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Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer and prognosis: cohort study and literature review

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Abstract

The abundance of tumour-infiltrating T-cells has been associated with microsatellite instability (MSI) and a favorable prognosis in colorectal cancer. Because molecular alterations in colon cancer including MSI, the CpG island methylator phenotype (CIMP), *BRAF* mutation and global DNA hypomethylation have been associated with clinical outcome, potential confounding by these molecular features needs to be controlled when assessing the prognostic significance of tumour-infiltrating T-cells. We utilized a database of clinically and molecularly-annotated colon and rectal carcinoma cases (N=768; stage I-IV) in two prospective cohort studies (the Nurses' Health Study and the Health Professionals Follow-up Study). Using tissue microarray and automated Ariol image analysis system, we quantified densities of CD3⁺, CD8⁺, CD45RO⁺ (PTPRC) and FOXP3⁺-cells within neoplastic epithelial areas. We used Cox proportional hazard models to compute mortality hazard ratio, adjusting for clinical and molecular features including *KRAS*, *BRAF*, and *PIK3CA* mutations, MSI, CIMP and LINE-1 hypomethylation. The densities of CD8⁺, CD45RO⁺ and FOXP3⁺-cells were significantly associated with patient survival in univariate analyses ($P_{\text{trend}} < 0.007$). In the multivariate model, tumour-infiltrating CD45RO⁺-cell density, but not that of CD3⁺, CD8⁺ or FOXP3⁺-cell, was significantly associated with survival ($p = 0.0032$). In multivariate linear regression analysis, MSI-high ($p < 0.0001$) and high-level tumour LINE-1 methylation ($p = 0.0013$) were independently associated with higher CD45RO⁺-cell

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density. Nonetheless, the survival benefit associated with CD45RO⁺-cells was independent of MSI and LINE-1 status. In conclusion, tumour-infiltrating CD45RO⁺-cell density is a prognostic biomarker associated with longer survival of colorectal cancer patients, independent of clinical, pathological and molecular features. In addition, MSI-high and tumour LINE-1 methylation level are independent predictors of CD45RO⁺-cell density. Our data offer a possible mechanism by which MSI confers an improved clinical outcome, and support efforts to augment host immune response in the tumour microenvironment as a strategy of targeted immunotherapy.

Keywords

colon cancer; reaction; mismatch repair; epigenetics; immunology; therapeutic target; immunity

Introduction

The abundance of tumour-infiltrating T-cells has been associated with improved clinical outcome of colorectal cancer patients (Table 1) [1-23]. Although the exact mechanism remains uncertain, the adaptive immune system may play an important role in suppressing tumour progression [18,24]. Tumour-infiltrating T-cells may be an indicator of host immune response to tumour and an attractive target for immunotherapy [25-28].

Tumour-infiltrating lymphocytes may also reflect specific molecular alterations associated with indolent tumour behavior. Previous studies have shown that lymphocytic infiltration is associated with microsatellite instability (MSI) in colorectal cancer [29-32]. Truncated peptides produced by frameshift mutations due to MSI may be immunogenic and contribute to host immune response [24,26,33]. However, little is known on the interrelationship between tumour-infiltrating T-cells, MSI and other tumour molecular features, including the CpG island methylator phenotype (CIMP), global DNA hypomethylation and *KRAS*, *BRAF* and *PIK3CA* mutations.

Previous studies have reported that MSI [34], CIMP [35], *BRAF* mutation [35,36], *PIK3CA* mutation [37], and tumour LINE-1 hypomethylation [38] are associated with prognosis, and that lymphocytic infiltration is associated with many of these molecular variables [32]. As such, to define the prognostic effect of tumour-infiltrating T-cells independent of those potential confounders, large studies of colorectal cancers with extensive molecular characterization are needed.

We, therefore, examined the prognostic role of tumour-infiltrating T-cell subsets in a database of 768 colorectal cancers from two prospective cohort studies. Because we concurrently assessed the densities of CD3⁺, CD8⁺, CD45RO⁺ (*PTPRC*) and FOXP3⁺-cells as well as other relevant molecular and pathologic features, we could evaluate the independent effect of each T-cell subset density on patient survival. Our data have shown that the density of CD45RO⁺-cells, but not that of CD3⁺, CD8⁺, or FOXP3⁺-cells, is an independent prognostic biomarker associated with longer survival of colorectal cancer patients.

Materials and methods

Study group

We utilized a database of colorectal cancer cases in the Nurses' Health Study (N=121,701 women followed since 1976) [39], and the Health Professionals Follow-up Study (N=51,529 men followed since 1986) [39]. Based on availability of follow-up data and adequate tissue specimens for tissue microarray (TMA), 768 colorectal cancers (diagnosed up to 2004) were

included (Table 2). Hospitals where patients underwent tumour resections were distributed throughout 48 States in the U.S. Patients were observed until death or June 30, 2009, whichever came first. Among our cohort studies, there was no significant difference in demographic features between cases with and without available tissue [39]. Tissue sections from all colorectal cancers were reviewed by a pathologist (S.O.) unaware of other data. Tumour grade was categorized as low vs. high ($\geq 50\%$ vs. $< 50\%$ gland formation). Written informed consent was obtained from all study subjects. Tissue collection and analyses were approved by the Institutional Review Boards.

***KRAS*, *BRAF* and *PIK3CA* sequencing, and MSI analysis**

DNA was extracted from tumour, and PCR-Pyrosequencing targeted for *KRAS* (codons 12-13) [40], *BRAF* (codon 600) [41] and *PIK3CA* (exons 9 and 20) were performed [42]. MSI analysis was performed, using BAT25, BAT26, BAT40, D2S123, D5S346, D17S250, D18S55, D18S56, D18S67 and D18S487 [43]. MSI-high was defined as instability in $\geq 30\%$ of the markers [43].

Analyses for CpG island methylation and LINE-1 methylation

Bisulfite DNA treatment and real-time PCR (MethyLight) were validated and performed [44]. We quantified DNA methylation in 8 CIMP-specific promoters [*CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*] [45-47]. CIMP-high, CIMP-low and CIMP-0 were defined as $\geq 6/8$, $1/8$ - $5/8$ and $0/8$ methylated promoters, respectively, according to the previously-established criteria [47]. To accurately quantify LINE-1 methylation, we utilized Pyrosequencing [48,49].

Immunohistochemistry and image analysis

Tissue microarrays (TMAs) were constructed [50]. For CD3, CD8 and FOXP3, antigen retrieval was performed, and deparaffinized tissue sections in EDTA Solution (pH 8.0) (Zymed, San Francisco, CA) were treated by a microwave for 30 sec in a pressure cooker. For CD45RO (the official gene symbol PTPRC), Citrate Buffer (pH 6.0) (Zymed) was used for antigen retrieval. Tissue sections were incubated with peroxidase block (5 min); protein block (20 min); and then, primary antibody against CD3 (rabbit polyclonal anti-human CD3 antibody, clone F7.2.38, 1:250 dilution, Dako Cytomation, Carpinteria, CA), CD8 (mouse monoclonal anti-human CD8 antibody, clone C8/144B, 1:100 dilution, Dako Cytomation), CD45RO (mouse monoclonal anti-human CD45RO antibody, clone UCHL1, 1:100 dilution, Dako Cytomation) or FOXP3 (mouse monoclonal anti-human FOXP3 antibody, clone 206D, 1:50 dilution; BioLegend, San Diego, CA) for 1 hr at room temperature. Next, we applied Envision System HRP-labeled polymer anti-rabbit (for CD3) or anti-mouse (for the other markers) (Dako Cytomation) for 30 min, diaminobenzidine (5 min) and hematoxylin counterstain (1 min).

After staining for each T-cell subset, TMA slides were scanned by an automated scanning microscope and Ariol image analysis system (Genetix, San Jose, CA) (Figure 1). The software was used to count the number of positive nuclei in each tissue core. We marked neoplastic epithelial areas, to exclude non-neoplastic areas (stroma, normal mucosa, and necrotic regions). We calculated the average density (cells/mm²) of each tumour-infiltrating T-cell subset. In addition, we analysed subset T-cell density in stromal areas as well as in whole TMA core (Supplementary Tables 1-2).

Immunohistochemical evaluation of T-cell subsets in cancer tissue has been a challenge, and there has been no standardized method. Pre-analytical variables such as tissue processing may have considerable impact on antigenicity of each T-cell subset, which may be substantially influenced by a subtle difference in conditions of immunohistochemical

procedure. We tried to minimize such measurement errors in a number of ways. We constructed TMA and performed the immunohistochemical procedure in a very similar condition for all specimens. We obtained two to four tumour tissue cores from each case, considering within-tumour heterogeneity.

Statistical analyses

We used SAS program (Version 9.1, SAS Institute, Cary, NC). All p values were two-sided, and statistical significance was set at $p=0.05$. Nonetheless, when multiple hypothesis testing was performed, a p-value for significance was adjusted to $p=0.0009$ ($=0.05/56$) or $p=0.0042$ ($=0.05/12$) by Bonferroni correction. To assess unadjusted relationship with each T-cell subset density, we used the Wilcoxon rank-sum test for dichotomous variables or Kruskal-Wallis test for variables with more than two categories.

For survival analyses, Kaplan-Meier method and the log-rank test were used (Figure 2). For analyses of colorectal cancer-specific mortality, deaths as a result of causes other than colorectal cancer were censored. To control for confounding, we used stage-stratified proportional hazards model to compute hazard ratio (HR) of death according to each T-cell subset status. Disease stage [I, IIA, IIB (indicating invasion of visceral serosal surface or adjacent organ), IIIA, IIIB, IIIC, IV, unknown] was used as a stratifying variable (by the “strata” option in the SAS “proc phreg” command) to avoid residual confounding and overfitting. The model initially included age (continuous), sex, BMI (≥ 30 vs. <30 kg/m²), family history of colorectal cancer, year of diagnosis (continuous), tumour location (proximal colon vs. distal colon vs. rectum), tumour grade, CIMP, MSI, *BRAF*, *KRAS*, *PIK3CA*, LINE-1 methylation (continuous) and other T-cell subsets (quartiles). A backward stepwise elimination with a threshold of $p=0.10$ was used to select variables, but all of T-cell subsets were forced into the final model. We assigned a “missing” indicator variable for those cases with missing data on CD3⁺ (5.3%), CD8⁺ (7.7%), CD45RO⁺ (3.9%) or FOXP3⁺ (8.2%). For cases with missing information in any of the other categorical variables [BMI (0.1%), tumour grade (0.4%), CIMP (1.8%), MSI (1.8%), *BRAF* (1.5%), *KRAS* (1.1%), and *PIK3CA* (10%)], we included those cases in a majority category of that variable. An interaction was assessed by the Wald test on the cross product of the CD45RO⁺ variable and another variable of interest (without data-missing cases).

To assess relationship with CD45RO⁺-cell density, a multivariate linear regression model was constructed. Because CD45RO⁺-cell density showed skewed distribution, we used “log_e(CD45RO⁺-cells/mm²)”, which distributed approximately normally. A backward stepwise elimination with a threshold of $p=0.10$ was used to select variables in the final model. After the final linear regression model was constructed, a distribution of residuals [observed minus predicted log(CD45RO⁺-cell density)] was visually inspected and confirmed that the assumption of residuals' normality and equal variance across predicted log(CD45RO⁺-cell density) was generally satisfied (data not shown). We assessed whether there was any influential case, by Cook's D statistics, a summary measure of influence, and found that there was no influential case (all Cook's D value <0.031).

Results

T-cell subsets in colorectal cancer

Utilizing tissue microarray (TMA), we quantified CD3⁺, CD8⁺, CD45RO⁺ and FOXP3⁺-cells in neoplastic epithelial areas by automated image analysis on 768 colorectal cancers. Density of each T-cell subset (cells/mm²) distributed as follows; CD3⁺ (mean 730; median 245; interquartile range 86-581), CD8⁺ (mean 806; median 237; interquartile range 77-646), CD45RO⁺ (mean 670; median 377; interquartile range 159-727), and FOXP3⁺ (mean 38;

median 26; interquartile range 14–48). Selected T-cell subset densities were positively associated with CIMP-high, MSI-high, *BRAF* mutation, and high-level global DNA methylation (as measured in LINE-1) (Table 2). Table 3 shows Spearman's correlation coefficients between T-cell subset densities in neoplastic epithelial areas.

Tumour-infiltrating T-cell subset density and patient survival

We examined density of each tumour-infiltrating T-cell subset and patient survival. There were 366 deaths including 229 colorectal cancer-specific deaths. Median follow-up for censored cases was 11.6 years. In Kaplan-Meier analysis (Figure 2), high CD45RO⁺ and FOXP3⁺ densities were associated with improved cancer-specific and overall survival (log-rank $p < 0.0004$). We performed univariate and multivariate Cox regression analyses (Table 4). In univariate analysis, CD8⁺, CD45RO⁺ and FOXP3⁺-cells were associated with longer cancer-specific survival ($P_{\text{trend}} < 0.007$) and overall survival ($P_{\text{trend}} \leq 0.04$). In multivariate analysis, however, only CD45RO⁺-cell density was significantly associated with overall survival ($P_{\text{trend}} = 0.015$), after adjusting for all known or suspected clinical and molecular features, including other T-cell subset densities. Compared to Q1 stratum, CD45RO⁺ Q4 stratum was associated with significantly longer cancer-specific survival [multivariate hazard ratio (HR), 0.51; 95% confidence interval (CI): 0.32–0.80; $p = 0.0032$] and overall survival (multivariate HR, 0.61; 95% CI: 0.43–0.85; $p = 0.0036$). In exploratory analyses, any of tumour-infiltrating CD3⁺/FOXP3⁺, CD8⁺/FOXP3⁺, and CD45RO⁺/FOXP3⁺ was not associated with survival (data not shown).

In addition, we performed survival analyses using each T-cell subset density in whole TMA tissue cores and in tumour stromal areas, which yielded results similar to Table 4 (Supplementary Tables 1–2).

Interaction between CD45RO⁺-cells and other variables in survival analysis

We examined whether the survival benefit associated with tumour-infiltrating CD45RO⁺-cells was modified by any of the clinical, pathologic and molecular variables (Figure 3). We did not observe a significant interaction between CD45RO⁺-cell density and any of the covariates (all $P_{\text{interaction}} > 0.10$), such as disease stage, MSI status, or CD3⁺, CD8⁺, or FOXP3⁺-cell density.

Multivariate linear regression analysis to assess relations with CD45RO⁺-cells

In view of the survival benefit specifically associated with tumour-infiltrating CD45RO⁺-cells, we constructed a multivariate linear regression model to define the clinical and molecular features that were independently associated with tumour-infiltrating CD45RO⁺-cells (Table 5). The presence of MSI-high was positively associated with CD45RO⁺-cell density ($p < 0.0001$), whereas LINE-1 hypomethylation was inversely associated with CD45RO⁺-cell density ($p = 0.0013$), adjusting for other potential factors. Nonetheless, as described above (Table 4), the influence of tumour-infiltrating CD45RO⁺-cells on patient survival was independent of tumour MSI and LINE-1 methylation status.

Discussion

We conducted this study to examine the independent prognostic effect of tumour-infiltrating T-cell subsets (CD3⁺, CD8⁺, CD45RO⁺ and FOXP3⁺) in a large population of colorectal cancer patients. We found that tumour-infiltrating CD45RO⁺-cell density was significantly associated with improved prognosis in colorectal cancers, independent of clinical, pathologic and molecular features, including densities of the other T-cell subsets. Thus, our findings suggest tumour-infiltrating CD45RO⁺-cell density as a prognostic biomarker in colorectal cancer. Furthermore, microsatellite instability (MSI)-high and high-level LINE-1

methylation were independently associated with tumour-infiltrating CD45RO⁺-cell density, raising potential roles of these molecular features in stimulating host immune response to tumour. Nonetheless, the survival benefit associated with tumour-infiltrating CD45RO⁺-cells was independent of tumour MSI and LINE-1 methylation status.

Examining tumour and host factors and patient outcome is important in cancer research [51-56]. Accumulating evidence suggests that effector/cytotoxic (CD3⁺ [2,12,15,16,33] and CD8⁺ [1-3,6-9,11,12,18-21]) and memory (CD45RO⁺ [3,12,18,19,23]) T-cells play important roles in antitumour immune response. With regard to regulatory T-cells, one specific marker is nuclear transcription factor FOXP3 [25,57,58]. FOXP3⁺ regulatory T-cell has been shown to modulate antitumour immune response [17-19,21-23,27,59-62] and suppress the activity of cytotoxic T-cells [18]. Thus, these specific subsets of effector/cytotoxic (CD3⁺ and CD8⁺), memory (CD45RO⁺) and regulatory (FOXP3⁺) T-cells are thought to be indicators of host immune response to tumour cells and might be a target for immunotherapy [25-27].

To examine T-cell subsets, we utilized a digitized, high-resolution image analysis, which could count the number of lymphocytes and minimise observer bias. The median densities of tumour-infiltrating T-cell subsets in the current study were compatible with data in the previous studies (CD3⁺ [12], CD8⁺ [21], CD45RO⁺ [18], and FOXP3⁺ [17,21,59]).

Previous studies have reported the relationship between MSI-high and density of CD3⁺ [2,16,33,59], CD8⁺ [2,7,8,18,59], CD45RO⁺ [18], or FOXP3⁺-cells [15,59]. Our current study has shown that densities of CD45RO⁺ and FOXP3⁺-cells, but not that of CD3⁺ or CD8⁺-cells, are significantly associated with MSI-high. The strong association between MSI and CD45RO⁺-cell density supports the hypothesis that truncated peptides produced by MSI and frameshift mutations may elicit host immune response and recruit CD45RO⁺-cells [24,33]. In most studies, MSI in colon cancer has been associated with improved survival [18,24,34,63-67], although the mechanism underlying this association is largely unknown. Our results suggest that stimulation of a host immune response to MSI-high cancer may offer one explanation. In addition, recent studies have reported a detrimental effect of adjuvant chemotherapy on MSI-high colon cancer patients [63,68], although there have been conflicting data [69]. It is possible that cytotoxic chemotherapy may attenuate the host immune response associated with MSI-high colon cancer. Further studies are needed to elucidate the mechanism of host immune response and modifying effect of chemotherapy.

In addition, we were able to detect an independent relationship between CD45RO⁺-cell density and high-level LINE-1 methylation, which has not been examined before. Previous studies have reported a link between LINE-1 hypomethylation, global DNA hypomethylation and chromosomal instability (CIN) [38,48,70]. Global DNA hypomethylation and CIN affect epigenetic status and gene copy numbers, respectively, in a genome-wide scale. Thus, it is not surprising that global DNA hypomethylation and/or CIN may affect expression levels of genes which contribute to interactions with host cells and regulation of host immune response to tumour. Additional studies are necessary to elucidate the exact mechanism of the relationship between LINE-1 methylation level and CD45RO⁺-cells.

In early-stage colorectal cancer, infiltration of CD45RO⁺-cells has been associated with an gene expression pattern reflective of T-helper 1 cells and cytotoxic T-cells [19]. Moreover, a recent study has reported that infiltration of CD45RO⁺-cells is associated with high expression of specific chemokines (CXCL9 and CXCL10), which may play roles in favorable prognosis of colorectal cancer patients [71]. Previous studies reported that tumour-infiltrating CD45RO⁺-cell density was associated with improved survival in colorectal

cancer [3,10,12,19,23]; however, none of those studies [3,10,12,19,23] examined the prognostic effect of CD45RO⁺-cells independent of established molecular alterations, including MSI, CIMP, *BRAF* mutation and LINE-1 methylation, all of which have been associated with colon cancer prognosis [34,35,38]. Because these molecular features are also associated with tumour-infiltrating T-cells, the independent effect of specific T-cell subsets on patient outcome can only be ascertained through a comprehensive, multivariate analysis model where data on clinical, pathologic, and molecular features are available simultaneously as in our current analyses.

A recent study by Salama et al. [18] reported that tumour-infiltrating FOXP3⁺-cell density was a prognostic factor in 445 stage II-III colorectal cancers in multivariate analysis which included clinical and pathological features, MSI, and CD8⁺ and CD45RO⁺ T-cell subsets. In contrast, our database of 768 stage I-IV colorectal cancers suggests that the survival benefit associated with tumour-infiltrating FOXP3⁺-cell density is absent after adjusting for CD45RO⁺ status and other clinical and molecular features. Of note, in contrast to the previous studies [15,59] and ours, Salama et al. [18] did not observe a significant relation between MSI and FOXP3⁺-cell density, and Le Gouvello et al. [61] found lower mRNA expression level of FOXP3 in MSI-high tumour tissue, perhaps reflecting differences in study populations and analysis methods on FOXP3 [15,59].

CD8⁺ and CD3⁺-cell densities have been associated with favorable prognosis in colorectal cancers [2,15,16,20]. Our analyses have shown that neither CD3⁺ nor CD8⁺-cell density is associated with improved survival in multivariate analysis. In particular, the attenuation in the effect of CD8⁺-cells in multivariate analysis is the result of adjusting for disease stage and CD45RO⁺-cells, suggesting that the improved outcome associated with CD8⁺-cells in previous studies [1,2,6-9,11,13,14,20] might be due, at least in part, to the confounding effect of CD45RO⁺-cells.

Recent studies have reported that the ratio of tumour-infiltrating CD3⁺/FOXP3⁺ [15] or CD8⁺/FOXP3⁺ [21] is associated with clinical outcome of colorectal cancers. However, in our current study, any of ratios of CD3⁺/FOXP3⁺, CD8⁺/FOXP3⁺, and CD45RO⁺/FOXP3⁺ was not associated with patient outcome (data not shown). Those previous studies had small sample sizes (N=94 [21], and N=101 [15]) and performed multiple hypothesis testing without validation cohort, and hence, generalisability of their findings may be limited.

Considering within-tumour heterogeneity, we evaluated two to four tumour tissue cores from each case in TMA. We admit that this approach might still fall short of capturing heterogeneity within tumour. Nonetheless, a misclassification, if any, in TMA analysis due to tumour heterogeneity would be non-differential and unrelated to patient outcome or molecular variables. Despite a possibility of such misclassified cases, we were able to demonstrate the prognostic significance of tumour-infiltrating CD45RO⁺-cells, because of our well-powered study design.

In summary, tumour-infiltrating CD45RO⁺-cell density is significantly associated with longer survival of colorectal cancer patients, independent of clinical, pathological and molecular features. In addition, MSI-high and LINE-1 methylation level are independent predictors of CD45RO⁺-cell density. Our findings suggest that tumour-infiltrating CD45RO⁺-cells may materially improve patient outcome, offer a possible mechanism by which MSI confers an improved patient outcome, and supports efforts to augment host immune response as a therapeutic strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CI	confidence interval
CIMP	CpG island methylator phenotype
HR	hazard ratio
MSI	microsatellite instability
MSS	microsatellite stable
OR	odds ratio

SD standard deviation
TMA tissue microarray

Figure 1.

Image analysis of CD3⁺, CD8⁺, CD45RO⁺ and FOXP3⁺-cells in colorectal cancer.

A. CD45RO⁺-cells in a tissue microarray (TMA) core (i). Neoplastic epithelial areas are selected, and there are CD45RO⁺-cells (in red color) in neoplastic epithelial areas (ii).

B. TMA images for CD3⁺, CD8⁺, CD45RO⁺ and FOXP3⁺-cells in colorectal cancer. Upper panels show high cell density cases, and lower panels show low cell density cases. In these TMA cores, neoplastic epithelial areas are subsequently selected as shown in panel A(ii) for image analysis.

Figure 2.

Kaplan-Meier survival curves with log-rank p values for colorectal cancer-specific survival (CS; left panel) and overall survival (OS; right panel) according to tumour-infiltrating T-cell subset density; CD3⁺-cells (A), CD8⁺-cells (B), CD45RO⁺-cells (C) and FOXP3⁺-cells (D). Q1-4, first to fourth quartile.

Figure 3.

Stratified analysis of tumour-infiltrating CD45RO⁺-cell density and colorectal cancer-specific mortality. $\log_e(\text{adjusted HR})$ with 95% confidence interval (CI) for CD45RO⁺-cell density Q4 (vs. Q1-3) in various strata is shown. There was no significant interaction (effect modification) by any of the variables including CD3⁺, CD8⁺, or FOXP3⁺-cell density. CIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-up Study; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stable; NHS, Nurses' Health Study; Q1-4, first to fourth quartile.

Table 1

Studies on tumour-infiltrating T-cell subsets and colorectal cancer patient survival

Ref.	Authors (year)	No. of hospitals	Sample size	No. of events		Disease stage	Additional therapy	High density of T-cells (vs. low density)					Variables examined in multivariate analysis
				OS	CS			Image analyzer	T-cell subset analyzed	5-year CS, OS or DFS, log-rank p value	CS, OS or DFS univariate HR (95% CI) ^a , p value	CS, OS or DFS multivariate HR (95% CI) ^a , p value	
[1]	Naito et al. (1998)	2	131	-	-	Duke's A-D	-	No	CD8 ⁺	p=0.0003 (OS)	-	0.61 (0.41-0.89) p=0.011 (OS)	inflammatory cells, invasive pattern, stage, tumour grade
[2]	Guidoboni et al. (2001)	1	109	37	-	II-III	No treatment or 5-FU adjuvant chemo therapy	No	CD3 ⁺	p=0.004 (OS)	-	0.40 (0.19-0.85) (OS)	age, sex, stage
									CD8 ⁺	p=0.0008 (OS)	-	0.33 (0.15-0.73) (OS)	
									GZMB ⁺	p=0.0001 (OS)	-	0.23 (0.10-0.50) (OS)	
[3]	Oberg et al. (2002)	1	93	59	47	Duke's C	No treatment or chemo or radiation therapy	No	CD8 ⁺	77% (vs. 38%) p=0.011 (CS)	-	-	-
									CD45RO ⁺	66% (vs. 33%) p=0.002 (CS)	-	-	
									CD68 ⁺	60% (vs. 37%) p=0.033 (CS)	-	-	
[4]	Petty et al. (2002)	2	72	-	-	I-IV	No treatment or chemo or radiation therapy	No	CD134 ⁺	p=0.02 (CS)	-	-	-
[5]	Diederichsen et al. (2003)	2	41	25	-	Duke's A-D	No treatment	No	CD4 ⁺ /CD8 ⁺ ratio	22% (vs. 61%) (OS)	-	p=0.046 (OS)	age, stage
[6]	Funada et al. (2003)	-	97	-	-	-	-	No	CD8 ⁺	-	p=0.01 (OS)	-	-
[7]	Prall et al. (2004)	1	152	-	-	III	5FU/LV adjuvant chemo or radiation therapy	No	CD8 ⁺	p<0.001 (CS)	-	-	-
[8]	Menon et al. (2004)	-	93	-	-	II-III	-	No	CD8 ⁺	p=0.03 (DFS)	-	0.56 (0.32-0.99) p=0.04 (DFS)	age, sex, location, stage, tumour grade
[9]	Chiba et al. (2004)	3	371	-	74	I-IV	-	No	CD8 ⁺	p<0.0001 (CS)	-	0.43 (0.23-0.83) p=0.01 (CS)	age, sex, tumour grade, invasive pattern, location, MMR protein, TIL, stage

Ref.	Authors (year)	No. of hospitals	Sample size	No. of events		Disease stage	Additional therapy	Image analyzer	High density of T-cells (vs. low density)			Variables examined in multivariate analysis	
				OS	CS				T-cell subset analyzed	5-year CS, OS or DFS, log-rank p value	CS, OS or DFS univariate HR (95% CI) ^a , p value		CS, OS or DFS multivariate HR (95% CI) ^a , p value
									node positive p=0.66 (CS) node negative p=0.006 (DFS) node positive p=0.62 (DFS)				
[17]	Frey et al. (2009)	-	1232	-	-	I-III	-	No	FOXP3 ⁺	MSS group1 62% (vs. 46%) p=0.004 (CS)	-	0.73 (0.60-0.90) p=0.019 (CS)	age, sex, stage (pT, pN), tumour grade, vascular invasion, tumour border configuration
										MSS group2 (CS) 60% (vs. 44%) p<0.001 (CS)	-	0.70 (0.60-0.90) p=0.007 (CS)	
										MSI 75%? (vs. 63%?) p=0.029 (CS)	-	0.63 (0.3-1.2) p=0.13 (CS)	
[18]	Salama et al. (2009)	1	445	-	-	II-III	5FU/LV based adjuvant chemo therapy	Yes	CD8 ⁺ CD45RO ⁺ FOXP3 ⁺	- - -	0.74 (0.67-0.82) p<0.0001 (CS) 0.74 (0.65-0.84) p<0.0001 (CS) 0.78 (0.70-0.87) p<0.0001 (CS)	NS (CS) NS (CS) 0.54 (0.38-0.77) p=0.001 (CS)	lymphocytic response, other T-cell subsets, MSI, stage, tumour grade, vascular, lymphatic and perineural invasion
[19]	Pages et al. (2009)	1	411	-	-	I-II	5FU/LV based adjuvant chemo therapy	Yes	CD8 ⁺ CD45RO ⁺ CD8 ⁺ plus CD45RO ⁺	p<0.0001(OS and DFS) p<0.0001(OS and DFS) p<0.0001(OS and DFS)	- - -	stage, bowel perforation	
[20]	Deschool meester et al. (2010)	-	209	100	100	I-IV	No treatment or adjuvant or neo-adjuvant chemo therapy	No	CD3 ⁺ CD8 ⁺ GZMB ⁺	p=0.04 (OS) p=0.04 (OS) -	- - -	0.54 (0.18-1.59) p=0.26 (OS) 2.06 (0.67-6.39) p=0.21 (OS) 1.18 (0.45-3.13) p=0.74 (OS)	age, sex, location, stage tumour grade, adjuvant treatment, other T-cell subsets
[21]	Suzuki et al. (2010)	1	94	-	-	I-IV	-	No	CD8 ⁺ /FOXP3 ⁺ ratio	p=0.01 (OS)	0.35 (0.15-0.81) p=0.014 (OS)	0.40 (0.17-0.94) p=0.035 (OS)	stage, venous invasion

Ref.	Authors (year)	No. of hospitals	Sample size	No. of events		Disease stage	Additional therapy	Image analyzer	High density of T-cells (vs. low density)			Variables examined in multivariate analysis	
				OS	CS				T-cell subset analyzed	5-year CS, OS or DFS, log-rank p value	CS, OS or DFS univariate HR (95% CI) ^a , p value		CS, OS or DFS multivariate HR (95% CI) ^a , p value
[22]	Correale et al. (2010)	-	57	-	-	-	FOLFOX chemo therapy	No	FOXP3 ⁺	-	p=0.0009 (DFS) p=0.0005 (OS)	-	-
[23]	Lee et al. (2010)	1	87	-	II	-		Yes	CD3 ⁺	p=0.010 (DFS) p=0.061 (OS)	p=0.003 (DFS) p=0.039 (OS)	0.20 (0.02-2.60) p=0.22 (DFS)	vascular invasion, neural invasion, other T-cell subsets?
									CD25 ⁺	p=0.013 (DFS) p=0.15 (OS)	p=0.002 (DFS) p=0.017 (OS)	0.22 (0.02-2.35) p=0.21 (DFS)	
									CD45RO ⁺	p=0.049 (DFS) p=0.16 (OS)	p=0.014 (DFS) p=0.037 (OS)	0.24 (0.02-1.10) p=0.014 (DFS)	
									FOXP3 ⁺	p=0.009 (DFS) p= 0.027 (OS)	p=0.005 (DFS) p=0.040 (OS)	0.14 (0.07-0.85) p=0.027 (DFS)	
									CD3 ⁺	79% (vs. 75%) Q4 (vs. Q1) ^b p=0.19 (CS)	0.73 (0.49-1.08) Q4 (vs. Q1) ^b p=0.070 ^c (CS)	1.30 (0.81-2.07) Q4 (vs. Q1) ^b p=0.16 ^c (CS)	
Current study	Many	768	366	229	I-IV	-		Yes	CD8 ⁺	78% (vs. 66%) Q4 (vs. Q1) ^b p=0.026 (CS)	0.61 (0.42-0.88) Q4 (vs. Q1) ^b p=0.007 ^c (CS)	0.81 (0.52-1.27) Q4 (vs. Q1) ^b p=0.34 ^c (CS)	age, sex, BMI, family history, year of diagnosis, location, stage, tumour grade, LINE-1, CIMP, MSI, BRAF, KRAS, PIK3CA, other T-cell subsets
									CD45RO ⁺	83% (vs. 68%) Q4 (vs. Q1) ^b p<0.0001 (CS)	0.40 (0.26-0.60) Q4 (vs. Q1) ^b p<0.0001 ^c (CS)	0.51 (0.32-0.80) Q4 (vs. Q1) ^b p=0.034 ^c (CS)	
									FOXP3 ⁺	80% (vs. 64%) Q4 (vs. Q1) ^b p<0.0001 (CS)	0.48 (0.32-0.70) Q4 (vs. Q1) ^b p<0.0001 ^c (CS)	0.89 (0.59-1.34) Q4 (vs. Q1) ^b p=0.76 ^c (CS)	

^a HR is based on comparing of high vs. low score of a given T-cell subset.

^b Quartile of density (Q1-4, first to fourth quartile).

^c P for trend.

BMI, body mass index; CI, confidence interval; CIMP, CpG island methylator phenotype; CS, colorectal cancer-specific survival; DFS, disease-free survival; 5-FU, 5-fluorouracil; GZMB, Granzyme B; HR, hazard ratio; L.V, leucovorin; MSI, microsatellite instability; MMR, mismatch repair; NS, not significant; OS, overall survival; TIL, tumour infiltrating lymphocyte; uPA, urokinase-type plasminogen activator.

Table 2
Subset T-cell density in neoplastic epithelial areas, according to various features in colorectal cancer

Clinical, pathologic or molecular feature	Total N	CD3 ⁺ (cells/mm ²) Median (25th -75th percentile)	P value	CD8 ⁺ (cells/mm ²) Median (25th -75th percentile)	P value	CD45RO ⁺ (cells/mm ²) Median (25th -75th percentile)	P value	FOXP3 ⁺ (cells/mm ²) Median (25th -75th percentile)	P value
All cases	768	245 (86-581)		237 (77-646)		377 (159-727)		26 (14-48)	
Age (years)			0.097		0.07		0.98		0.74
<65	286 (37%)	225 (77-532)		270 (87-843)		392 (154-694)		26 (14-47)	
≥65	482 (63%)	257 (96-603)		214 (72-565)		373 (164-754)		26 (13-49)	
Gender			0.12		0.0025		0.0029		0.17
Men	283 (37%)	264 (85-735)		304 (88-917)		299 (153-611)		25 (13-46)	
Women	485 (63%)	234 (92-517)		193 (70-517)		425 (169-839)		27 (14-49)	
Body mass index (BMI, kg/m ²)			0.69		0.70		0.87		0.88
<30	632 (82%)	244 (86-572)		248 (75-685)		376 (156-718)		26 (14-48)	
≥30	135 (18%)	254 (94-590)		200 (87-548)		393 (181-783)		25 (11-48)	
Family history of colorectal cancer in any first degree relative			0.52		0.90		0.62		0.93
(-)	589 (77%)	244 (92-584)		247 (75-615)		372 (156-718)		26 (14-49)	
(+)	179 (23%)	248 (81-548)		215 (80-748)		399 (163-805)		27 (12-46)	
Year of diagnosis			<0.0001		<0.0001		0.077		<0.0001
Prior to 1995	291 (38%)	159 (71-447)		300 (127-892)		402 (205-756)		22 (11-37)	
1995 to 2004	477 (62%)	276 (121-640)		188 (62-542)		357 (142-699)		30 (15-53)	
Tumour location			0.72		0.74		0.030		0.30
Rectum	153 (20%)	255 (95-590)		200 (76-655)		284 (125-656)		22 (12-48)	
Distal colon	233 (30%)	227 (87-551)		255 (80-663)		374 (175-681)		27 (14-44)	
Proximal colon	382 (50%)	247 (84-570)		239 (73-609)		417 (174-857)		27 (14-49)	
Tumour grade			0.49		0.80		0.0051		0.89
Low grade	690 (90%)	241 (85-568)		234 (77-647)		373 (158-688)		26 (14-48)	
High grade	75 (10%)	288 (143-717)		245 (70-634)		493 (183-1737)		26 (15-48)	
Disease stage			0.29		0.14		0.37		<0.0001
I	166 (21%)	284 (103-588)		219 (80-608)		409 (156-847)		34 (18-52)	
II	236 (31%)	239 (76-593)		274 (69-836)		397 (181-805)		25 (14-47)	
III	217 (28%)	227 (102-520)		250 (91-737)		377 (145-680)		25 (12-48)	

Clinical, pathologic or molecular feature	Total N	CD3 ⁺ (cells/mm ²) Median (25th -75th percentile)	P value	CD8 ⁺ (cells/mm ²) Median (25th -75th percentile)	P value	CD45RO ⁺ (cells/mm ²) Median (25th -75th percentile)	P value	FOXP3 ⁺ (cells/mm ²) Median (25th -75th percentile)	P value
IV	112 (15%)	196 (81-468)		141 (56-475)		345 (135-548)		20 (10-32)	
Unknown	37 (5%)	292 (79-620)		300 (55-588)		361 (220-1207)		42 (17-103)	
CIMP status			0.39		0.56		<0.0001		0.19
CIMP-0/low	630 (84%)	240 (86-524)		232 (77-636)		348 (154-678)		25 (13-46)	
CIMP-high	124 (16%)	252 (83-737)		274 (70-710)		538 (255-1314)		28 (14-56)	
MSI status			0.55		0.58		<0.0001		0.0017
MSI-low/MSS	630 (84%)	233 (86-525)		234 (73-647)		339 (143-655)		25 (13-46)	
MSI-high	123 (16%)	267 (73-714)		270 (83-597)		616 (316-1415)		34 (18-57)	
BRAF mutation			0.91		0.58		0.0006		0.059
(-)	642 (85%)	241 (87-558)		232 (76-654)		348 (154-684)		25 (13-47)	
(+)	114 (15%)	256 (82-615)		264 (77-592)		529 (255-1023)		33 (15-57)	
KRAS mutation			0.13		0.066		0.0043		0.036
(-)	481 (63%)	245 (81-559)		205 (71-559)		412 (175-832)		28 (14-50)	
(+)	278 (37%)	241 (123-568)		265 (83-832)		315 (146-627)		23 (13-40)	
PIK3CA mutation			0.94		0.033		0.12		0.45
(-)	576 (84%)	248 (86-586)		226 (75-645)		364 (146-692)		27 (14-48)	
(+)	112 (16%)	229 (105-528)		304 (122-864)		425 (203-798)		23 (13-42)	
LINE-1 methylation			0.31		0.39		0.0035		0.25
≥60%	438 (59%)	249 (94-588)		246 (83-650)		409 (191-797)		28 (13-50)	
<60%	306 (41%)	216 (81-486)		215 (66-636)		290 (137-676)		25 (14-42)	

To assess a difference in distributions of T-cell subset densities, we used the Wilcoxon rank sum test for dichotomous variables or Kruskal-Wallis test for variables with more than two categories. A p value for significance was adjusted for multiple hypothesis testing to $p=0.0009$ ($=0.05/56$) by Bonferroni correction. CIMP, CpG island methylator phenotype; MSI, microsatellite instability; MSS, microsatellite stable.

Table 3
Correlation between tumour-infiltrating T-cell subset densities (*r*; Spearman's correlation coefficient)

	CD3 ⁺ -cell	CD8 ⁺ -cell	CD45RO ⁺ -cell	FOXP3 ⁺ -cell
CD3 ⁺ -cell		$r = 0.47$ $p < 0.0001$	$r = 0.33$ $p < 0.0001$	$r = 0.061$ $p = 0.11$
CD8 ⁺ -cell	$r = 0.47$ $p < 0.0001$		$r = 0.24$ $p < 0.0001$	$r = -0.022$ $p = 0.58$
CD45RO ⁺ -cell	$r = 0.33$ $p < 0.0001$	$r = 0.24$ $p < 0.0001$		$r = 0.23$ $p < 0.0001$
FOXP3 ⁺ -cell	$r = 0.061$ $p = 0.11$	$r = -0.022$ $p = 0.58$	$r = 0.23$ $p < 0.0001$	

Table 4
Subset T-cell density in neoplastic epithelial areas and patient mortality in colorectal cancer

T-cell subset	Quartile	Colorectal cancer-specific survival			Overall survival	
		Univariate HR (95% CI)	Multivariate HR (95% CI)	1 (referent)	Univariate HR (95% CI)	Multivariate HR (95% CI)
CD3 ⁺ -cell (N=727)	Q1 (0-85/mm ²)	1 (referent)	1 (referent)	1 (referent)	1 (referent)	1 (referent)
	Q2 (86-244 /mm ²)	1.10 (0.77-1.58)	1.29 (0.89-1.88)	1.04 (0.78-1.39)	1.04 (0.77-1.42)	
	Q3 (245-579 /mm ²)	0.89 (0.62-1.30)	1.40 (0.93-2.10)	0.91 (0.67-1.22)	1.19 (0.86-1.65)	
	Q4 (≥580 /mm ²)	0.73 (0.49-1.08)	1.30 (0.81-2.07)	0.94 (0.70-1.26)	1.37 (0.96-1.96)	
	P for trend ^a	0.070	0.16	0.50		0.051
CD8 ⁺ -cell (N=709)	Q1 (0-75 /mm ²)	1 (referent)	1 (referent)	1 (referent)	1 (referent)	1 (referent)
	Q2 (76-235 /mm ²)	0.70 (0.49-1.01)	0.75 (0.51-1.11)	0.83 (0.62-1.12)	0.78 (0.57-1.06)	
	Q3 (236-645 /mm ²)	0.64 (0.44-0.92)	0.79 (0.53-1.18)	0.77 (0.57-1.04)	0.83 (0.60-1.14)	
	Q4 (≥646 /mm ²)	0.61 (0.42-0.88)	0.81 (0.52-1.27)	0.74 (0.54-0.99)	0.85 (0.60-1.20)	
	P for trend ^a	0.0068	0.34	0.040		0.47
CD45RO ⁺ -cell (N=738)	Q1 (0-158 /mm ²)	1 (referent)	1 (referent)	1 (referent)	1 (referent)	1 (referent)
	Q2 (159-376 /mm ²)	0.64 (0.45-0.92)	0.81 (0.56-1.18)	0.74 (0.56-0.98)	0.79 (0.59-1.06)	
	Q3 (377-725 /mm ²)	0.80 (0.57-1.12)	1.18 (0.81-1.70)	0.73 (0.55-0.97)	0.96 (0.71-1.30)	
	Q4 (≥726 /mm ²)	0.40 (0.26-0.60)	0.51 (0.32-0.80)	0.52 (0.38-0.70)	0.61 (0.43-0.85)	
	P for trend ^a	<0.0001	0.034	<0.0001		0.015
FOXP3 ⁺ -cell (N=705)	Q1 (0-12 /mm ²)	1 (referent)	1 (referent)	1 (referent)	1 (referent)	1 (referent)
	Q2 (13-25 /mm ²)	0.65 (0.46-0.93)	0.75 (0.52-1.09)	0.85 (0.65-1.12)	1.07 (0.81-1.43)	
	Q3 (26-47 /mm ²)	0.44 (0.30-0.64)	0.74 (0.49-1.11)	0.52 (0.38-0.71)	0.85 (0.62-1.17)	
	Q4 (≥48 /mm ²)	0.48 (0.32-0.70)	0.89 (0.59-1.34)	0.48 (0.35-0.66)	0.80 (0.57-1.14)	
	P for trend ^a	<0.0001	0.76	<0.0001		0.095

The multivariate, stage-matched Cox regression model initially included age, sex, body mass index, family history, year of diagnosis, tumour location, tumour grade, CIMP, MSI, BRAF, KRAS, PIK3CA, LINE-1 methylation and CD3⁺, CD8⁺, CD45RO⁺ and FOXP3⁺-cells. A backward stepwise elimination with a threshold of p=0.10 was used to select variables in the final model.

^aThe density of each T-cell subset categorical variable was used as an ordinal variable.

HR, hazard ratio; CI, confidence interval; Q1-4, first to fourth quartile.

Table 5
Multivariate linear regression analysis to predict tumour-infiltrating CD45RO⁺-cell density [log_e(CD45RO⁺ cells/mm²)] in colorectal cancers

Variables in the final model	Adjusted β coefficient [change in log _e (CD45RO ⁺ -cells/mm ²) by a given variable]	95% confidence limits	P value (T-test)
MSI-high (vs. low/MSS)	0.53	0.31, 0.76	<0.0001
LINE-1 hypomethylation (for a unit of 30% decrease in methylation level)	-0.43	-0.69, -0.17	0.0013

Considering plausible causal relationship, the multivariate linear regression model to predict log_e(CD45RO⁺-cells/mm²) initially included age, sex, body mass index, family history of colorectal cancer, tumour location, tumour grade, CIMP, MSI, KRAS, BRAF, PIK3CA and LINE-1 methylation. A backward stepwise elimination with a threshold of p=0.10 was used to select variables in the final model. Adjusted β coefficient represents a change in log_e(CD45RO⁺-cells/mm²) by a given variable, assuming that all other variables remain constant. A p value for significance was adjusted for multiple hypothesis testing to p=0.0042 (=0.05/12) by Bonferroni correction.

MSI, microsatellite instability; MSS, microsatellite stable.