Cancer Management and Research

Dovepress

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ORIGINAL RESEARCH

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

This article was published in the following Dove Press journal:
Cancer Management and Research

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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

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DovePress
http://doi.org/10.2147/CMAR.S212094

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.[10] Naito et al[11] 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 metachronous metastasis in CRC.[12,13] CD45RO+ TILs have also been reported to have prognostic significance. Pages et al[14] 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.[15] Regulatory T-cells engage in the maintenance of immunological self-tolerance by actively suppressing self-reactive lymphocytes.[16,17] 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.[18] 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.[19,20] Li et al[21] 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.[22] 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 software[23] (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/mm^2) 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, 0178–0.784) 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;

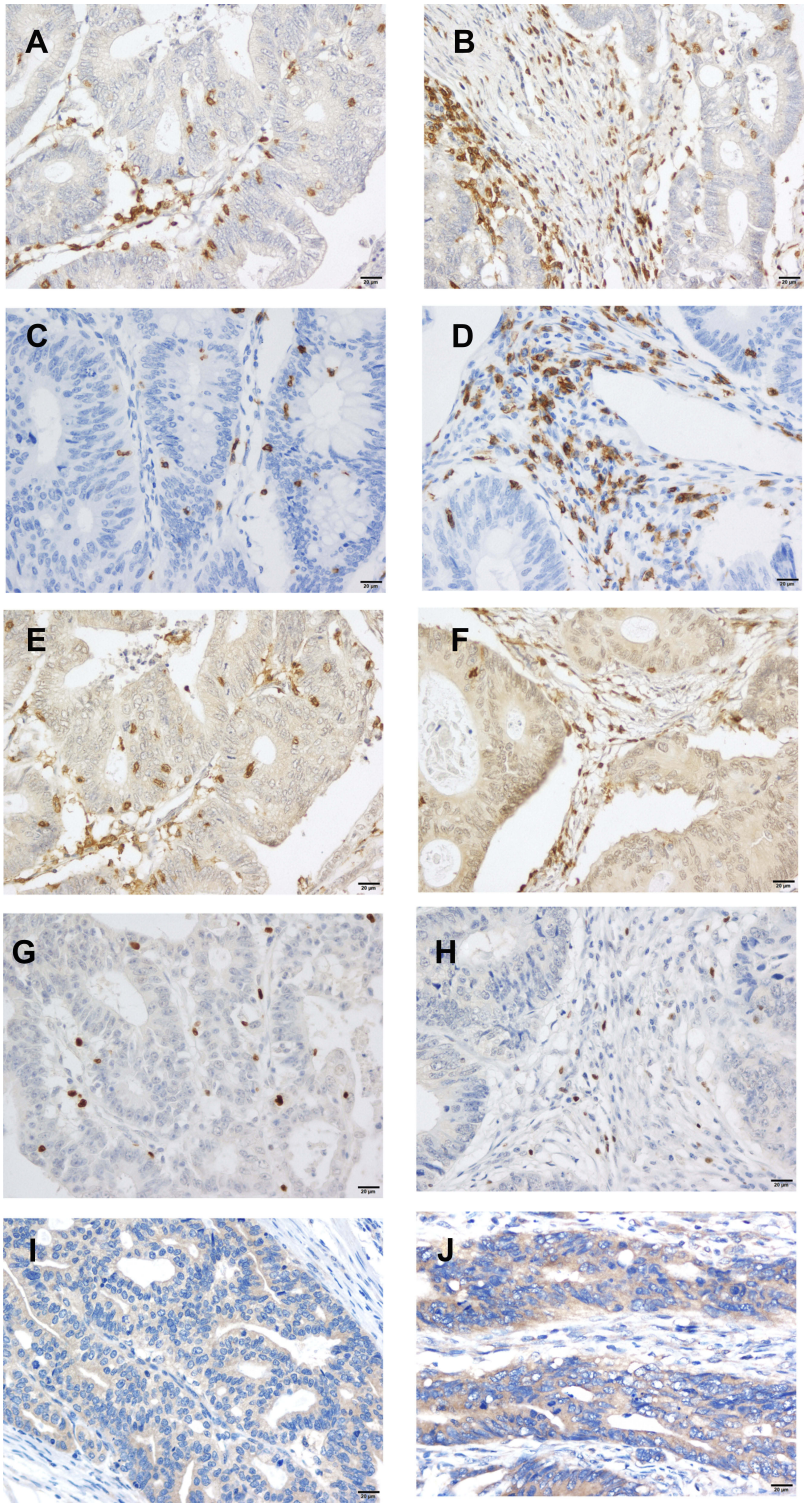

Figure 1 Representative examples of immunohistochemical findings for CD3, CD8, CD45RO, FOXP3, and PD-L1 (original magnification, ×400). (**A,B**) Positive for intraepithelial and stromal CD3; (**C,D**) positive for intraepithelial and stromal CD8; (**E,F**) positive for intraepithelial and stromal CD45RO; (**G,H**) positive for intraepithelial and stromal FOXP3; (**I,J**) positive for cytoplasmic and membranous PD-L1.

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.

Table 1 Clinicopathological and molecular features according to the densities of tumor-infiltrating lymphocytes and PD-L1 expression

Variables	Subgroup	No. of patients													
		CD3e			CD8e			CD45ROe			FOXP3e			PD-L1		
		L	H	p	L	H	p	L	H	p	L	H	p	L	H	p
Gender	Male	63	33	0.518	70	26	0.920	66	30	0.924	66	30	0.924	43	53	0.637
	Female	43	29		53	19		49	23		49	23		29	43	
Age	<60	49	33	0.426	58	24	0.492	53	29	0.323	54	28	0.510	32	50	0.352
	≥60	57	29		65	21		62	24		61	25		40	46	
CEA	<5.2ng/mL	64	50	0.010	78	36	0.061	71	43	0.013	73	41	0.079	49	65	0.962
	≥5.2ng/mL	42	12		45	9		44	10		42	12		23	31	
Chemotherapy	No	41	31	0.196	55	17	0.483	47	25	0.503	42	30	0.019	27	45	0.271
	Yes	65	31		68	28		68	28		73	23		45	51	
Location	Colon	52	38	0.150	59	31	0.023	56	34	0.069	62	28	0.896	39	51	0.893
	Rectum	54	24		64	14		59	19		53	25		33	45	
Histology type	A	94	58	0.417	110	42	0.563	103	49	0.778	101	51	0.097	64	88	0.601
	MA	12	4		13	3		12	4		14	2		8	8	
Grade	Poor	6	0	0.086	6	0	0.194	6	0	0.178	6	0	0.178	4	2	0.404
	Well /moderate	100	62		117	45		109	53		109	53		68	94	
Vascular invasion	No	99	56	0.553	115	40	0.337	108	47	0.350	106	49	0.950	68	87	0.400
	Yes	7	6		8	5		7	6		9	4		4	9	
Neural invasion	No	82	51	0.556	98	35	0.831	86	47	0.043	90	43	0.838	55	78	0.450
	Yes	24	11		25	10		29	6		25	10		17	18	
pT	pT3	76	40	0.388	91	25	0.025	82	34	0.373	79	37	0.884	54	62	0.178
	pT4	30	22		32	20		33	19		36	16		18	34	
LNH	<12	26	12	0.567	32	6	0.097	33	5	0.005	27	11	0.843	17	21	0.853
	≥12	80	50		91	39		82	48		88	42		55	75	
MSI status	Low/MSS	74	43	0.212	89	28	0.017	84	33	0.002	81	36	0.103	51	66	0.121
	high	5	7		5	7		3	9		5	7		2	10	
APC mutation	Wild-type	27	17	0.983	32	12	0.979	29	15	0.844	28	16	0.694	18	26	0.977
	Mutant	52	33		62	23		58	27		58	27		35	50	

(*Continued*)

Table 1 (Continued).

Variables	Subgroup	No. of patients														
		CD3e			CD8e			CD45ROe			FOXP3e			PD-L1		
		L	H	p	L	H	p	L	H	p	L	H	p	L	H	p
BRAF mutation	Wild type Mutant	73 6	48 2	0.483	88 6	33 2	0.889	80 7	41 1	0.273	79 7	42 1	0.268	49 4	72 4	0.716
KRAS mutation	Wild type Mutant	41 38	28 22	0.718	51 43	18 17	0.844	47 40	22 20	0.861	44 42	25 18	0.575	31 22	38 38	0.374
NRAS mutation	Wild type Mutant	75 4	47 3	1.000	90 4	32 3	0.388	81 6	41 1	0.426	81 5	41 2	1.000	49 4	73 3	0.445
ERBB2 mutation	Wild type Mutant	73 6	44 6	0.536	88 6	29 6	0.086	83 4	34 8	0.019	82 4	35 8	0.020	51 2	66 10	0.121
POLE mutation	Wild type Mutant	74 5	44 6	0.335	88 6	30 5	0.168	81 6	37 5	0.336	80 6	38 5	0.505	50 3	68 8	0.524
PIK3CA mutation	Wild type Mutant	64 15	40 10	0.887	76 18	28 7	0.913	69 18	35 7	0.643	68 18	36 7	0.640	46 7	58 18	0.176
PTEN mutation	Wild type Mutant	75 4	43 7	0.106	89 5	29 6	0.068	81 6	37 5	0.336	81 5	37 6	0.178	49 4	69 7	0.739
TP53 mutation	Wild type Mutant	22 57	18 32	0.337	24 70	16 19	0.034	21 66	19 23	0.025	24 62	16 27	0.316	13 40	27 49	0.246

Variables	Subgroup	No. of patients											
		CD3s			CD8s			CD45ROs			FOXP3s		
		L	H	p	L	H	p	L	H	p	L	H	p
Gender	Male Female	58 46	38 26	0.748	62 49	34 23	0.742	54 48	42 24	0.203	61 44	35 28	0.750
Age	<60 ≥60	52 52	30 34	0.752	50 61	32 25	0.194	45 57	37 29	0.156	47 68	35 28	0.203
CEA	<5.2ng/mL ≥5.2ng/mL	66 38	48 16	0.130	71 40	43 14	0.163	67 35	47 19	0.501	64 41	50 13	0.017
Chemotherapy	No Yes	41 63	31 33	0.265	46 65	26 31	0.625	42 60	30 36	0.634	40 65	32 31	0.112

(*Continued*)

Table 1 (Continued).

Variables	Subgroup	No. of patients											
		CD3s			CD8s			CD45ROs			FOXP3s		
		L	H	p	L	H	p	L	H	p	L	H	p
Location	Colon Rectum	57 47	33 31	0.751	63 48	27 30	0.258	52 50	38 28	0.432	61 44	29 34	0.151
Histology type	A MA	91 13	61 3	0.111	98 13	54 3	0.267	90 12	62 4	0.286	92 13	60 3	0.173
Grade	Poor Well /moderate	6 98	0 64	0.084	5 106	1 56	0.665	5 97	1 65	0.405	5 100	1 62	0.412
Vascular invasion	No Yes	99 5	56 8	0.081	105 6	50 7	0.133	93 9	62 4	0.571	98 7	57 6	0.557
Neural invasion	No Yes	82 22	51 13	0.896	93 18	40 17	0.046	80 22	53 13	0.847	80 25	53 10	0.151
pT	pT3 pT4	73 31	43 21	0.732	74 37	42 15	0.383	72 30	44 22	0.612	72 33	44 19	0.863
LNH	<12 ≥12	26 78	12 52	0.448	23 88	15 42	0.440	24 78	14 52	0.851	25 80	13 50	0.706
MSI status	Low/MSS high	70 7	47 5	0.920	77 7	40 5	0.752	73 5	44 7	0.217	77 6	40 6	0.346
APC mutation	Wild type Mutant	26 51	18 34	0.921	26 58	18 27	0.334	30 48	14 37	0.255	30 53	14 32	0.565
BRAF mutation	Wild type Mutant	71 6	50 2	0.473	78 6	43 2	0.713	73 5	48 3	0.903	76 7	45 1	0.258
KRAS mutation	Wild type Mutant	38 39	31 21	0.283	46 38	23 22	0.715	43 35	26 25	0.719	43 40	26 20	0.713
NRAS mutation	Wild-type Mutant	72 5	50 2	0.701	79 5	43 2	1.000	73 5	49 2	0.703	79 4	43 3	0.699
ERBB2 mutation	Wild type Mutant	73 4	44 8	0.066	79 5	38 7	0.109	75 3	42 9	0.012	76 7	41 5	0.754

(*Continued*)

Table 1 (Continued).

Variables	Subgroup	No. of patients									
		CD3s			CD8s			CD45ROs			FOXP3s		
		L	H	p	L	H	p	L	H	p	L	H	p
POLE mutation	Wild type	70	48	1.000	77	41	1.000	74	44	0.111	77	41	0.520
	Mutant	7	4		7	4		4	7		6	5	
PIK3CA mutation	Wild type	58	46	0.073	65	39	0.248	61	43	0.496	65	39	0.487
	Mutant	19	6		19	6		17	8		18	7	
PTEN mutation	Wild type	71	47	0.765	77	41	0.914	73	45	0.341	77	41	0.520
	Mutant	6	5		7	4		5	6		6	5	
TP53 mutation	Wild type	22	18	0.561	25	15	0.694	22	18	0.439	28	12	0.430
	Mutant	55	34		59	30		56	33		55	34	

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 stability.

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 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 al[26] 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 2 Univariate analyses of factors associated with relapse-free and overall survival

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	0.558	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
BRAF mutation, M vs WT	2.111	0.912–4.888	0.081	4.399	1.507–12.842	0.007
KRAS mutation, M vs WT	1.110	0.687–1.792	0.671	0.870	0.399–1.894	0.725
NRAS mutation, M vs WT	0.795	0.250–2.531	0.698	0.045	0.000–71.101	0.410
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; MSS, 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.

0.751 and 0.757 for RFS and OS, respectively. 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+[12,13] and CD8+[11,27]), memory T-cells (CD45RO+[14,15]), and regulatory T-cells (FOXP3+[16,18]) 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.[28,29] 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+,[10,30] CD8+,[18,31] CD45RO+,[18,32] and FOXP3+[30,31]). Previous studies have demonstrated the high density of CD3+, CD8+, CD45RO+, or FOXP3+ TILs with MSI-high.[18,30,33,34] In the current study, high

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

DFS				OS			
Prognostic features	**HR**	**95% CI**	**p**	**Prognostic features**	**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	0.436	0.199–0.956	0.038
				PTEN mutation, M vs WT	6.526	2.149–19.815	0.001

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.

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 al[21] 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's[22] 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 carcinogenesis[35] 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.

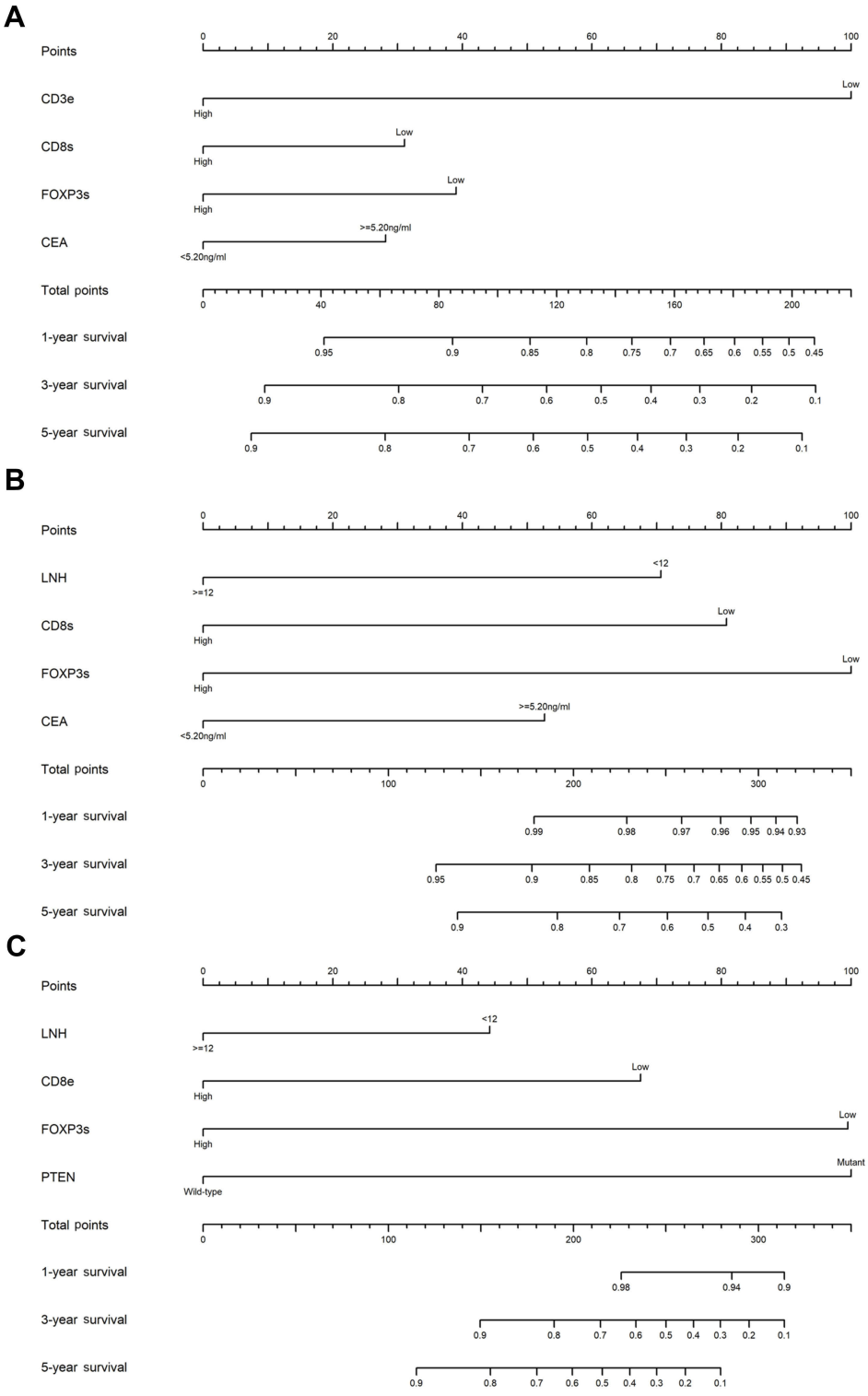

Figure 2 Nomograms for 1-, 3-, and 5-year probabilities of survival. (**A**) Nomogram A predicting relapse-free survival based on Model A, with a c-index of 0.751; (**B**) nomogram B predicting overall survival based on Model A, with a c-index of 0.757; (**C**) nomogram C predicting overall survival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

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.

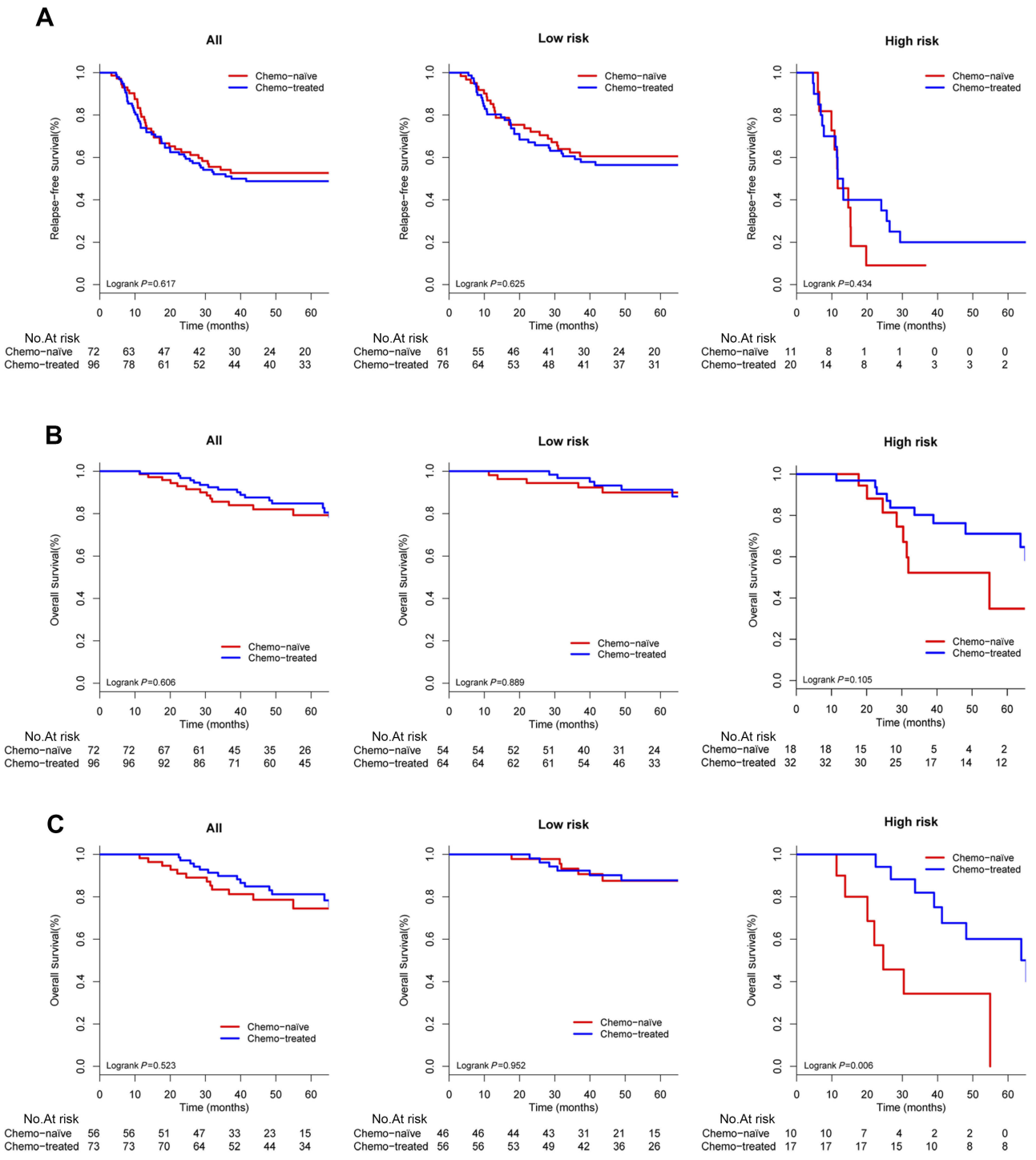

Figure 3 Relationship between risk groups and benefit from adjuvant chemotherapy in stage II colorectal cancer patients. (**A**) Relapse-free survival based on nomogram A classification; (**B**) overall survival based on nomogram B classification; (**C**) overall survival based on nomogram C classification.

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.

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; 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.

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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Supplementary material

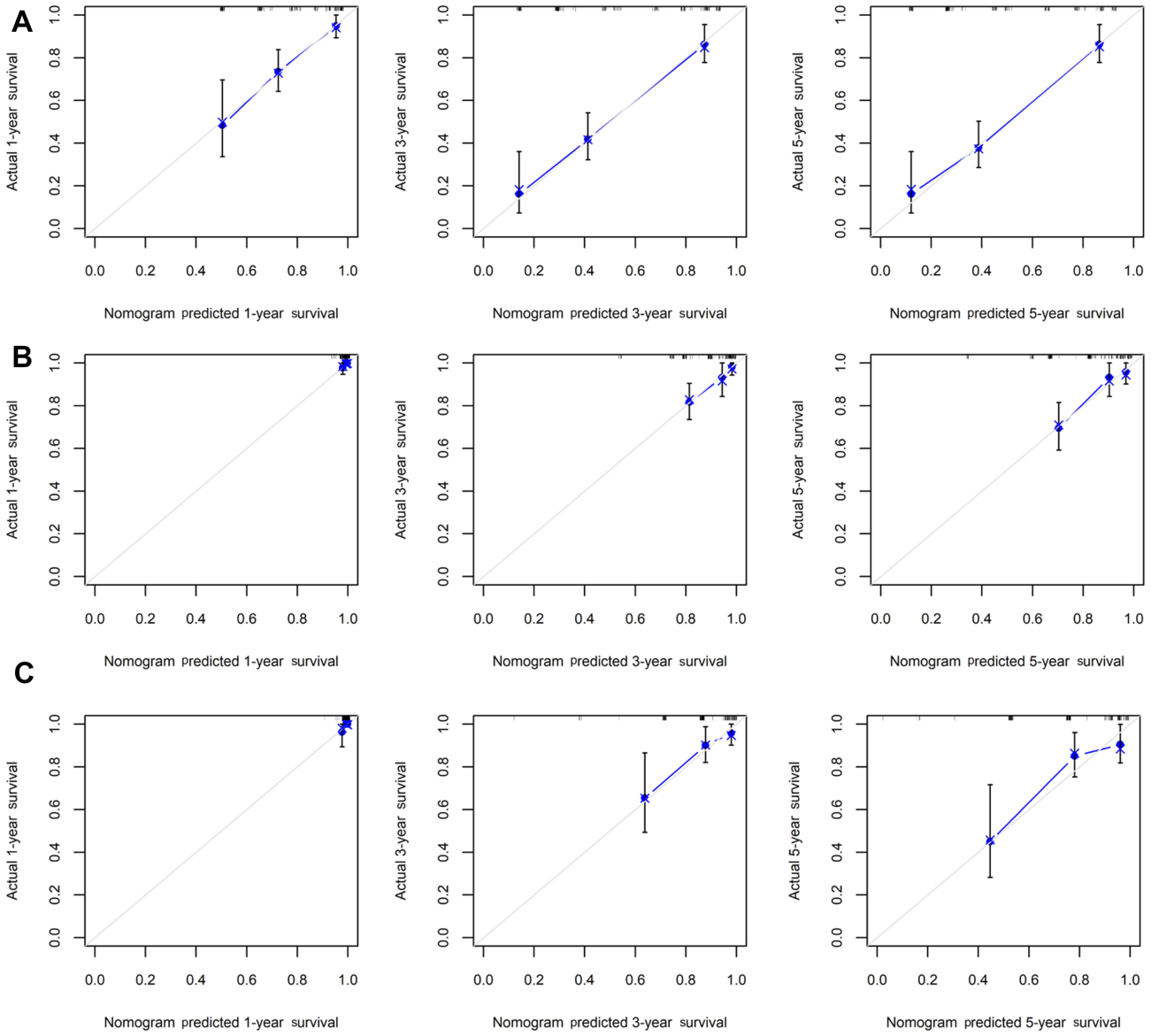

Figure S1 Calibration of the nomograms for 1-, 3-, and 5-year probabilities of survival. The x-axis shows the nomogram-predicted survival at 1, 3, and 5 years, and the y-axis shows the observed actual survival and 95% confidence intervals. (**A**) Calibration of nomogram A; (**B**) calibration of nomogram B; (**C**) calibration of nomogram C.

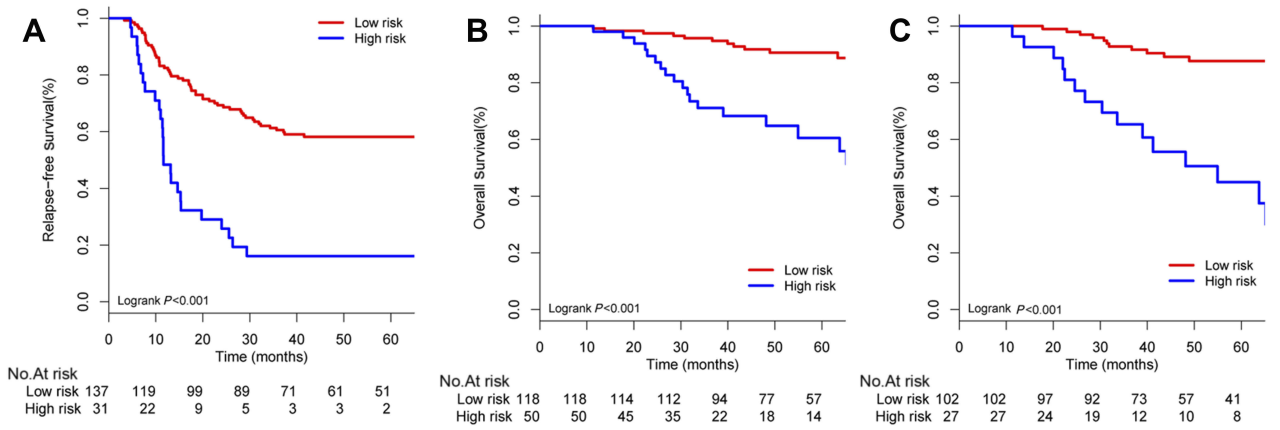

Figure S2 Survival curves comparing different risk groups. The patients were stratified into two groups according to the cutoff values generated by X-tile program. (**A**) Relapse-free survival based on nomogram A classification; (**B**) Overall survival based on nomogram B classification; (**C**) overall survival based on nomogram C classification.