Immunological nomograms predicting prognosis and guiding adjuvant chemotherapy in stage II colorectal cancer

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Background: The type, abundance, and location of tumor-infiltrating lymphocytes (TILs) have been associated with prognosis in colorectal cancer (CRC). This study was conducted to assess the prognostic role of TILs and develop a nomogram for accurate prognostication of stage II CRC.

Methods: Immunohistochemistry was conducted to assess the densities of intraepithelial and stromal CD3+, CD8+, CD45RO+, and FOXP3+ TILs, and to estimate PD-L1 expression in tumor cells for 168 patients with stage II CRC. The prognostic roles of these features were evaluated using COX regression model, and nomograms were established to stratify patients into low- and high-risk groups and compare the benefit from adjuvant chemotherapy.

Results: In univariate analysis, patients with high intraepithelial or stromal CD3+, CD8+, CD45RO+ and FOXP3+ TILs were associated significantly with better relapse-free survival (RFS) and overall survival (OS), except for stromal CD45RO+ TILs. In multivariate analysis, patients with high intraepithelial CD3+ and stromal FOXP3+ TILs were associated with better RFS (p<0.001 and p=0.032, respectively), while only stromal FOXP3+ TILs was an independent prognostic factor for OS (p=0.031). The nomograms were well calibrated and showed a c-index of 0.751 and 0.757 for RFS and OS, respectively. After stratifying into low- and high-risk groups, the high-risk group exhibited a better OS from adjuvant chemotherapy (3-year OS of 81.9% vs 34.3%, p=0.006).

Conclusion: These results may help improve the prognostication of stage II CRC and identify a high-risk subset of patients who appeared to benefit from adjuvant chemotherapy.

Keywords: CD3, CD8, FOXP3, stage II, adjuvant chemotherapy

Introduction

5-fluorouracil-based adjuvant chemotherapy has been well established for patients with stage III colorectal cancer (CRC), but in stage II CRC, adjuvant chemotherapy is still hotly disputed considering the cost, toxicity, and limited survival benefit.1–4 A number of clinicopathological features (poor histological differentiation, T4 stage, <12 nodes harvested, high preoperative carcinoembryonic antigen (CEA) level, intestinal obstruction or perforation, and the presence of lymphovascular or perineural invasion) have been identified assisting the decision for adjuvant chemotherapy in stage II disease.1,5,6 However, only T4 stage has been proven to help identify a specific subset of stage II CRC patients who could achieve survival benefit from adjuvant chemotherapy.7 Besides, some polygene signatures have been widely explored,8,9 but there is still a long way to put these results into clinical
practice. Identifying novel biomarkers to filter out the high-risk group of stage II CRC which could benefit from adjuvant chemotherapy is badly needed.

Adaptive immune response has been proven to influence the biological behavior of tumor cells, and the immune microenvironment formed by the type, abundance, and location of immune cells within tumor tissues were found to be a better predictor of patient survival than traditional clinicopathological features. First demonstrated that the infiltration of tumor nests by CD8+ T-cells was a novel prognostic factor contributing to a better survival in CRC. Thereafter, CD3+ tumor-infiltrating lymphocytes (TILs) have been identified to be associated with favorable prognosis and a lower risk of metastatic metastasis in CRC. CD45RO+ TILs have also been reported to have prognostic significance. revealed that high levels of CD45RO+ TILs were correlated with the absence of signs of early metastatic invasion, a less advanced pathological stage, and increased survival. In early-stage CRC, patients with a strong infiltration of CD45RO+ T-cells exhibited an increased expression of T-helper 1 and cytotoxicity-related genes and helped predict tumor recurrence and survival. Nuclear transcription factor FOXP3, as a key regulatory gene for the development of regulatory T-cells, has been proven to be associated with immunological self-tolerance by actively suppressing self-reactive lymphocytes. Nuclear transcription factor FOXP3, as a key regulatory gene for the development of regulatory T-cells, has been proven to be associated with improved survival in CRC. Therapeutic antibodies targeting the programmed cell death 1 protein (PD-1) and the programmed death-ligand 1 protein (PD-L1) have been proven to be effective in a number of cancer types. Revealed higher expressions of PD-1 and PD-L1 correlated with better prognosis of CRC patients. The objective of the current study was to assess and compare the prognostic role of PD-L1 and different types of TILs in stage II CRC and construct a nomogram for better prognostication, and to identify the subgroup of stage II CRC patients who can actually benefit from chemotherapy.

Methods

Study group

We 1:1 matched 84 recurrent stage II CRC patients to patients without recurrence, rendering 168 patients for analysis in our study. CRC tissue blocks were sent for next-generation sequencing (NGS) at Burning Rock Dx Corporation, Shanghai. No patients received preoperative therapy before radical surgery. Patients did not tolerate adequate course of adjuvant chemotherapy was excluded. All patients were regularly followed-up with a median follow-up time at 54.4 months (range 11.3–95.8 months). Informed consent had been obtained and this study was approved by the institutional review board of the Fudan University Shanghai Cancer Center.

Immunohistochemistry (IHC)

Immunohistochemically staining was performed according to standard protocol. Briefly, paraffin-embedded samples were cut into 4 μm sections and placed on polylysine-coated slides. Paraffin sections were baked overnight at 58°C, dewaxed in xylene, rehydrated through a graded series of ethanol, quenched for endogenous peroxidase activity in 0.3% hydrogen peroxide for 15 mins. Antigen retrieval was performed by high-pressure cooking in citrate buffer (pH=6.0) for about 20 mins, then allowed to cool to room temperature, blocking the nonspecific antibody binding sites in 5% normal goat serum for 2 hrs. Sections were incubated at 37°C for 1.5 hrs with rabbit polyclonal antibody against CD3 (1:400, Abcam, ab16669, USA), CD8 (1:400, Cell Signaling Technology, 70306S, USA), CD45RO (1:400, Dako, DK-2600 Glostrup, Denmark), FOXP3 (1:400, Abcam, ab20034, USA), and PD-L1 (1:100, Abcam, ab205921), in a moist chamber. Biotinylated secondary antibody was performed using the EnVision+System-HRP (AEC) (K4005, Dako, Glostrup, Denmark). Subsequently, sections were counterstained with hematoxylin (Sigma-Aldrich, St Louis, MO, USA). TMA slides were scanned by an automated scanning microscope and counted by Image-Pro Plus software (IPP; produced by Media Cybernetics Corporation, USA). Epithelial and stromal areas were calculated separately. Five independent visual fields (at ×400 magnification), representing the most abundant lymphocytic infiltrates, were selected for each patient sample, and we used the mean density to stratify variables into dichotomous data for statistical analysis. PD-L1 expression score was the sum of the cytoplasmic and membrane scores. Cytoplasmic expression level was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (strong), and membrane expression level was scored as 0 (absent) or 1 (present). PD-L1 scores 2/3/4 were counted as high, scores 0/1 as low.

Statistical analysis

We used chi-square tests or Fisher’s exact test to compare immunological biomarkers expression levels. Univariate and
multivariate analyses were conducted using the Cox regression model. Nomograms were established by R software and the model performance for predicting outcome was evaluated by Harrell’s concordance index (c-index). X-tile 3.6.1 software23 (Yale University, New Haven, CT, USA) was used to determine the optimal cutoff values, stratifying the patients into low- and high-risk groups. Kaplan–Meier curves were drawn and log-rank tests were used to compare the survival data between different groups. p-values were accepted at <0.05 and all analyses were performed with the R 2.15.3 software.

Results

Immunohistochemical characteristics

Epithelial and in stromal TILs were evaluated separately. Utilizing tissue microarray (TMA), we quantified CD3+, CD8+, CD45RO+, and FOXP3+ cells by automatic imaging analysis on 168 stage II CRC samples. Representative immunohistochemical findings are demonstrated in Figure 1. Densities of each T-cell subset (cells/mm²) were distributed as follows: intraepithelial CD3+ (mean 84; range 0–352), stromal CD3+ (mean 376; range 0–1380), intraepithelial CD8+ (mean 60; range 0–344), stromal CD8+ (mean 220; range 0–1120), intraepithelial CD45RO+ (mean 76; range 0–384), stromal CD45RO+ (mean 344; range 0–1600), intraepithelial FOXP3+ (mean 16; range 0–132), and stromal FOXP3+ (mean 132; range 0–600). Seventy-two patients were identified as PD-L1 low, and 96 patients were identified as PD-L1 high.

Correlation of immune biomarkers with clinicopathological and molecular features

Molecular features were available in 129 patients who successfully underwent NGS. As shown in Table 1, patients with high intraepithelial CD3+, CD45RO+, and stromal FOXP3+ TILs had a significantly higher incidence of normal preoperative CEA (p=0.010, 0.013, and 0.017, respectively). Patients with high intraepithelial FOXP3+ TILs underwent less adjuvant chemotherapy (p=0.019). More colon disease was observed in patients with high intraepithelial CD8+ TILs. Patients with high intraepithelial CD45RO+ and stromal CD8+ TILs had a significantly lower incidence of neural invasion (p=0.043 and 0.046, respectively). More T4 tumors were found in patients with high intraepithelial CD8+ TILs (p=0.025). Patients with high intraepithelial CD45RO+ TILs had a significantly higher incidence of adequate lymph nodes harvested (p=0.005). Patients with high intraepithelial CD8+ and CD45RO+ TILs had a significantly higher incidence of MSI-high (p=0.017 and 0.002, respectively). More ERBB2 mutation were observed in patients with high intraepithelial CD45RO+, FOXP3+, and stromal CD45RO+ TILs (p=0.019, 0.020, and 0.012, respectively). More TP53 mutation were found in patients with high intraepithelial CD8+ and CD45RO+ TILs (p=0.034 and 0.025, respectively). No significant differences were observed for gender, age, histology type, grade, vascular invasion, APC mutation, BRAF mutation, KRAS mutation, NRAS mutation, POLE mutation, PIK3CA mutation, and PTEN mutation.

Prognostic factors

In univariate analysis (Table 2), for tumor features, CEA was significantly associated with better relapse-free survival (RFS) and overall survival (OS) (p<0.001 and p=0.015, respectively). Number of lymph nodes harvested (LNH) were significantly associated with better OS (p=0.012). Grade reached marginal significance for both RFS and OS (p=0.055 and p=0.068, respectively). For molecular features, BRAF and PTEN mutation were found to be significantly associated with better OS (p=0.007 and p=0.034, respectively), whereas BRAF mutation only reached marginal significance for RFS (p=0.081). For Immune biomarkers, high intraepithelial or stromal CD3+, CD8+, CD45RO+, FOXP3+ TILs were significantly associated with better RFS and OS (all p<0.05), except for high stromal CD45RO+ TILs (p=0.110). PD-L1 was not associated with RFS or OS (p=0.574 and p=0.820, respectively). A multivariate model was developed to test independent prognostic factors for RFS and OS (Table 3). In the first model (Model A, n=168), only tumor features and immune biomarkers with a p<0.100 in univariate analysis were included. CEA (p=0.040; RR, 1.591; 95% CI, 1.022–2.495), intraepithelial CD3+ TILs (p=0.001; RR, 0.192; 95% CI, 0.094–0.395), and stromal FOXP3+ TILs (p=0.032; RR, 0.526; 95% CI, 0.292–0.974) were found to be the strongest prognostic factors for RFS, whereas LNH (p=0.010; RR, 0.374; 95% CI, 0.178–0.784) and stromal FOXP3+ TILs (p=0.031; RR, 0.249; 95% CI, 0.071–0.878) were proven to be independent prognostic factors for OS. The second model added molecular features (Model B, n=129) for analysis, intraepithelial CD3+ (p=0.001; RR, 0.179; 95% CI, 0.082–0.391) and stromal FOXP3+ TILs (p=0.015;
RR, 0.425; 95% CI, 0.214–0.845) retained significance for RFS. While for OS, stromal FOXP3+ TILs (p=0.016; RR, 0.155; 95% CI, 0.034–0.703), LNH (p=0.038; RR, 0.436; 95% CI, 0.199–0.956), and PTEN mutation (p=0.001; RR, 6.526; 95% CI, 2.149–19.815) were the strongest prognostic factors.

Figure 1 Representative examples of immunohistochemical findings for CD3, CD8, CD45RO, FOXP3, and PD-L1 (original magnification, ×400). (A,B) Positive for intraepithelial and stromal CD3; (C,D) positive for intraepithelial and stromal CD8; (E,F) positive for intraepithelial and stromal CD45RO; (G,H) positive for intraepithelial and stromal FOXP3; (I,J) positive for cytoplasmic and membranous PD-L1.
| Variables         | Subgroup | No. of patients | CD3e | CD8e | CD45ROe | FOXP3e | PD-L1 |
|-------------------|----------|----------------|------|------|---------|--------|-------|
|                   |          |                | L    | H    | p       | L      | H    |
|                   |          |                | L    | H    | p       | L      | H    | p       | L    | H    | p       | L    | H    | p       | L    | H    | p       | L    | H    | p       | L    | H    | p       | L    | H    | p       |
| Gender            | Male     | 63             | 33   | 0.518 | 0.920 | 66     | 0.924 | 43   | 0.637 |
|                   |          | 43             | 29   |       | 0.920 | 66     | 0.924 | 29   | 0.637 |
|                   | Female   | 49             | 33   | 0.426 | 0.492 | 53     | 0.323 | 54   | 0.510 |
|                   |          | 57             | 29   |       | 0.492 | 53     | 0.323 | 61   | 0.466 |
| Age               | <60      | 49             | 33   | 0.426 | 0.492 | 53     | 0.323 | 54   | 0.510 |
|                   | ≥60      | 57             | 29   |       | 0.492 | 53     | 0.323 | 61   | 0.466 |
| CEA               | <5.2ng/mL| 64             | 50   | 0.010 | 0.061 | 71     | 0.013 | 49   | 0.962 |
|                   | ≥5.2ng/mL| 42             | 12   |       | 0.061 | 71     | 0.013 | 49   | 0.962 |
| Chemotherapy      | No       | 41             | 31   | 0.196 | 0.483 | 47     | 0.503 | 42   | 0.271 |
|                   | Yes      | 65             | 31   |       | 0.483 | 47     | 0.503 | 42   | 0.271 |
| Location          | Colon    | 52             | 38   | 0.150 | 0.023 | 56     | 0.069 | 62   | 0.893 |
|                   | Rectum   | 54             | 24   |       | 0.023 | 56     | 0.069 | 62   | 0.893 |
| Histology type    | A        | 94             | 58   | 0.417 | 0.563 | 103    | 0.778 | 101  | 0.601 |
|                   | MA       | 12             | 4    |       | 0.563 | 103    | 0.778 | 101  | 0.601 |
| Grade             | Poor     | 6              | 0    | 0.086 | 0.194 | 6      | 0.178 | 6    | 0.178 |
|                   | Well /moderate | 100  | 62   | 0.194 | 0.539 | 109    | 0.539 | 109  | 0.539 |
| Vascular invasion | No       | 99             | 56   | 0.553 | 0.337 | 108    | 0.350 | 106  | 0.400 |
|                   | Yes      | 7              | 6    |       | 0.337 | 108    | 0.350 | 106  | 0.400 |
| Neural invasion   | No       | 82             | 51   | 0.556 | 0.831 | 86     | 0.043 | 90   | 0.838 |
|                   | Yes      | 24             | 11   |       | 0.831 | 86     | 0.043 | 90   | 0.838 |
| pT                | pT3      | 76             | 40   | 0.388 | 0.025 | 82     | 0.373 | 79   | 0.884 |
|                   | pT4      | 30             | 22   |       | 0.025 | 82     | 0.373 | 79   | 0.884 |
| LNH               | <12      | 26             | 12   | 0.567 | 0.097 | 33     | 0.005 | 27   | 0.853 |
|                   | ≥12      | 80             | 50   |       | 0.097 | 33     | 0.005 | 27   | 0.853 |
| MSI status        | Low/MSS  | 74             | 43   | 0.212 | 0.017 | 84     | 0.002 | 81   | 0.121 |
|                   | high     | 5              | 7    |       | 0.017 | 84     | 0.002 | 81   | 0.121 |
| APC mutation      | Wild-type| 27             | 17   | 0.983 | 0.979 | 29     | 0.844 | 28   | 0.977 |
|                   | Mutant   | 52             | 33   |       | 0.979 | 29     | 0.844 | 28   | 0.977 |

(Continued)
| Variables     | Subgroup    | No. of patients |
|---------------|-------------|----------------|
|               |             | CD3e           |
|               |             | CD8e           |
|               |             | CD45ROe        |
|               |             | FOXP3e         |
|               |             | PD-L1          |
|               |             | L  | H  | p  | L  | H  | p  | L  | H  | p  | L  | H  | p  |
| BRAF mutation | Wild type   | 73 | 48 | 0.483 | 88 | 33 | 0.889 | 80 | 41 | 0.273 | 79 | 42 | 0.268 |
|               | Mutant      | 6  | 2  |        | 6  | 2  |        | 7  | 1  |        | 7  | 1  |        |
| KRAS mutation | Wild type   | 41 | 28 | 0.718 | 51 | 18 | 0.844 | 47 | 22 | 0.861 | 44 | 25 | 0.575 |
|               | Mutant      | 38 | 22 |        | 43 | 17 |        | 40 | 20 |        | 42 | 18 |        |
| NRAS mutation | Wild type   | 75 | 47 | 1.000 | 90 | 32 | 0.388 | 81 | 41 | 0.426 | 81 | 41 | 1.000 |
|               | Mutant      | 4  | 3  |        | 4  | 3  |        | 6  | 1  |        | 5  | 2  |        |
| ERBB2 mutation| Wild type   | 73 | 44 | 0.536 | 88 | 29 | 0.086 | 83 | 34 | 0.019 | 82 | 35 | 0.020 |
|               | Mutant      | 6  | 6  |        | 6  | 6  |        | 6  | 8  |        | 4  | 8  |        |
| POLE mutation | Wild type   | 74 | 44 | 0.335 | 88 | 30 | 0.168 | 81 | 37 | 0.336 | 80 | 38 | 0.505 |
|               | Mutant      | 5  | 6  |        | 6  | 5  |        | 6  | 5  |        | 6  | 5  |        |
| PIK3CA mutation| Wild type  | 64 | 40 | 0.887 | 76 | 28 | 0.913 | 69 | 35 | 0.643 | 68 | 36 | 0.640 |
|               | Mutant      | 15 | 10 |        | 18 | 7  |        | 18 | 7  |        | 18 | 7  |        |
| PTEN mutation | Wild type   | 75 | 43 | 0.106 | 89 | 29 | 0.068 | 81 | 37 | 0.336 | 81 | 37 | 0.336 |
|               | Mutant      | 4  | 7  |        | 5  | 6  |        | 6  | 5  |        | 5  | 6  |        |
| TPS3 mutation | Wild type   | 22 | 18 | 0.337 | 24 | 16 | 0.034 | 21 | 19 | 0.025 | 24 | 16 | 0.316 |
|               | Mutant      | 57 | 32 |        | 70 | 19 |        | 66 | 23 |        | 62 | 27 |        |

(Continued)
Table 1 (Continued).

| Variables          | Subgroup      | No. of patients |   |   |   |
|--------------------|---------------|-----------------|---|---|---|
|                    |               | CD3s            | CD8s | CD45ROs | FOXP3s |
|                    |               | L   | H   | p   | L   | H   | p   | L   | H   | p   |
| Location           | Colon         | 57  | 33  | 0.751 | 63  | 27  | 0.258 | 52  | 38  | 0.432 |
|                    | Rectum        | 47  | 31  |        | 48  | 30  |        | 50  | 28  |        |
| Histology type     | A             | 91  | 61  | 0.111 | 98  | 54  | 0.267 | 90  | 62  | 0.286 |
|                    | MA            | 13  | 3   |        | 13  | 3   |        | 12  | 4   |        |
| Grade              | Poor          | 6   | 0   | 0.084 | 5   | 1   | 0.665 | 5   | 1   | 0.405 |
|                    | Well /moderate| 98  | 64  |        | 106 | 56  |        | 97  | 65  |        |
| Vascular invasion  | No            | 99  | 56  | 0.081 | 105 | 50  | 0.133 | 93  | 62  | 0.571 |
|                    | Yes           | 5   | 8   |        | 6   | 7   |        | 9   | 4   |        |
| Neural invasion    | No            | 82  | 51  | 0.896 | 93  | 40  | 0.046 | 80  | 53  | 0.847 |
|                    | Yes           | 22  | 13  |        | 18  | 17  |        | 22  | 13  |        |
| pT                 | pT3           | 73  | 43  | 0.732 | 74  | 42  | 0.383 | 72  | 44  | 0.612 |
|                    | pT4           | 31  | 21  |        | 37  | 15  |        | 30  | 22  |        |
| LNH                | <12           | 26  | 12  | 0.448 | 23  | 15  | 0.440 | 24  | 14  | 0.851 |
|                    | ≥12           | 78  | 52  |        | 88  | 42  |        | 78  | 52  |        |
| MSI status         | Low/MSS       | 70  | 47  | 0.920 | 77  | 40  | 0.752 | 73  | 44  | 0.217 |
|                    | high          | 7   | 5   |        | 7   | 5   |        | 5   | 7   |        |
| APC mutation       | Wild type     | 26  | 18  | 0.921 | 26  | 18  | 0.334 | 30  | 14  | 0.255 |
|                    | Mutant        | 51  | 34  |        | 58  | 27  |        | 48  | 37  |        |
| BRAF mutation      | Wild type     | 71  | 50  | 0.473 | 78  | 43  | 0.713 | 73  | 48  | 0.903 |
|                    | Mutant        | 6   | 2   |        | 6   | 2   |        | 5   | 3   |        |
| KRAS mutation      | Wild type     | 38  | 31  | 0.283 | 46  | 23  | 0.715 | 43  | 26  | 0.719 |
|                    | Mutant        | 39  | 21  |        | 38  | 22  |        | 35  | 25  |        |
| NRAS mutation      | Wild-type     | 72  | 50  | 0.701 | 79  | 43  | 1.000 | 73  | 49  | 0.703 |
|                    | Mutant        | 5   | 2   |        | 5   | 2   |        | 5   | 2   |        |
| ERBB2 mutation     | Wild type     | 73  | 44  | 0.066 | 79  | 38  | 0.109 | 75  | 42  | 0.012 |
|                    | Mutant        | 4   | 8   |        | 5   | 7   |        | 3   | 9   |        |

(Continued)
Nomogram construction, risk group stratification, and benefit from adjuvant chemotherapy

Variables with a $p$-value $<0.10$ in the multivariate analysis were included in nomogram construction. Three nomograms were constructed based on variables for RFS (nomogram A) and OS (nomogram B) in Model A and variables for OS (nomogram C) in Model B (see Figure 2), we did not establish a nomogram for RFS in Model B due to limited variables in the final model. Calibration curves were exhibited in Figure S1. For Model A, the nomograms were well calibrated and showed a c-index of 0.751 and 0.757 for RFS and OS, respectively. For Model B, the nomogram for OS was well calibrated and reached a c-index of 0.768. X-tile software was used to select the optimal cutoff values. After stratifying into low- and high-risk groups (Figure S2), for nomogram A, high-risk patients had a significantly worse RFS than low-risk patients (5-year RFS, 16.1% vs 58.2%, $p<0.001$). For nomogram B and nomogram C, worse OS was observed in high-risk group compared with low-risk group (5-year OS, 60.5% vs 90.6%, $p<0.001$; 5-year OS, 45.0% vs 87.7%, $p<0.001$, respectively). The relationship between risk groups and benefit from adjuvant chemotherapy is illustrated in Figure 3. No significant differences for RFS were observed between chemo-treated and chemo-naïve patients in different risk groups ($p=0.625$ and 0.434, respectively). For nomogram B, in high-risk group, chemo-treated patients had a better OS versus chemo-naïve patients, which reached marginal significance (5-year OS, 71.1% vs 34.8%, $p=0.105$). For nomogram C, better OS was observed in chemo-treated patients compared with chemo-naïve patients (3-year OS, 81.9% vs 34.3%, $p=0.006$).

Discussion

The therapeutic success of 5-fluorouracil-based adjuvant chemotherapy has been validated in stage III CRC, but not for patients with stage II disease.24,25 Up to now, only one nomogram predicting recurrence in stage II CRC has been constructed in literature by Hoshino et al26 which included sex, carcinoembryonic antigen, tumor location, tumor depth, lymphatic invasion, venous invasion, and number of lymph nodes studied, rendering a c-index of 0.64. In our study, we first introduced immune biomarkers into nomogram construction, achieving a c-index of overwhelming

### Table 1 (Continued)

| Variables | Subgroup | No. of patients | CD3s | CD8s | CD45ROs | FOXP3s |
|-----------|----------|-----------------|------|------|---------|--------|
|           |          |                 | L    | H    | L       | H      |
| POLE      | Wild type| 70              | 48   | 1.000| 46      | 0.073  |
|           | Mutant   | 7               | 4    | 0.073| 4       | 0.073  |
| PIK3CA    | Wild type| 58              | 46   | 0.073| 43      | 0.248  |
|           | Mutant   | 19              | 6    | 0.248| 4       | 0.248  |
| PTEN      | Wild type| 71              | 47   | 0.765| 41      | 0.313  |
|           | Mutant   | 6               | 5    | 0.313| 4       | 0.313  |
| TP53      | Wild type| 22              | 18   | 0.561| 15      | 0.864  |
|           | Mutant   | 34              | 22   | 0.561| 30      | 0.561  |

Note: Molecular features were available in only 129 patients.

Abbreviations: CD3e, intraepithelial CD3+ cells; CD3s, stromal CD3+ cells; CD8e, intraepithelial CD8+ cells; CD8s, stromal CD8+ cells; CD45ROe, intraepithelial CD45RO+ cells; CD45ROs, stromal CD45RO+ cells; FOXP3e, intraepithelial FOXP3+ cells; FOXP3s, stromal FOXP3+ cells; L, low; H, high; CEA, carcinoembryonic antigen; A, adenocarcinoma; MA, mucinous adenocarcinoma; LNH, number of lymph nodes harvested; MSI, microsatellite instability; MSS, microsatellite stable.
In the current study, high and CD8+, CD45RO+, and FOXP3+ cells may benefit from adjuvant chemotherapy. Besides, the risk classification based on nomogram could identify a special high-risk subset of stage II CRC patients who may benefit from adjuvant chemotherapy.

Accumulating evidence suggests that effector/cytotoxic T-cells (CD3+, CD8+, and CD45RO+), memory T-cells (CD45RO+), and regulatory T-cells (FOXP3+) play important roles in antitumor immune response. Thus, the specific subsets of these TILs are thought to be indicators of host immune response to tumor cells and might be a target for immunotherapy. In the current study, we utilized a digitized, high-resolution image analysis system to count the number of TILs, and the mean densities of T-cell subsets were comparable with previous studies (CD3+, CD8+, CD45RO+, and FOXP3+). Previous studies have demonstrated the high density of CD3+, CD8+, CD45RO+, or FOXP3+ TILs with MSI-high. In the current study, high

| Variables                          | RFS                     |           | OS                      |           |
|------------------------------------|-------------------------|-----------|-------------------------|-----------|
|                                    | HR 95% CI    | p        | HR 95% CI    | p        |
| Tumor features                     |            |          |            |          |
| Gender, female vs male             | 0.829       | 0.534–1.287 | 0.742 | 1.371      | 0.661–2.843 | 0.396 |
| Age, ≥60 vs <60                    | 1.258       | 0.814–1.942 | 0.301 | 1.679      | 0.793–3.554 | 0.176 |
| CEA, ≥5.2 ng/mL vs <5.2 ng/mL      | 2.274       | 1.472–3.515 | <0.001 | 2.468      | 1.189–5.122 | 0.015 |
| Adjuvant chemotherapy, yes vs no   | 1.118       | 0.722–1.732 | 0.618 | 0.825      | 0.396–1.716 | 0.606 |
| Location, rectum vs colon          | 1.335       | 0.867–2.054 | 0.189 | 1.188      | 0.573–2.462 | 0.643 |
| Histology type, MA vs A            | 0.827       | 0.381–1.795 | 0.631 | 0.654      | 0.155–2.754 | 0.563 |
| Grade, well/moderate vs poor        | 0.411       | 0.166–1.021 | 0.055 | 0.328      | 0.099–1.085 | 0.068 |
| Vascular invasion, yes vs no        | 0.780       | 0.340–1.791 | 0.538 | 0.773      | 0.183–3.256 | 0.726 |
| Neural invasion, yes vs no          | 0.934       | 0.548–1.592 | 0.802 | 0.403      | 0.122–1.332 | 0.136 |
| pT, T4 vs T3                        | 0.993       | 0.621–1.587 | 0.976 | 1.065      | 0.485–2.340 | 0.876 |
| LNH, ≥12 vs <12                     | 0.756       | 0.464–1.231 | 0.261 | 0.389      | 0.186–0.085 | 0.012 |
| Molecular features                  |            |          |            |          |
| MSI status, high vs low/MSS         | 0.770       | 0.310–1.915 | 0.574 | 0.699      | 0.165–2.962 | 0.627 |
| APC mutation, M vs WT               | 0.988       | 0.593–0.645 | 0.962 | 2.173      | 0.819–5.765 | 0.119 |
| KRAS mutation, M vs WT              | 2.111       | 0.912–4.888 | 0.081 | 4.399      | 1.507–12.842 | 0.007 |
| NRAS mutation, M vs WT              | 1.110       | 0.687–1.792 | 0.671 | 0.870      | 0.399–1.894 | 0.725 |
| ERBB2 mutation, M vs WT             | 0.833       | 0.335–2.074 | 0.695 | 0.326      | 0.044–2.410 | 0.272 |
| POLE mutation, M vs WT              | 0.994       | 0.430–2.299 | 0.988 | 1.531      | 0.523–4.480 | 0.437 |
| PIK3CA mutation, M vs WT            | 0.663       | 0.338–1.298 | 0.231 | 0.862      | 0.325–2.287 | 0.765 |
| PTEN mutation, M vs WT              | 1.061       | 0.459–2.456 | 0.889 | 2.873      | 1.080–7.640 | 0.034 |
| TP53 mutation, M vs WT              | 1.187       | 0.698–2.019 | 0.527 | 1.173      | 0.493–2.792 | 0.718 |
| Immune biomarkers, high vs low      |            |          |            |          |
| CD3e                               | 0.132       | 0.066–0.265 | <0.001 | 0.276      | 0.105–0.726 | 0.009 |
| CD8e                               | 0.210       | 0.101–0.437 | <0.001 | 0.253      | 0.076–0.835 | 0.024 |
| CD45ROe                            | 0.247       | 0.131–0.467 | <0.001 | 0.287      | 0.100–0.825 | 0.020 |
| FOXP3e                             | 0.211       | 0.109–0.410 | <0.001 | 0.195      | 0.059–0.644 | 0.007 |
| PD-L1                              | 1.134       | 0.731–1.761 | 0.574 | 0.918      | 0.442–1.910 | 0.820 |
| CD3s                               | 0.375       | 0.224–0.638 | <0.001 | 0.356      | 0.145–0.874 | 0.024 |
| CD8s                               | 0.361       | 0.209–0.623 | <0.001 | 0.191      | 0.058–0.630 | 0.007 |
| CD45ROs                            | 0.497       | 0.307–0.805 | 0.004 | 0.514      | 0.228–1.162 | 0.110 |
| FOXP3s                             | 0.257       | 0.148–0.444 | <0.001 | 0.148      | 0.045–0.488 | 0.002 |

Note: Cox proportional hazards regression model, molecular features were available in only 129 patients.

Abbreviations: RFS, relapse-free survival; OS, overall survival; M, mutant; WT, wild type; CEA, carcinoembryonic antigen; A, adenocarcinoma; MA, mucinous adenocarcinoma; LNH, number of lymph nodes harvested; MSI, microsatellite instability; MS, microsatellite stability; CD3e, intraepithelial CD3+ cells; CD3s, stromal CD3+ cells; CD8e, intraepithelial CD8+ cells; CD8s, stromal CD8+ cells; CD45ROe, intraepithelial CD45RO+ cells; CD45ROs, stromal CD45RO+ cells; FOXP3e, intraepithelial FOXP3+ cells; FOXP3s, stromal FOXP3+ cells.
densities of CD45RO+ and CD8+ cells, but not that of CD3+ or FOXP3+ cells, are significantly associated with MSI-high. We used multivariate analysis to assess the prognostic roles of these immune biomarkers and found intraepithelial CD3+ TILs and stromal FOXP3+ TILs were the strongest prognostic factors for RFS, whereas only stromal FOXP3+ TILs were an independent prognostic factor for OS. Our study revealed patients with high intraepithelial CD3+ and stromal FOXP3+ TILs had a significantly higher incidence of normal preoperative CEA, which partially explained the good prognosis associated with these biomarkers. Although Li et al21 concluded PD-L1 correlated with better prognosis in CRC patients, our study did not prove the prognostic role PD-L1, which is in agreement with Masugi’s22 study. Despite numerous studies have demonstrated the prognostic roles of immune-related biomarkers using IHC, seldom have these studies involved molecular features for analysis. In our study, 129 patients successfully underwent NGS and classic mutations for CRC were evaluated for their prognostic roles. KRAS mutation and PTEN mutation were found to be significant factors for OS in univariate analysis, while only PTEN mutation was demonstrated as an independent prognostic factor in multivariate analysis after adjusting for clinicopathological features and immune biomarkers. PTEN is a candidate tumor suppressor and key negative regulator of the PI3K pathway, involving in cell proliferation, migration, and survival.35 Somatic mutations in PTEN were detected in about 6% of sporadic CRC, and PTEN mutation was found to be associated with proximal tumors, mucinous histology, MSI-H, CIMP-high, and BRAF mutation.36 In our study, 8.5% PTEN mutation was observed, 36.4% of MSI-high patients were observed in PTEN mutation group compared with 6.8% in the wild-type group, which is in consistence with previous studies.36,37 Recent reports suggest that PTEN exerts an important tumor suppressor role in colorectal carcinogenesis35 and correlative analyses have associated loss of PTEN with poorer survival,38,39 which is in agreement with our study.

Our study is limited as a retrospective study in nature, further validations from other institutions are merited. Secondly, we did not separate colon and rectal cancer for further study due to limited sample size. Moreover, considering intratumoral heterogeneity, we admit that our study might still fall short of capturing heterogeneity within tumor. Despite of these shortcomings, this is the largest study elucidating the prognostic roles of the densities of various types of TILs focusing on stage II CRC, and we first used nomogram to visualize the results and stratify patients into low- and high-risk groups. More importantly, it is easier for clinical use than signatures or other risk classification systems.

Table 3 Multivariate Cox proportional model for predictors of relapse-free and overall survival

| DFS                      | HR   | 95% CI   | p     | OS                     | HR   | 95% CI   | p     |
|--------------------------|------|----------|-------|------------------------|------|----------|-------|
| Model A (N=168)          |      |          |       | Model A (N=168)        |      |          |       |
| CEA, ≥5.2 ng/mL vs <5.2 ng/mL | 1.591 | 1.022–2.475 | 0.040 | CEA, ≥5.2 ng/mL vs <5.2 ng/mL | 2.080 | 0.995–4.349 | 0.052 |
| CD3e, high vs low        | 0.192 | 0.094–0.395 | <0.001| LNH, ≥12 vs <12         | 0.374 | 0.178–0.784 | 0.010 |
| CD8s, high vs low        | 0.600 | 0.338–1.064 | 0.080 | CD8s, high vs low       | 0.325 | 0.093–1.143 | 0.080 |
| FOXP3s, high vs low      | 0.526 | 0.292–0.974 | 0.032 | FOXP3s, high vs low     | 0.249 | 0.071–0.878 | 0.031 |
| Model B (N=129)          |      |          |       | Model B (N=129)         |      |          |       |
| CD3e, high vs low        | 0.179 | 0.082–0.391 | <0.001| CD8e, high vs low       | 0.282 | 0.067–1.178 | 0.083 |
| FOXP3s, high vs low      | 0.425 | 0.214–0.845 | 0.015 | FOXP3s, high vs low     | 0.155 | 0.034–0.703 | 0.016 |
| LNH, ≥12 vs <12          |      |          |       | LNH, ≥12 vs <12         | 0.436 | 0.199–0.956 | 0.038 |
| PTEN mutation, M vs WT   | 6.526 | 2.149–19.815 | 0.001 | PTEN mutation, M vs WT  |      |          |       |

Notes: Cox proportional hazards regression model. Model A included tumor features and immune biomarkers with a p<0.10 in univariate analysis (N=168). Model B included tumor features, immune biomarkers, and molecular features with a p<0.10 in univariate analysis (N=129). A backward LR (likelihood ratio) elimination with a threshold of p=0.10 was presented in the final model.

Abbreviations: RFS, relapse-free survival; OS, overall survival; M, mutant; WT, wild type; CEA, carcinoembryonic antigen; LNH, number of lymph nodes harvested; CD3e, intraepithelial CD3+ cells; CD8e, intraepithelial CD8+ cells; CD8s, stromal CD8+ cells; FOXP3s, stromal FOXP3+ cells.

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In summary, we constructed nomograms which may help to predict RFS and OS in patients with stage II CRC. Furthermore, we identified a high-risk subset of stage II CRC patients who appeared to benefit from adjuvant chemotherapy.

Figure 2. Nomograms for 1-, 3-, and 5-year probabilities of survival. (A) Nomogram A predicting relapse-free survival based on Model A, with a c-index of 0.751; (B) nomogram B predicting overall survival based on Model A, with a c-index of 0.757; (C) nomogram C predicting overall survival based on Model B, with a c-index of 0.768.

**Abbreviations:** CEA, carcinoembryonic antigen; LNH, number of lymph nodes harvested; CD3e, intraepithelial CD3+ cells; CD8s, stromal CD8+ cells; CD8e, intraepithelial CD8+ cells; FOXP3s, stromal FOXP3+ cells; PTEN, wild-type.
Ethics approval and consent to participate

Informed consent had been obtained and this study was approved by the institutional review board of the Fudan University Shanghai Cancer Center. The patient consent was written informed consent, and that this study was conducted in accordance with the Declaration of Helsinki.

Figure 3 Relationship between risk groups and benefit from adjuvant chemotherapy in stage II colorectal cancer patients. (A) Relapse-free survival based on nomogram A classification; (B) overall survival based on nomogram B classification; (C) overall survival based on nomogram C classification.
Abbreviation list
TILs, tumor-infiltrating lymphocytes; CRC, colorectal cancer; dMMR, deficient mismatch repair; pMMR, proficient mismatch repair; CEA, carcinoembryonic antigen; PD-1, programmed cell death 1 protein; PD-L1, programmed death-ligand 1 protein; NGS, next-generation sequencing; TMA, tissue microarray; RFS, relapse-free survival; OS, overall survival; LNH, lymph nodes harvested; NCCN, National Comprehensive Cancer Network; MSI, microsatellite instability; MSS, microsatellite stability; CD3\(e\), intraepithelial CD3\(e\) cells; CD3\(s\), stromal CD3\(e\) cells; CD8\(e\), intraepithelial CD8\(+\) cells; CD8\(s\), stromal CD8\(+\) cells; CD45RO\(e\), intraepithelial CD45RO\(+\) cells; CD45RO\(s\), stromal CD45RO\(+\) cells; FOXP3\(e\), intraepithelial FOXP3\(+\) cells; FOXP3\(s\), stromal FOXP3\(+\) cells.

Author contributions
All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure
The abstract for this paper was accepted as poster presentation at the 2018 ASCO conference. The authors report no other potential conflicts of interest in this work.

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Figure S1 Calibration of the nomograms for 1-, 3-, and 5-year probabilities of survival. The x-axis shows the nomogram-predicted survival at 1, 3, and 5 years, and the y-axis shows the observed actual survival and 95% confidence intervals. (A) Calibration of nomogram A; (B) calibration of nomogram B; (C) calibration of nomogram C.
Figure S2 Survival curves comparing different risk groups. The patients were stratified into two groups according to the cutoff values generated by X-tile program. (A) Relapse-free survival based on nomogram A classification; (B) Overall survival based on nomogram B classification; (C) overall survival based on nomogram C classification.