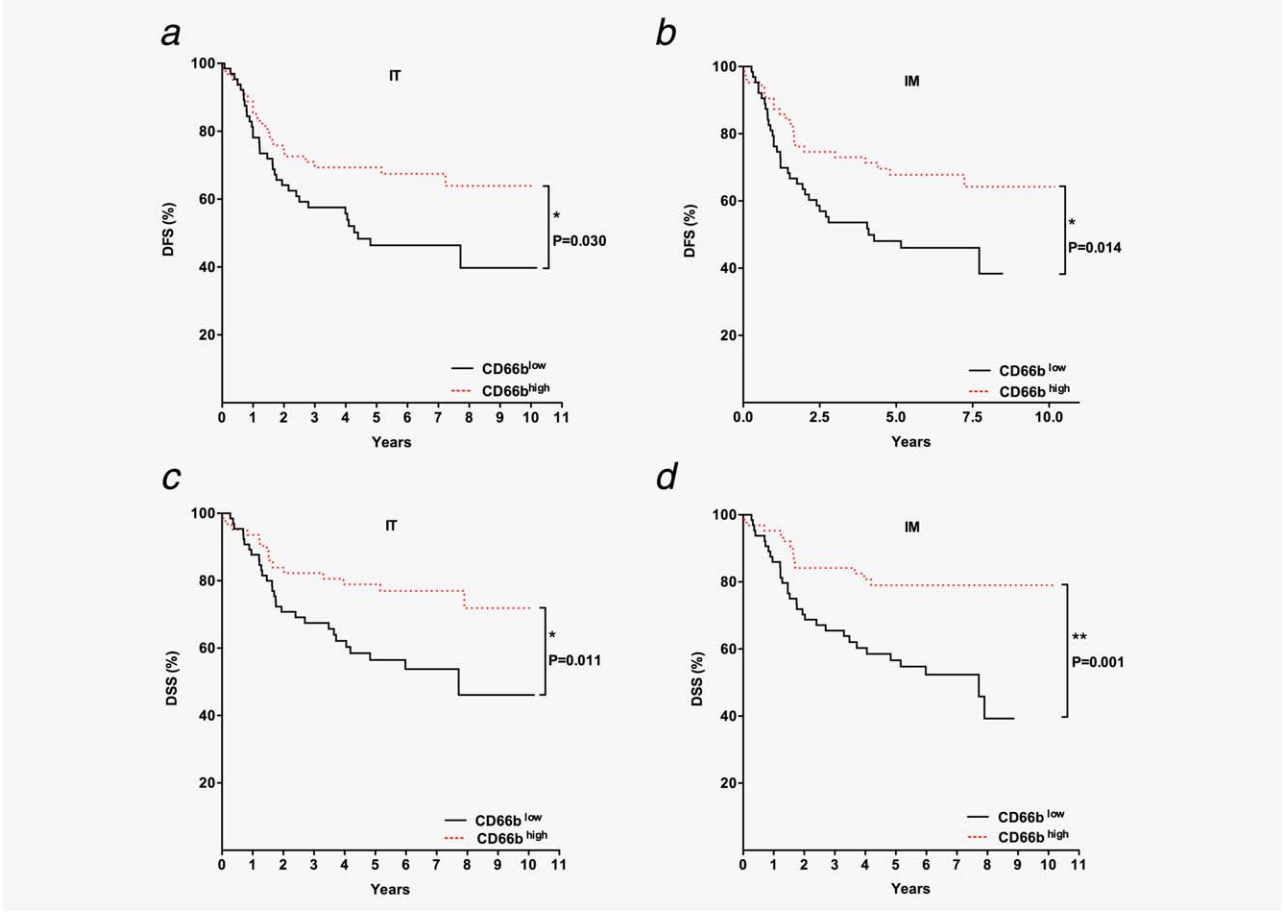

Figure 2. Prognostic significance of CD66b+ IRA in patients with Stages I–IV CRC. (*a–d*) Kaplan–Meier survival curves show DFS (*a*, *b*) and DSS (*c, d*) for patients ($n = 128$) presenting a high or low density of neutrophils ($CD66b^{high}$ or $CD66b^{low}$, respectively) in the IM or in the IT. Median values were employed to divide tumors into high and low $CD66b^{+}$ immunoreactive area. $*p \leq 0.05$, $**p \leq 0.01$; log-rank test.

high IM and IT TAN densities were significantly associated with better DFS ($p = 0.02$ for both IM and IT) and DSS ($p = 0.002$ and $p = 0.01$, respectively) (Table 2). Results were paralleled by Kaplan–Meier curves, showing a significantly better outcome in patients whose tumors had high $CD66b^{+}$ cells infiltration (Fig. 2).

To assess whether the detected potential prognostic value of TANs could be influenced by other variables, we incorporated densities of $CD66b^{+}$ TANs in a multivariate Cox proportional hazard model. At multivariate analysis, only pathological stage and vascular invasion were independently associated with poor DFS and DSS (Table 2), while both IM and IT TAN densities were not significantly associated with a likelihood of different outcome. However, in the model, the interactions between pathological stage, 5-FU treatment and TAN densities ($p = 0.15$ and 0.76 for IT and IM TAN densities, respectively) suggested that the prognostic significance of IT TAN but not of IM TAM, could be modified in the different settings of these two parameters (*i.e.*, pathological stage and 5-FU-based chemotherapy) across the study cohort (Table 2). Consistently, a subgroup analysis restricted to stages in which 5-FU-based chemotherapy was administered (*i.e.*, patients with Stages II–IV CRC), showed that the 5-FU-based chemotherapy interacted significantly with IT TAN density ($p = 0.04$), but not with IM TAN density ($p = 0.63$) (Table 2).

Predictive significance of TAN for response to chemotherapy

Based upon the significant interaction between TAN IT infiltration, TNM stage and 5-FU treatment, we next focused on the potential value of IT TAN density as outcome predictors in patients with Stage III MSS CRC, who are expected to gain the highest clinical benefit from adjuvant chemotherapy.[35,36]

In Stage III patients, high tumor grade, the presence of vascular invasion, the extent of nodal involvement, and $BRAF^{c.1799T>A}$ mutation were significantly associated with worse DFS (Table 3), at univariate regression Cox analysis. Nonsignificance for *KRAS* status was likely due to the sample size of our cohort ($n = 178$), precluding the observation of the reported effect in larger data sets ($n > 2000$).[37,38] In contrast, the use of 5-FU-based chemotherapy was associated with better DFS (HR = 0.61; $p = 0.06$; Table 3), as expected.[35,36] High TAN IT infiltration *per se* was not associated with a better outcome (HR = 0.61; $p = 0.14$; Table 3). However, multivariate Cox analysis revealed a significant interaction between 5-FU treatment and IT TAN densities

Table 3. Univariate and multivariate analysis for DFS of different prognostic factors in 178 Stage III MSS CRC patients

		Univariate analysis		Multivariate analysis	
	N	HR (95% CI)	*p*	HR (95% CI)	*p*
Age at surgery (years)[1]					
<68	97	1.00 Ref.			
≥68	81	1.69 (1.02–2.78)	**0.04**		
Gender					
Male	108	1.00 Ref.			
Female	70	1.04 (0.63–1.73)	0.87		
Anatomical location					
Colon DX	50	1.00 Ref.			
Colon SX	75	0.68 (0.37–1.27)	0.22		
Rectum	53	1.10 (0.59–2.03)	0.77		
Tumor grade					
G1–G2	144	1.00 Ref.		1.00 Ref.	
G3	34	2.35 (1.37–4.04)	**0.002**	1.93 (1.12–3.37)	**0.02**
Tumor cell type					
Adenocarcinoma	165	1.00 Ref.			
Variants	13	1.76 (0.80–3.86)	0.16		
Vascular invasion					
No	123	1.00 Ref.		1.00 Ref.	
Yes	55	2.48 (1.51–4.10)	**<0.001**	2.00 (1.20–3.36)	**0.008**
Nodal status					
N1	115	1.00 Ref.		1.00 Ref.	
N2	63	2.55 (1.55–4.20)	**<0.001**	2.12 (1.27–3.53)	**0.004**
KRAS and BRAF status					
Wild-type	114	1.00 Ref.			
Mutated *KRAS*[1]	59	1.27 (0.75–2.14)	0.37		[2,3]
Mutated *BRAF*[4]	4	3.45 (1.06–11.2)	**0.04**		[5]
5-FU therapy					
No	52	1.00 Ref.			
Yes	126	0.61 (0.36–1.02)	0.06		[4,6]
CD66b$^+$IRA IT					
Low ≤ 1.26	134	1.00 Ref.			
High > 1.26	44	0.61 (0.32–1.17)	0.14		[6]

Age entered as a continuous variable. $p \leq 0.05$
[1]Assessed at *KRAS* codons 12, 13 and 61.
[2]Assessed at $BRAF^{c.1799T>A}$ mutational hot-spot.
[3]Multivariate analysis for *BRAF*: variable not entered due to the low number of events <10.
[4]Interaction between *KRAS* status and 5-FU $p = 0.87$.
[5]Interaction between *KRAS* status, 5-FU and CD66b $^+$ IRA IT $p = 0.31$.
[6]Interaction between 5-FU and CD66b $p = 0.003$.
Abbreviations: IRA, Immunoreactive area; IM, invasive margin; IT, intratumoral compartment; HR, hazard ration; CI, confidence interval.

($p = 0.003$; Table 3). These results suggest that the prognostic significance of IT TAN in the whole cohort could be modified by the postsurgical treatment.

Following the assessment of the significant interaction between IT TAN density and 5-FU treatment, we compared the significance of IT TAN in treated *versus* untreated patients, as to their outcome. In this subgroup analysis, high density of IT TAN was associated with better DFS (HR = 0.42; $p = 0.01$; Fig. 3b) in treated patients, but with worse DFS (HR = 3.041; $p = 0.07$; Fig. 3c) in untreated ones. Together, these results indicate the dual clinical significance of IT TAN in Stage III CRC patients, depending on the administration of 5-FU-based

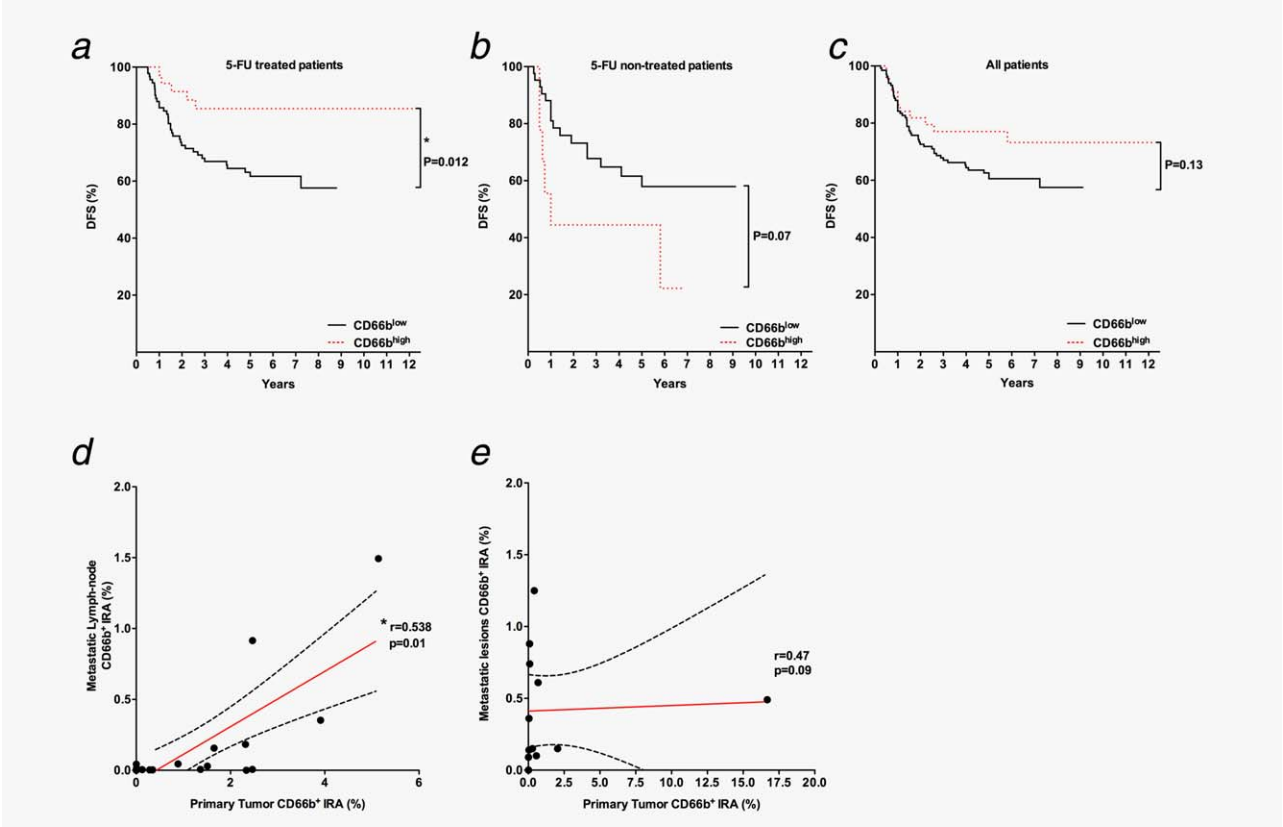

Figure 3. Predictive significance of CD66b$^+$ IRA for 5-FU chemotherapy response. (*a–c*) Kaplan–Meier survival curves show DFS for stage III CRC patients with high density of neutrophils or low density of neutrophils (CD66b^{high} or CD66b^{low}, respectively) found in the IT. Curves were plotted for 5-FU treated and nontreated patients ($n = 178$) (*a*), 5-FU treated patients ($n = 126$) (*b*) and 5-FU nontreated patients ($n = 52$) (*c*) *$p \leq 0.05$, Log-rank Test, (*d*) Correlation between the CD66b$^+$ IRA found in primary tumor and in metastatic lymph nodes in patients with Stage III CRC. (*e*) Correlation between the CD66b$^+$ IRA found in primary tumor and in metastatic liver lesions in patients with Stage IV CRC. (*d–e*) *$p \leq 0.05$; Spearman's correlation.

chemotherapy. Consistently, IT TAN was not associated with patients' outcome in the entire set of Stage III patients (HR = 0.64; $p = 0.13$; Fig. 3a).

We compared TAN IT density in primary tumors and metastatic lymph nodes. The CD66b$^+$ IT IRA found in the metastatic lymph node and in the primary tumor were significantly correlated ($p = 0.01$, Fig. 3 days), so that the densities of neutrophils in the metastatic lymph nodes closely mirror the densities found in primary tumors. In contrast, no correlation was found when comparing IT TAN density between the primary tumors and matched metastatic liver lesions in Stage IV patients ($p = 0.09$, Fig. 3e), suggesting that the density of neutrophils found in distant metastatic lesions does not mirror the densities of TAN found in primary tumors.

Discussion

Inflammatory cells are an essential component of the tumor microenvironment.[5,6] In particular, cells of the monocyte-macrophage lineage have been shown to promote angiogenesis, tissue remodeling, metastasis and suppression of adaptive immune responses.[25,39] Neutrophils have long been overlooked as a component of the inflammatory tumor microenvironment. Evidence has now accumulated that TAN can represent an essential constituent of cancer-related inflammation.[14,19]

CRC has served as a paradigm for the connection between inflammation and cancer.[7,40] Today, the prognostic significance of the worldwide used TNM system is questioned and efforts have been made to identify new biomarkers related to the tumor microenvironment and to infiltrating immune and inflammatory cells.[41] For instance, it has been shown that T cell infiltration correlated with better prognosis in CRC,[4,9–12] a finding consistent with a protective function of adaptive immune response in this tumor. However, the complexity and heterogeneity of CRC ask for new additional markers, which should improve mortality prediction and identify the most adequate treatment for individual cancer patient.

The occurrence and significance of neutrophil infiltration in CRC have been the object of conflicting reports.[2,30–32] In the present study, we set out to rigorously assess the presence and significance of TAN in CRC. One reason for controversy is that there is no consensus on the method to identify neutrophils in CRC tissues. By comparing MPO and CD66b, two markers known to stain neutrophils in tissues, we found that CD66b is a specific marker for neutrophils in CRC tissues

and should be used in further studies. Staining with MPO in CRC is not neutrophil specific and MPO is expressed during mononuclear phagocyte differentiation.[42] In particular, granules containing MPO are formed in bone marrow pro-monocytes, found in mature monocytes and lost during maturation into macrophages in tissues.[42] CD15 is used to stain neutrophils in flow cytometry. However, in addition to infiltrating leukocytes, we observed the expression of CD15 on colorectal epithelial tumor cells (data not shown). These data are consistent with previous studies showing that a valuable percentage of $CD15^+$ cells in CRC tissues were negative for MPO.[30,43] Therefore, the use of MPO and CD15 as neutrophil markers may have contributed to conflicting results on TAN infiltration in CRC.[30] Our results suggest that CD66b is a reliable marker for TAN identification in CRC.

Here, we used a rigorous methodological approach that combines a computer-assisted image analysis system with the immunological detection of neutrophils ($CD66b^+$ cells), located at the invasive tumor margin and in the IT, in whole tissue sections.[10] In line with previous findings based on tissue microarray data,[2] we showed that infiltration of neutrophils in CRC evolved overtime, being consistent form Stages I–III followed by a significant reduction in Stage IV. As proposed for T cell, the significant decrease in neutrophil infiltration observed in Stage IV CRC could be linked to immune escape in patients with advanced disease.[11] We observed that higher densities of TAN were significantly associated with better clinical outcome in patients with CRC (Stages I–IV). Importantly, an interaction was observed between clinical stage, TAN infiltration and 5-fluorouracil(5-FU)-based chemotherapy treatment. Predictive markers for response to chemotherapy are needed and successful chemotherapy might depend on both innate and adaptive immunity.[44,45] Importantly, in Stage III patients, infiltration of TAN was associated with better responses to 5-FU-based chemotherapy and with poor prognosis in untreated patients, indicating the dual clinical significance of IT TAN, depending on the administration of 5-FU-based chemotherapy.

The mechanism responsible for the correlation observed between high TAN infiltration and response to 5-FU-containing therapeutic regimens remains elusive. Chemotherapeutic agents have been shown to affect the polarization of innate immune cells and to trigger an antitumor immune response.[44,46–48] Regarding the chemotherapeutic agent 5-FU, the anticancer properties were related to its ability to selectively kill the immunosuppressive myeloid-derived suppressor cells (MDSC), comprising the subset of granulocytic MDSC.[49,50] In addition, 5-FU activates caspase-1 on MDSC, leading to inflammasome activation and IL-1β secretion,[51] which triggered $CD8^+$ T-cell-dependent anticancer immunity.[52] Therefore, further investigations should determine whether 5-FU based chemotherapy eliminates immunosuppressive G-MDSC and promotes the recruitment of inflammatory TAN in CRC tissues sustaining an adaptive immune response against cancer. Consistently, previous studies showed that 5-FU treatment increased neutrophil phagocytic function and induced the recruitment of neutrophils in the colon, suggesting that 5-FU-based chemotherapy might recruit and activate neutrophils in CRC tissues.[53,54] In addition, we found in preliminary experiments that neutrophils displayed a cytostatic activity towards CRC cell lines that may boost the cytotoxicity of 5-FU (data not shown). Thus, the association observed here between high TAN and response to 5-FU containing regimens may reflect a regulation of neutrophil function by this agent and/or sensitization of tumor cells to the antitumor activity of neutrophils. In addition, we showed that the density of neutrophils found in metastatic lymph nodes mirrored their density in the primary tumor, suggesting that neutrophils can act in the metastatic niche, as previously suggested in mouse.[55]

Genetic and epigenetic alterations were proposed to improve prognostic stratification of patients with CRC. In particular, specific mutations in genes involved in cellular pathways, such as *BRAF* and *KRAS,* which are both involved in the mitogen-activated protein kinases (MAPK) pathway, were associated with worse patients' outcome.[37,38] In addition, it has long been known that the MSI/MSS status impacts on the response to 5-FU and that MSI patients are unlikely to benefit from 5-FU. In stage III CRC, high TAN infiltration was associated with better response to 5-FU-containing chemotherapeutic regimens. MSI/MSS status and mutations in *BRAF* and *KRAS* are tumor cell intrinsic properties and the results reported here indicate that a component of the inflammatory microenvironment of CRC is a key determinant of the response to 5-FU regimens. The lack of relevant interactions between *KRAS* status, 5-FU and IT TAN ($p = 0.31$) does not support a correlation between *KRAS* mutation, IT TAN and response to chemotherapy in our series (Table 3). Further prospective studies are needed to assess whether TAN infiltration can be useful in an integrated model to personalized treatment of CRC patients with 5-FU and/or targeted therapies and immunotherapy.

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