The intratumoural subsite and relation of CD8\(^+\) and FOXP3\(^+\) T lymphocytes in colorectal cancer provide important prognostic clues

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Background: To find improved tools for prognostic evaluation in patients with colorectal cancer (CRC), we have analysed how infiltration of cytotoxic T lymphocytes (CD8\(^+\)) and regulatory T lymphocytes (FoxP3\(^+\)) correlates to prognosis, not only according to quantity and relation, but also to subsite within tumours of different molecular characteristics (microsatellite instability and CpG island methylator phenotype status).

Methods: CD8 and FOXP3 expression was evaluated by immunohistochemistry in 426 archival tumour tissue samples from patients surgically resected for CRC. The average infiltration of CD8\(^+\) and FOXP3\(^+\) cells was assessed along the tumour invasive front, in the tumour centre and within the tumour epithelium (intraepithelial).

Results: We found that infiltration of CD8\(^+\) T lymphocytes within the tumour epithelium provided the strongest prognostic information \((P<0.001)\). At the tumour invasive front and tumour centre, FOXP3 expression withheld the strongest association to prognosis \((P<0.001)\), suggesting FOXP3\(^+\) T-lymphocyte infiltration to be a better prognostic tool than CD8\(^+\) T lymphocytes at these intratumoural subsites. We further analysed the possible prognostic impact of the relation between these T-cell subsets, finding that a high intraepithelial CD8 expression was associated with a better patient outcome, independent of FOXP3 infiltration. In groups of low intraepithelial CD8 expression, however, a high infiltration rate of FOXP3\(^+\) cells at the tumour invasive front, significantly improved prognosis.

Conclusions: Analyses of intraepithelial infiltration of CD8\(^+\) T lymphocytes, infiltration of FOXP3\(^+\) T lymphocytes at the tumour front or centre, and the relation between these subsets, may be a valuable tool for predicting prognosis in colon cancer.

Colorectal cancer (CRC) is one of the four most common types of cancer in the western world (Jemal \textit{et al}, 2011). Overall 5-year survival rates are reported to be between 50 and 60\%, corresponding closely to disease progression. The success scores for accurate prediction of patient prognosis remain discouraging (Mlecnik \textit{et al}, 2011). One variable that has been shown critical to prognosis in CRC is the state of the tumour-associated immune response (Hanahan and Weinberg, 2011), where a high number of tumour-infiltrating lymphocytes (TILs) has been associated with a better prognosis (Maccarty, 1922; Svennevig \textit{et al}, 1984; Ropponen \textit{et al}, 1997). The interactions between the local immune infiltration and tumour cells are complex and describe a balance between tumour-controlling and tumour-promoting effects (Deschoolmeester \textit{et al}, 2011). Also, an increased number of TILs can be counteracted by immune deviation and/or lymphatic inactivation. The immune infiltrates are found to be heterogeneous between tumour types and patients (Fridman \textit{et al}, 2012) and their effect on prognosis varies in different cancers.

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The prognostic importance regarding intratumoural subsite, T-cell subsets and infiltration rate has been investigated in earlier studies (Naito et al., 1998; Galon et al., 2006), but questions still remain as to which variables bear the strongest prognostic information (Ogino et al., 2011; Fridman et al., 2012). The introduction of immune scoring into clinical practice of CRC classification is under progress, work initiated by Galon et al. (2012), striving to find a standardised methodology. In the light of this it is of utmost importance to find the most informative prognostic factor/factors regarding immune cell infiltration in CRC.

Cytotoxic T lymphocytes (CTLs; CD8+), are established as important players in anti-tumour immunity in CRC (Naito et al., 1998; Deschoolmeester et al., 2011). Cytotoxic T lymphocytes have the ability to kill target cells upon being exposed to a tumour cell antigen/HLA1 complex for which their T-cell receptor is specific (Deschoolmeester et al., 2011). Regulatory T lymphocytes (Tregs; FOXP3+) can suppress the activity of CTLs and have therefore been thought to down-modulate tumour-specific immunity. In some studies, Tregs have been shown to correlate to reduced activation of conventional T cells (Svensson et al., 2012), but the data are conflicting. In line with their suggested suppressive functions, Tregs have been associated with adverse outcomes in ovarian, breast and hepatocellular carcinomas but were not found to be significantly prognostic in others, like oesophageal carcinomas (Ladoire et al., 2011a; Deschoolmeester et al., 2011). A number of studies have even found FOXP3+ T lymphocytes to be associated with a favourable prognosis in some cancers, notably CRC (Ladoire et al., 2011b).

Host cells and cancer cells interact constantly with each other in the tumour microenvironment so to understand the immunologic milieu of the cancer, both host and tumour factors need to be considered (Ogino et al., 2011). Three major pathways of CRC tumorigenesis have been well described (Jass, 2007), the microsatellite stable (MSS), the microsatellite instability (MSI) (Boland et al., 1998; Pino and Chung, 2010) and, more recently, the CpG island methylator phenotype (CIMP) pathways. Immune infiltration has been shown to vary according to MSI status, with MSI tumours more often being highly infiltrated by immune cells, which might explain the better prognosis in these patients (Popat et al., 2005; Deschoolmeester et al., 2011). However, most studies on the prognostic impact of immune infiltrates have generally not accounted for the molecular status of the tumour.

We have previously studied the relation between lymphocytic infiltration (CD3+ and molecular characteristics (i.e., MSI screening status and CIMP status) in a cohort of CRC patients (Dahlin et al., 2011). In the present study, tumour progression in this CRC cohort is related to, not only quantity and the relationship between subsets of T lymphocytes (CD8+ and FOXP3+), but also to their subsite within the tumour, to find out where the most important prognostic information of these lymphocyte markers can be found and how they best could be implemented in a future clinical setting. In addition, we compare the prognostic importance of CD8+ and FOXP3+ T lymphocytes in CRC of different MSI screening and CIMP status.

**MATERIALS AND METHODS**

**Study population.** The tumour tissue used in this study comes from The Colorectal Cancer in Umeå study (CRUMS) (Dahlin et al., 2010). Clinical specimens from patients surgically resected for CRC were collected between 1995 and 2003 at the department of Surgery, Umeå University Hospital, Umeå, Sweden. An informed consent was retrieved from the patients and the handling of patient data as well as tissue samples was approved by the research ethical committee at Umeå University Hospital (Regional Ethical Review Board in Umeå, Sweden). Formalin-fixed paraffin-embedded tissue was sampled from all patients and pathological variables were characterised by one pathologist by reviewing routinely stained sections. By reviewing the patient records and the Swedish population registry during autumn 2012, clinical data, including survival data, were obtained by a surgeon. The median follow-up time for patients still alive at the end of follow-up was 113 months. Exclusion criteria included unavailable or insufficient tumour sample, or lack of clinical information. A total of 426 patients (268 colon cancers, 155 rectal cancers, and 3 not specified site within the colorectum) were included in the study. Because of missing data of either CD8 or FOXP3 expression, 409 specimens for CD8 and 412 specimens for FOXP3 were available for analysis. Preoperative radiation therapy was administered to 90 (58%) rectal cancer patients of whom 70 received 5 × 5 Gy, and 20 received 25 × 2 Gy. For survival analyses, 31 patients were excluded due to incomplete follow-up data or due to death by perioperative complications (death within 30 days of and due to operation).

**Immunohistochemistry.** According to routine procedures, CRC specimens obtained after primary tumour resection were fixed in 4% formaldehyde and embedded in paraffin. From each patient, one 4-μm section was cut, dried, de-waxed and rehydrated. For immunohistochemical procedures, a staining machine (Ventana BenchMark Ultra, Ventana Medical Systems, Inc., Tucson, AZ, USA) was used with the CC1 standard pretreatment and the iVIEW DAB detection kit (Ventana Medical Systems, Inc.) for visualization. Anti-CD8 polyclonal antibody (Clone C8/144B: Dako, Stockholm, Sweden) was used at a dilution of 1:50, and anti-FOXP3 monoclonal antibody (Clone 236A/E7: AbCam, Cambridge, UK) was used at a dilution of 1:100. The slides were counterstained with haematoxylin.

Immunohistochemical staining was evaluated under light microscope as most representative area at different subsites: the invasive tumour front, tumour centre and within the tumour epithelium (intraepithelial expression), as illustrated in Figure 1. In more detail, tumour front was identified as the stromal area along the invasive margin defined by a depth of two high-power fields (×40 objective magnification) underneath the invasive margin. Tumour centre was identified as the stromal area within the
Infiltration of CD8<sup>+</sup> and FOXP3<sup>+</sup> cells at the invasive tumour front, tumour centre and within the tumour epithelium of CRC.

The infiltration of CD8<sup>+</sup> and FOXP3<sup>+</sup> cells was assessed using immunohistochemical evaluation of CD8 and FOXP3 expression, respectively. CD8<sup>+</sup> and FOXP3<sup>+</sup> cells were semi-quantitatively evaluated in specimens from 426 CRC patients according to a previously documented four-graded scale (Dahlin et al., 2011). The T-lymphocyte markers were assessed at three different subsites: invasive tumour front, tumour centre and within the tumour epithelium (intraepithelial expression) (Figure 1). Approximately 85% of all tumours displayed a modest to massive infiltration of CD8<sup>+</sup> and FOXP3<sup>+</sup> cells (total score 5–12), whereas the remaining showed weak or no infiltration (total score 3–4). The majority of CD8<sup>+</sup> and FOXP3<sup>+</sup> cells were found at tumour stromal sites (invasive tumour front and tumour centre), with the highest density along the invasive tumour front. CD8 expression was detected intraepithelially in 100% of the cases. FOXP3 expression was not, or just sporadically, present within the tumour epithelium.

The frequencies of infiltrating CD8<sup>+</sup> and FOXP3<sup>+</sup> cells using total score as a representative of all subsites, are presented in a cross-tabulation in Table 1A. Infiltrating cells expressing CD8<sup>+</sup> or FOXP3 were highly and positively correlated (P<0.001). The amount of CD8<sup>+</sup> cells, however, was frequently higher than that of FOXP3<sup>+</sup> cells.

**Results**

**Table 1A. Cross-tabulation between total score for CD8 and FOXP3 expression in CRC**

| CD8     | 3–4 | 5–6 | 7–12 | P-value |
|---------|-----|-----|------|---------|
| 3–4     | 17  | 28  | 5.0  | <0.001* |
| 5–6     | 25  | 69  | 90   | <0.001* |
| 7–12    | 27  | 27  | 90   | <0.001* |

*Exact linear-by-linear association test.

**Associations between infiltrating CD8<sup>+</sup> and FOXP3<sup>+</sup> cells and clinicopathological parameters.** The scores of infiltrating CD8<sup>+</sup> and FOXP3<sup>+</sup> cells in CRC specimens were correlated to clinicopathological variables (Table 1B). As no great difference could be seen when relating these parameters to lymphocyte infiltration at different sites compared with infiltration as a total, we chose to present the results for total score (clinicopathological characteristics in relation to CD8 intraepithelial expression and FOXP3 expression at the tumour front can be found in Supplementary Information Table S1). CD8 expression was significantly associated with age (P<0.001), localisation (P<0.001) and growth pattern (P<0.001). CD8 expression further showed a strong association with preoperative radiation therapy (P<0.001), where radiated tumours more often were less infiltrated. An inverse association with tumour stage was found for both CD8<sup>+</sup> (P<0.001) and FOXP3<sup>+</sup> (P<0.001) infiltration.

**Prognostic importance of infiltrating CD8<sup>+</sup> and FOXP3<sup>+</sup> cells in colon cancer.** We compared overall cancer-specific survival in patients with different scores of infiltrating CD8<sup>+</sup> or FOXP3<sup>+</sup> cells. As several of the patients in the rectal cancer group had received preoperative radiation therapy, which affects the infiltration rate significantly for CD8<sup>+</sup> cells (Table 1B), we separated this group from the colon cancer patients. Figure 2 shows Kaplan–Meier plots of cancer-specific survival in colon cancer patients with different levels of infiltrating CD8<sup>+</sup> and FOXP3<sup>+</sup> cells. An increased infiltration of CD8<sup>+</sup> cells (total score) was highly significantly associated with an improved prognosis (Log-rank P=0.008) (Figure 2A). A similar, but even stronger association was seen for FOXP3<sup>+</sup> cells (Log-rank P<0.001) (Figure 2A). When analysed as continuous variables in a multivariable model adjusting for stage, age, sex and localisation (proximal vs distal), the prognostic significance of CD8 total score was lost (HR 0.82; 95% CI 0.60–1.13; P=0.220), whereas for FOXP3 total score remained significant (HR 0.68; 95% CI 0.51–0.90; P=0.008).

When comparing infiltration rate and overall cancer-specific survival according to intratumoural subsite, the most prominent association for CD8<sup>+</sup> cells was seen for a high intraepithelial infiltration (Log-rank P<0.001) (Figure 2B). CD8 infiltration at the invasive tumour front or tumour centre showed weaker associations to prognosis (Log-rank P=0.015 and 0.021, respectively). When analysed as a continuous variable in the multivariable model, the risk estimate decreased significantly with
increasing intraepithelial infiltration of CD8⁺ cells (HR 0.71; 95% CI 0.55–0.92; P = 0.010). Significance for a high CD8 infiltration at the tumour invasive front and centre was lost in multivariate models. This further supports the importance of intratumoural subsite for evaluation of the prognostic impact of CD8⁺ infiltration. Such discrepancy between sites could not be seen, increasing intraepithelial infiltration of CD8⁺ cells in the front to that in the centre. An increased infiltration of FOXP3⁺ cells in both front and centre was highly and significantly associated with an improved prognosis (Log-rank \( P < 0.001 \)) (Figure 2B). The prognostic importance of infiltrating FOXP3⁺ cells, according to intratumoural subsite retained significant in the multivariate analysis (FOXP3 at the tumour front; HR 0.73; 95% CI 0.55–0.96; \( P = 0.022 \); and tumour centre; HR 0.62; 95% CI 0.49–0.80; \( P < 0.001 \)). This suggests that FOXP3 is a stronger indicator of prognosis than CD8 at these subsites.

**Prognostic impact of CD8⁺ cells in relation to FOXP3⁺ cells in colon cancer.** In order to find out whether the proportion of infiltrating CTLs in relation to that of the Tregs had any impact on prognosis in our colon cases, we compared overall cancer-specific survival in patients with different CD8 to FOXP3 density ratios. We related immune cell density to prognosis both according to total score and intratumoural subsite. We restricted the analyses to comparison of CD8 intraepithelial expression (due to its most pronounced prognostic effect). FOXP3 infiltration showed a significant correlation to prognosis both in the centre and the invasive front, but we chose to henceforth look at the front as regulatory T cells are generally distantly acting. When comparing total score, there was no association between the amounts of CD8⁺ and FOXP3⁺ cells and survival (Log-rank \( P = 0.707 \)) (Figure 3A). Interestingly, when we instead compared intratumoural subsites, we saw that a higher intraepithelial CD8 to FOXP3 front ratio had the better prognostic association (Log rank \( P = 0.039 \)) (Figure 3B), which further strengthens the importance of intratumoural subsite assessment. We also compared the prognostic value of different groups of CD8 or FOXP3 low (scores 1 and 2) or high (scores 3 and 4) expression according to subsites. The best prognosis was found in patients with a high density of intraepithelial CD8⁺ cells, irrespective of FOXP3 infiltration. FOXP3 density at the tumour invasive front was on the other hand of prognostic significance in cases with a low number of intraepithelial CD8⁺ T cells (Log-rank \( P = 0.017 \)) (Figure 3C), suggesting that FOXP3 by itself holds important prognostic information. The poorest prognosis was
found in cases with a low infiltration rate of both CD8\(^+\) and FOXP3\(^+\) cells.

**Prognostic importance of infiltrating CD8\(^+\) and FOXP3\(^+\) cells in rectal cancer.** No significant association was seen between infiltrating CD8\(^+\) or FOXP3\(^+\) cells (total score) and overall survival, in rectal cancer patients (Log-rank \(P=0.338\) and 0.106, respectively) (Supplementary Information Figure S1a). A significant correlation of CD8 and FOXP3 expression to prognosis was however seen when comparing sites. A high intraepithelial infiltration rate of CD8\(^+\) cells was associated with an improved prognosis (Log-rank \(P=0.034\)) as was a high infiltration rate of FOXP3\(^+\) cells at the tumour front (Log-rank \(P=0.008\)) (Supplementary Information Figure S1b).

**Association between infiltrating CD8\(^+\) and FOXP3\(^+\) cells and molecular parameters.** When relating infiltrating CD8\(^+\) or FOXP3\(^+\) cells to molecular parameters (Table 2), CD8 expression presented as total score was not found to be significantly associated with MSI screening status (Log-rank \(P=0.128\)). However, significances were found at the different subsites, with the highest significance found for MSI tumours more often being highly infiltrated by intraepithelial CD8\(^+\) T lymphocytes (Log-rank \(P<0.001\)). FOXP3 expression (total score) was significantly associated with MSI screening status (Log-rank \(P=0.035\)), whereas no association was found at the different subsites. Furthermore, no correlation of CD8\(^+\) or FOXP3\(^+\) infiltration was found to CIMP status.

**Prognostic importance of infiltrating CD8\(^+\) and FoxP3\(^+\) cells according to MSI screening status and CIMP status.** To further analyse the prognostic value of infiltrating T-lymphocyte subsets, we compared overall cancer-specific survival within different subgroups of colon cancer defined by MSI screening status and CIMP status. We restricted the analyses to comparison of CD8 intraepithelial expression and FOXP3 expression at the tumour invasive front.

CD8\(^+\) intraepithelial infiltration was not found to be a prognostic factor in MSI cases (Log-rank \(P=0.832\)), however, it was so in MSS cases (Log-rank \(P<0.001\)) (Figure 4A and B). FOXP3\(^+\) infiltration at the tumour front was significant for prognosis in subgroups of both MSI (Log-rank \(P<0.001\)) and MSS (Log-rank \(P<0.001\)) (Figure 4A and B), where MSI or MSS cases with low infiltration of FOXP3\(^+\) cells displayed the worst prognosis.

A high amount of CD8\(^+\) and FOXP3\(^+\) cells was found to be associated with a better prognosis in subgroups of colon cancer arranged by CIMP status, particularly in groups of CIMP-negative and CIMP-low cases (Supplementary Information Figure S2).

In a multivariate model adjusting for MSI and CIMP status, the prognostic importance of a high infiltration rate of intraepithelial CD8\(^+\) cells and FOXP3\(^+\) cells at the tumour invasive front respectively, remained significant and hazard ratio was mainly unaffected. This suggests that T-cell infiltration is independent of these molecular characteristics.

**DISCUSSION**

Tumours of CRC are generally immunogenic and often infiltrated by T lymphocytes (Sherwood et al, 2013). An increased amount of T lymphocytes infiltrating the tumour in CRC has repeatedly been proven to be associated with a better prognosis (Galon et al, 2006; Dahlin et al, 2011; Roxburgh and McMillan, 2012). Additional information regarding different intratumoural subsites, T-lymphocyte subsets and how infiltration correlates to prognosis in different...
In this study, we analyse the T-cell subsets likely to bear the most important effector functions in tumour control, that is, the cytotoxic T cells and their counterpart, the regulatory T cells. We combine analyses of density, relation, different intratumoural subtypes and tumour molecular characteristics to try to find out how these prognostic tools best could be implemented in the clinical setting.

By using an immunohistochemical approach we evaluated the degree of infiltrating CD8+ and FOXP3+ cell subsets in 426 archival tumour tissue samples from patients surgically resected for CRC. Colon carcinomas were separated from rectal cancers due to the reducing effect that radiation therapy given to many patients in the latter group, has on T-cell infiltration. In univariate analyses we observed significant associations of a high amount of infiltrating CD8+ cells with improved survival in patients with colon cancer. In multivariate analysis, adjusting for stage, age, sex and localisation (proximal vs distal), the prognostic effect of intraepithelial infiltration of CD8 cells remained significant whereas the significance for CD8 total score, tumour invasive front and centre was lost.

No prognostic discrepancy between intratumoural subtypes could be seen when comparing infiltration of FOXP3+ cells in the tumour invasive front to that in the centre, a high infiltration rate in both subsites being significantly associated with a better prognosis. The prognostic effect of FOXP3+ cell infiltration for total score, at the tumour invasive front and in the centre remained significant in multivariate analysis, suggesting that at these intratumoural subtypes, evaluation of FOXP3 is the better prognostic indicator. When comparing a possible effect of different relations between CD8+ and FOXP3+ cells on prognosis, we found that having a high infiltration rate of intraepithelial CD8+ cells was associated with a good overall survival, regardless of FOXP3+ density at the tumour front. However, in colon cancers with low infiltration of intraepithelial CD8+ cells, a better overall survival was found in patients with a high infiltration of FOXP3+ cells compared with those having a low infiltration rate. These results are in line with a study by Yoon et al (2012) who also observed FOXP3+ cells to be a prognostic indicator in patients with a low infiltration of CD8 positive lymphocytes, in CRC.

The biological explanation to why intraepithelial CTLs would have a greater impact on prognosis than the ones located in the front or centre is likely due to their direct anti-tumour effects. The CTLs infiltrating the tumour epithelium, being in the immediate proximity, are most likely specifically directed against the tumour, and could therefore be considered more effective. Earlier work by Pages et al (2005) has shown a correlation between high tumour immune cell infiltration and absence of perineural invasion and lymphatic emboli. The hypothesis that the sublocalisation of CTLs bears prognostic importance has gained momentum from studies by, for example, Naito et al (1998) and Galon et al (2006). An intra-epithelial location of CTLs could possibly mean a more effective protection in that aspect. Tregs have been known to counteract the anti-tumour immune response in some cancers (Deschoolmeester et al, 2011; Mathai et al., 2012), but its role cannot be assessed as unambiguous as we still cannot see the whole picture. In this study, a high infiltration of FOXP3+ cells seems to be a protective factor, associated with a very good patient outcome, whereas the reverse can be said for patients with a very low infiltration, results that are consistent with the works of Salama et al (2009), Ladoire et al (2011b) and Frey et al (2010). This raises the question of whether there is something that singles out the FOXP3+ cells in the colon from their non-colon counterparts. In a study by Martin et al (2010), it was shown that some tumour-infiltrating FOXP3+ cells are not Tregs but conventional T cells transiently expressing FOXP3 upon TCR activation. In another study, by Miyara et al (2009), they identified subgroups of FOXP3+ cells secreting pro-inflammatory cytokines IL-2 and IFN-γ.
Subsite and relation of CD8 and FOXP3 in CRC

Table 2. CD8 and FOXP3 total score and expression at intratumoural subsites in relation to molecular characteristics in colon cancers

| Total score | CD8 | FOXP3 | P-value | CD8 | FOXP3 | P-value |
|-------------|-----|-------|---------|-----|-------|---------|
|             | 3–4 | 5–6   | 7–12    | 3–4 | 5–6   | 7–12    |
| MSI         |     |       |         |     |       |         |
| MSI         | 3 (6.1) | 10 (20.4) | 36 (73.5) | 14 (26.9) | 18 (34.6) | 20 (38.5) |
| MSS         | 19 (9.7) | 64 (32.7) | 113 (57.7) | 37 (18.8) | 108 (54.8) | 52 (26.4) |
| CIMP status |     |       |         |     |       |         |
| CIMP-negative | 10 (9.7) | 30 (29.1) | 63 (61.2) | 19 (18.4) | 54 (52.4) | 30 (29.1) |
| CIMP-low     | 9 (8.7) | 32 (30.8) | 63 (60.6) | 24 (22.9) | 51 (48.6) | 30 (28.6) |
| CIMP-high    | 3 (6.8) | 14 (31.8) | 27 (61.4) | 10 (21.3) | 22 (46.8) | 15 (31.9) |
| Front        |     |       |         |     |       |         |
| MSI         | 1 (2.0) | 5 (10.2) | 21 (42.9) | 9 (17.3) | 21 (40.4) | 16 (30.8) |
| MSS         | 13 (6.6) | 57 (29.1) | 79 (40.3) | 47 (24.0) | 102 (51.8) | 57 (28.9) |
| CIMP status |     |       |         |     |       |         |
| CIMP-negative | 9 (8.7) | 26 (25.2) | 39 (37.9) | 29 (28.2) | 55 (53.4) | 29 (28.2) |
| CIMP-low     | 4 (3.8) | 27 (26.0) | 48 (46.2) | 25 (24.0) | 48 (45.7) | 33 (31.4) |
| CIMP-high    | 1 (2.3) | 10 (22.7) | 15 (34.1) | 18 (40.9) | 22 (46.8) | 14 (29.8) |
| Centre       |     |       |         |     |       |         |
| MSI         | 3 (6.0) | 14 (28.0) | 21 (42.0) | 12 (24.0) | 13 (24.5) | 19 (35.8) |
| MSS         | 16 (8.0) | 82 (40.8) | 85 (42.3) | 18 (9.0) | 27 (13.4) | 98 (48.8) |
| CIMP status |     |       |         |     |       |         |
| CIMP-negative | 6 (5.6) | 42 (39.3) | 50 (46.7) | 9 (8.4) | 13 (12.4) | 53 (50.5) |
| CIMP-low     | 10 (9.5) | 38 (36.2) | 44 (41.9) | 13 (12.4) | 20 (18.5) | 45 (41.7) |
| CIMP-high    | 3 (6.7) | 19 (42.2) | 14 (31.1) | 9 (20.0) | 9 (19.1) | 20 (42.6) |
| Intraepithelial |     |       |         |     |       |         |
| MSI         | 14 (28.0) | 11 (22.0) | 14 (28.0) | 11 (22.0) | 17 (32.1) | 4 (7.5) |
| MSS         | 93 (46.3) | 59 (29.4) | 39 (19.4) | 10 (5.0) | 64 (31.8) | 12 (6.0) |
| CIMP status |     |       |         |     |       |         |
| CIMP-negative | 44 (41.1) | 32 (29.9) | 24 (22.4) | 7 (6.5) | 98 (8.6) | 9 (15.6) |
| CIMP-low     | 48 (45.7) | 29 (27.6) | 20 (19.0) | 8 (7.6) | 7 (8.6) | 9 (15.6) |
| CIMP-high    | 12 (37.8) | 10 (22.2) | 11 (24.4) | 7 (15.6) | 7 (8.6) | 9 (15.6) |

Abbreviations: CIMP = CpG island methylator phenotype; MSI = microsatellite unstable; MSS = microsatellite stable.

Unless otherwise indicated, χ² test was used for categorical variables.

aAbbreviates cases lacking nuclear staining of tumour cells for at least one of MLH1, MSH2, MSH6 or PMS2 were considered to have a positive MSI screening status.

bPhenotype determined according to hypermethylation of an eight-gene panel with the following number of hypermethylated genes found for CIMP-negative, 0 genes; CIMP-low, 1–5 genes; CIMP-high, 6–8 genes.

Other studies though have reached the conclusion that CRC-derived FOXP3^+ cells, do mediate immune suppression (Kryczek et al, 2009; Sherwood et al, 2013). Possibly the answer partly lies in the organ specific differences, attributed to the intestinal environment. The fact that the colon is a barrier organ, constantly confronted with foreign antigens, could make it an exception to the rule, with regulatory T cells blocking tumour promoting inflammation (Ladoire et al, 2011b). In gastric cancer, a high amount of Tregs proved to be associated with a better prognosis, leading Haas et al (2009) to suggest this potentially protective role. Gastrointestinal bacteria can trigger cascades of pro-inflammatory cytokines, with tumour promoting effects (Whiteside, 2012). Having a high amount of regulatory T cells, repressing microbe induced inflammation, could mean a protection not only by hindering the development of cancer in the colorectal epithelium, but also by preventing tumour growth. Bacteria and bacterial products could also potentially aid to sustain the activity of the tumour immune response. As there seem to be a strong correlation between the different subsets, regarding infiltration rate, a high amount of FOXP3^+ cells can be an indication for an active and potent immune response, thereby explaining the association to better prognosis.

In this study, we can see that a high infiltration rate of FOXP3^+ cells is associated with a high infiltration of CD8^+ cells. Both subsets express ligands enabling T-cell extravasation and tumour infiltration (Ohmichi et al, 2011; West et al, 2013), possibly potentiating immune cell infiltration synergistically.

With some exceptions, many other studies on the prognostic impact on immune infiltrates have generally not accounted for the molecular status of the tumour. In this study, lymphocyte infiltration was evaluated in molecular subgroups of CRC defined by MSI screening and CIMP status. We found that even though MSI tumours are more highly infiltrated, the prognostic importance of lymphocye infiltration is likely independent of these molecular characteristics.

Due to the convincing prognostic importance of immune cell infiltration, Galon et al (2012) have taken on the urgent task to implement an immuno-score as a component of cancer
classification. The method advocated is a quantitative automated immunohistochemistry score that grades CD3+ and CD8+ infiltration at the invasive margin and tumour centre. By this Galon and colleagues wish to pursue assay uniformity and in the future a globally accepted rapid, inexpensive and powerful scoring method of immune cell infiltration. In this study, we have instead analysed the tumour sections semi-quantitatively, which has the disadvantage of observer variability. On the other hand, considering the heterogenic density of T cells within a single section, our method has the benefit of more accurately identifying tumour compartments and excluding necrotic areas (Richards et al. 2014). Furthermore, our method enables a more comprehensive view of the prognostic importance of immune cells at different intratumoural subsites, which might add important information as to how different markers are best implemented in a standardised immunoscore used in clinic.

In conclusion, analysis of the intratumoural subsite of infiltrating T cells is valuable for predicting prognosis in CRC. Here we found that evaluation of CD8 expression within the tumour epithelium of colon cancers bears the most important prognostic information. At other intratumoural subsites, such as tumour front adds additional prognostic information. These results may be valuable when defining a prognostic immunoscore in the clinical setting.

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