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Multiplex immunohistochemistry indicates biomarkers in colorectal cancer

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Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, whose survival ratio and indicating biomarkers are limited. The rapid development of multiple immunofluorescences gives rise to widespread applications of this new advanced technology called multiplex immunohistochemistry (mIHC), which makes it possible to detect several fluorescent proteins on the same tumor tissue microarray (TMA) within the same time and spatial organization. By taking advantage of this mIHC technology, we detected three tumor-associated antigens (TAA) including the human epidermal growth factor receptor 2 (HER2), the cluster of differentiation 133 (CD133), the programmed death ligand-1 (PD-L1), and one immune-associated macrophage marker, the cluster of differentiation 68 (CD68) in cancer tissues versus para-carcinomatous normal tissues derived from a cohort of 84 CRC patients. All four markers were upregulated in cancer tissue compared with normal tissues. And the expressions of CD133, HER2, PD-L1, and CD68 were correlated with pathological grade, T stage, tumor size, metastasis, respectively. Accordingly, CD133 and PD-L1 could be applied as potential diagnostic biomarkers for CRC at an early stage, while the enrichment of HER2 might act as an advanced indicator in aggressive cancer status of CRC; whereas, CD68 could be potentially considered as an advanced diagnostic indicator in CRC patients, as well as a metastatic promoter in CRC-related TME. The differential expression of these four proteins, as well as their clinicopathological correlation, indicates that these four proteins could be utilized as specific diagnostic and prognostic biomarkers in CRC patients.

Key words: multiplex immunohistochemistry (mIHC), tumor tissue microarray (TMA), colorectal cancer (CRC), clinicopathological characteristics, biomarkers

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, accounting for approximately 9.7% of total cancer cases and approximately 8.5% of cancer deaths worldwide [1]. In China, CRC is the fifth leading cause of cancer-related death, with a total of 191,000 deaths in 2015 [2].

Despite novel insights into the molecular basis of CRC, currently available therapies do not significantly improve the overall survival (OS) of CRC patients. A considerable proportion of CRC patients develops local recurrence and distant metastasis within 5 years after surgical treatment. Although recent advances have been achieved in the multidisciplinary management and treatment of CRC, the disease-free survival of CRC still remains poor [3]. Moreover, using disease risk stratification based on tumor size, lymph node, or distant metastases (TNM staging) and histological grading is not sufficient for the prognosis of individual CRC patients [4]. Besides, even though the serological markers (e.g., CEA) currently used for diagnosis are useful to detect disease recurrence after treatment, they are not so reliable as their changes are not always detectable in patients with CRC. Therefore, additional clinicopathological and prognostic biomarkers are urgently needed.

In light of this, we attempted to analyze the expressions of three tumor-associated antigens (TAA) including the human epidermal growth factor receptor 2 (HER2), the cluster of differentiation 133 (CD133), the programmed death ligand-1 (PD-L1), and one immune-associated macrophage marker, the cluster of differentiation 68 (CD68), as well as their potential clinicopathological value in CRC. Specifically, relying on fluorescent multiplex immunohistochemistry (mIHC) technology on tumor tissue microarray (TMA), we

explored the expressions of these markers in cancer tissues versus their para-carcinomatous normal tissues derived from a cohort of 84 CRC patients.

Patients and methods

Patients. The HColA180Su14 tumor tissue microarray (TMA) (Xinchao, Shanghai, China) consisted of paired colorectal adenocarcinoma tissues and matched adjacent normal tissues randomly derived from 90 CRC patients, of which a cohort of 84 cases with integral information was taken into final analyses and 6 cases with censored data were excluded. Patients underwent surgery from January 2009 to October 2009, and the follow-up information was available from February 2009 to July 2015. The study was conducted under the approval of the Institutional Ethics Committee. All procedures were performed in accordance with the relevant guidelines and regulations. The clinicopathological characteristics of 84 patients are summarized in Table 1.

Sample and tissue microarray (TMA) preparation. TMAs were made based on the pathology diagnosis of each tissue. Firstly, formalin-fixed, paraffin-embedded tumor samples were identified and specimens were reviewed on hematoxylin and eosin (H&E) staining by an independent surgical pathologist to confirm the presence of a tumor and adjacent normal tissue [5]. Then the pathologist circled at least two representative tumor areas from each donor block. Next, core cylinders (1 mm) were punched from each of these areas and deposited into a recipient paraffin block to form the TMAs. Finally, consecutive 6 mm-thick TMA sections were cut and placed on charged poly-L-lysine-coated slides for subsequent IHC analyses [6].

Fluorescent mIHC of TMA. For mIHC staining, the multiplex IHC antibodies for the CD133, HER2, PD-L1, and CD68 were optimized by concentration and application order, meantime, the spectral library was built based on the single-stained slides [7]. Then, the multiplex immunofluorescence staining and multispectral imaging of the four proteins were obtained on one TMA slide by using PANO 7-plex IHC Kit (cat. 0004100100, Panovue, Beijing, China). Briefly, the slide was deparaffinized by xylene 10 min for three times, followed by 100% ethanol, 95% ethanol, 85% ethanol, and 75% ethanol for 5 min, respectively. After rinsing in distilled water for 5 min, the slide was pretreated with 100 ml antigen retrieval solution (citric acid solution, pH 6.0/pH 9.0) by microwaving method (45 seconds on 100% power, then 15 min on 20% power) and transferred to 1× TBST containing slide jar to mix well. The slide was then blocked in 10% blocking solution for 10 min, and followed by staining with the primary antibody against HER2, CD133, PD-L1, or CD68, respectively for 1 h at room temperature. After washing the slide with 1× TBST 3 min twice, the slide was incubated with polymer HRP-anti-mouse/rabbit IgG secondary antibody for 10 min at RT. Then the slide was covered by tyramide (TSA)-conjugated fluorophore (TSA Fluorescence Kits, Panovue,

Table 1. Clinicopathological characteristics of a cohort of 84 CRC patients.

Clinicopathological Characteristics (n=84)	Number	Proportion (%)
Gender		
Male	45	53.57
Female	39	46.43
Age (years)		
≤65	44	52.38
>65	40	47.62
Tumor Size (cm)		
V≤5 cm^3	11	13.10
V>5 cm^3	73	86.90
L≤5 cm	35	41.67
L>5 cm	49	58.33
T Stage		
T1	1	1.19
T2	10	11.90
T3	47	55.95
T4	26	30.95
Lymph Node (N Stage)		
Negative (N0)	57	67.86
Positive (N1a, b-N2a, b)	27	32.14
Metastasis (M Stage)		
Negative (M0)	81	96.43
Positive (M1a, b)	3	3.57
TNM Stage		
I	10	11.90
II A	34	40.48
II B	10	11.90
II C	1	1.19
III A	1	1.19
III B	17	20.24
III C	8	9.52
IV A	1	1.19
IV B	2	2.38
Pathological Grade		
I	5	5.95
II	65	77.38
III	13	15.48
IV	1	1.19
Histology		
Adenocarcinoma	72	85.71
Canalicular adenoma	7	8.33
Mucinous adenocarcinoma	4	4.76
Signet-ring cell carcinoma	1	1.19
Differentiation		
Well	19	22.62
Moderate	60	71.43
Poor	5	5.95
Disease status at last follow-up		
Survival	51	60.71
Death	33	39.29

Beijing, China) at 1:100 dilution and incubated for 10 min at RT. Finally, TSA was vacuumed off, and the slide was washed with 1× TBST 3 min twice for the next staining procedure. For every additional marker in the multiplex immunofluorescence assay [8], the process was repeated by microwave heat-treating the slide for antigen retrieval, followed directly by one primary antibody staining in each procedure circle ordered as HER2, CD133, PD-L1, CD68, respectively, and then downstream procedures as mentioned above. Nuclei were counterstained with 4'-6'-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, D9542) after all the human antigens had been labeled. The detailed information for primary antibodies is summarized in Supplementary Table S1.

Multispectral imaging. To obtain multispectral images, the stained slide was scanned using the Polaris System (PerkinElmer, Waltham, MA, USA), which captures the fluorescent spectra at 20 nm wavelength intervals from 420 to 720 nm with identical exposure time; the scans were combined to build a single stack image with high contrast and accuracy.

Scoring multispectral images. InForm Image Analysis Software (Perkin Elmer, Waltham, MA, USA) was used to batch analysis of multispectral images from the experiment [9]. First, the images of unstained and single-stained sections were used to extract the spectrum of autofluorescence of tissues and each fluorescein, respectively. Then the extracted images were further used to establish a spectral library required for multispectral unmixing by InForm image analysis software. Finally, reconstructed images of sections with the autofluorescence removed were obtained by using this spectral library. For scoring, three to six representative regions of interest for high-powered (200×) imaging from all cases were selected. To build an algorithm for segment tissues and cells, a few representative multispectral images from the experiment were loaded into InForm software. Then, two tissue categories of tumor and stroma were trained according to DAPI signals intensity. Next, the detected tissue compartments were selected and quantified for each stained protein in the slide. Corresponding numbers of total cells and positive cells were counted as well. 4-bin (0, 1+, 2+, 3+) scoring system was used to quantify proteins levels by calculating H-score with cell stains. H-score was calculated using the percentages in each bin and ranges from 0 to 300. Thereby the score results were shown by the percentage positivity of cells with each bin, which included four levels (0–1, 1–2, 2–3, 3–) so as to measuring protein expressions into negative, low, medium, and high levels, respectively. To simplify, H-score with 0–1 and 1–2 (0, 1+) was considered as the low expression, while 2–3 and 3– (2+, 3+) were as the high expression.

Statistics. The expression difference of four proteins in patient specimens was determined by the Mann-Whitney U test. Clinical correlation was analyzed by χ^2-test and Spearman analysis. Overall survival rates were assessed by the Kaplan-Meier analysis, and the log-rank test was used to plot survival curves; *$p<0.05$ was considered to be significant, **$p<0.01$ and ***$p<0.0001$ were considered to be strongly significant. All the analyses were performed with statistics software GraphPad Prism 8.0 and SPSS 17.0.

Results

Demographics. For this cohort of 84 CRC patients, a follow-up was carried out until 2015 to evaluate a seven-year survival. Among nine clinicopathological characteristics including gender, age, tumor size, T stage, lymph node, metastasis, TNM stage, pathological grade, and differentiation, the patients' survival was associated with six of them. Patients with small tumor size (L<5 cm), negative lymph nodes (N0), negative metastasis (M0), early TNM stage (TNM 1–2), slight pathological grade (Grade I–II), and well differentiation have a relatively better prognosis than those with large tumor (L≥5 cm), positive lymph nodes (N1–2), distant metastasis (M1), late TNM stage (TNM 3–4), advanced pathological grade (Grade III–IV), and moderate/poor differentiation ($p<0.05$, Supplementary Table S2, Supplementary Figure S1).

Fluorescent mIHC profile on TMA slides of CRC patients. To obtain multiple fluorescent images, the TMA slides were firstly scored according to DAPI signals intensity, then detected tissues compartments were selected for each stained protein in slides. After all the four antibodies of HER2, CD133, PD-L1, and CD68 have been performed, the protein expressions were quantified by the scoring system to calculate H-score with cell fluorescence. The images of monochromatic proteins in detected tissue compartments and cells were displayed in the upper and middle row ordered as CD133, PD-L1, HER2, and CD68 behind H&E staining and DAPI image (Figure 1). The multispectral fluorescence of HER2, CD68, PD-L1, and CD133 with DAPI was merged as shown in the bottom big image. The selected images displayed tissue from the sigmoid (Figure 1A) and ascending colon (Figure 1B), respectively.

Fluorescent mIHC determines the significant markers in CRC patients. To explore the potential biomarker in CRC, we compared the expression levels of CD133, PD-L1, HER2, and CD68 between cancer tissue and para-carcinomatous normal tissue in a cohort of 84 CRC patients. To be more specific, we first analyzed all cells in the total category, then we divided cells into tumor and stromal cells, and performed the same analysis again. No matter monochromatic CD133, PD-L1, HER2, or CD68, or multiple stained combinations, such as bichromatic CD133/PD-L1, CD133/HER2, CD133/CD68, HER2/PD-L1, HER2/CD68, and PD-L1/CD68; and trichromatic CD133/HER2/PD-L1, CD133/PD-L1/CD68, CD133/HER2/CD68, and HER2/PD-L1/CD68; all of them presented higher levels in cancer tissues than in normal tissues (Table 2; Figure 2). As for panchromatic staining of CD133/PD-L1/HER2/CD68, there is no significance between cancer and normal tissues as these four detected proteins were not co-expression in the CRC samples. Meanwhile, the expression trend in tumor cells was consistent with that in

total cells, and most of the expressions in stromal cells were of no differences (Supplementary Figures S2, S3).

The correlation between four proteins and clinicopathological characteristics. To explore the correlation between four proteins (CD133, PD-L1, HER2, CD68) and nine clinicopathological factors (gender, age, tumor size, T stage, lymph node, metastasis, TNM stage, pathological grade, and differentiation, as listed in Table 1), several statistical analyses were performed. Firstly, all clinicopathological features were divided into two subgroups, and the Mann-Whitney U test was used to figure out the expression difference of four proteins in clinical subtypes, respectively. All four markers were found to be significantly expressed in one or two specific clinicopathological subtypes among three classifications of cells (Supplementary Table S3; Figure 3). To be specific, the expression of HER2 was significantly higher in advanced T stage as T4 (n=26) than in early T stages as T1–3 (n=58) in both total cells (p=0.022, Figure 3A) and tumor cells (p=0.0263, Figure 3E). PD-L1 expression was associated with tumor size, but unusually low in tumor with maximum length <5 cm (n=49) yet high in length >5 cm (n=35) in total cells (p=0.0408, Figure 3B). As for CD133, its expression was significantly higher in CRC patients at early pathological Grade I and II (n=70) than those who were judged as advanced Grade III and IV (n=14) in both total cells (p=0.013, Figure 3C) and tumor cells (p=0.01, Figure 3F). Notably, the expression of CD68 was also associated with pathological grading in total cells, but in contrast to CD133, CD68 was significantly expressed in Grade III and IV (n=14) than patients as Grade I and II (n=70, p=0.0462, Figure 3D). Furthermore, CD68 was the only marker with significant expression related to clinical features in stromal cells, which was significantly highly expressed in patients with positive tumor metastasis (n=3) compared to patients with negative metastasis (n=81, p=0.0327, Figure 3G). Secondly, we classified H-score 0 to 1+ as the low expression level, while H-score 2+ to 3+ as the high expression level for each protein, respectively. To obtain an overall clinical correlation between clinicopathological subtypes and protein expressions, the Chi-square test was applied to analyze it

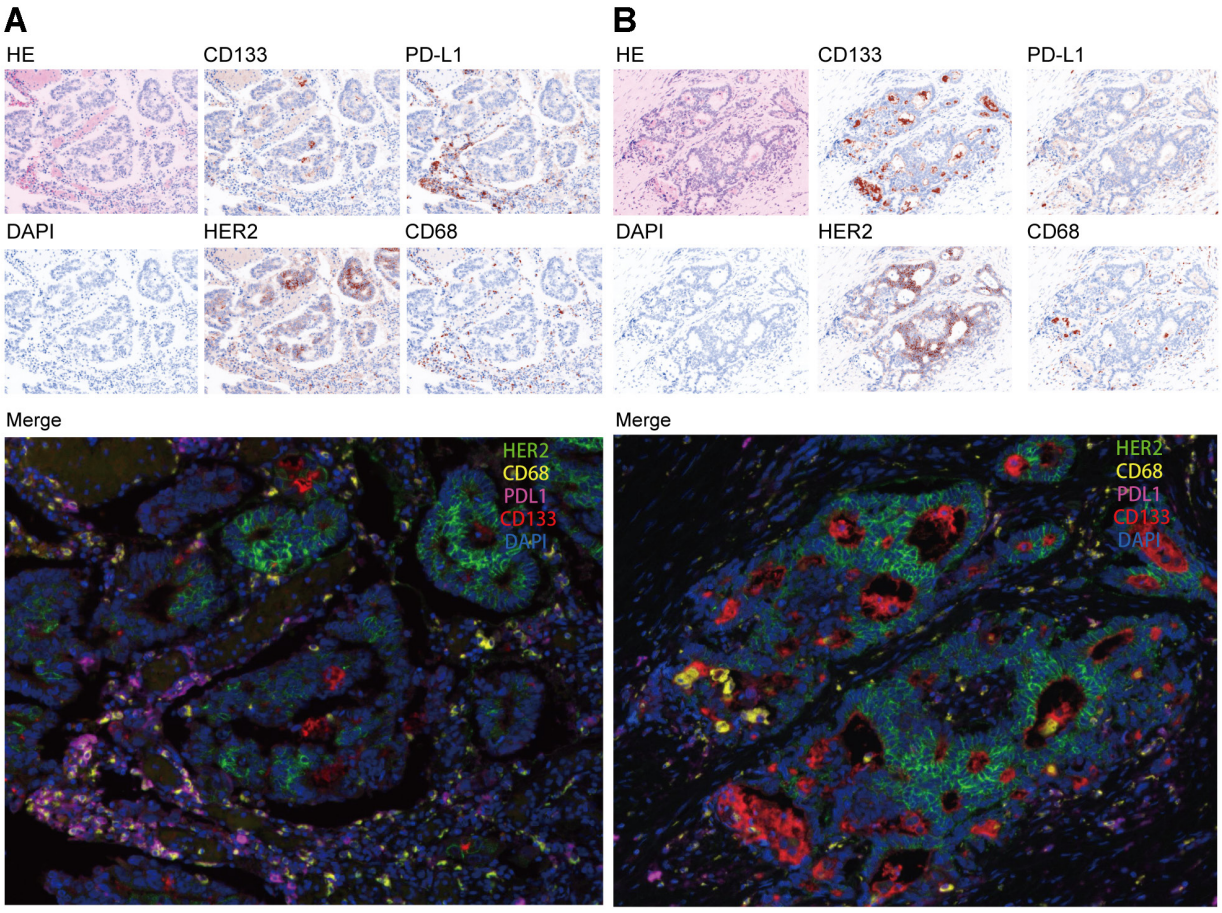

Figure 1. Mono- and pan-chromatic mIHC profile of CRC tissue. A, B) Representative images for single and multiple staining in cancer tissues obtained from sigmoideum (A) and ascendens (B) of the colon. The upper and middle small images show the selected tissue compartments stained by H&E, the raw scanned cell image by DAPI, and the single stained proteins as HER2, CD68, PD-L1, and CD133. The bottom large image shows a merged multispectral fluorescence from HER2, CD68, PD-L1, CD133, and DAPI.

only in total cells of cancer tissue for the 84 CRC patients (Table 3). Finally, Spearman analysis was performed to explore the correlation of clinical single variables with each protein expression (Table 4). Consistently, the PD-L1 expression was negatively correlated with tumor length (R=–0.245, p=0.024). While especially, the HER2 expression was negatively correlated with patients situating in subgroups of pathological grade (I–II vs. III–IV, R=–0.238, p=0.029), and positively correlated with survival or dead status (R=0.222, p=0.042). These results suggested that CD133 and PD-L1 probably are the early markers in CRC occurrence, while HER2 and CD68 might make a clue of more aggressive cancer status during CRC progression.

Prognostic markers associated with clinicopathological characteristics in a cohort of 84 CRC patients. Further clinical analyses were then performed to explore the prognostic potential of the four markers in CRC patients with follow-up time from 1 month at least to 78 months at most during 2009–2015. At first, the univariate analyses of four proteins were performed in total, tumor and stromal cells of CRC cancer tissues, respectively. In both tumor and stromal cells, there were no proteins exerting prognostic significance among the 84 CRC patients (p>0.05, Supplementary Table S4). While in total cells, the only one protein with significant prognostic correlation was CD68 (HR (95 CI%)=4.343 (0.4361–43.25); p=0.0076; Figure 4D), compared with insignificant CD133 (p=0.3999, Figure 4A), HER2 (p=0.6427, Figure 4B), and PD-L1 (p=0.9036, Figure 4C). It demonstrated that CRC patients whose tumors featured high CD68 (n=80) had a longer OS time than those whose tumors featured low CD68 (n=4, p<0.01, Figure 4D). The result supported our hypothesis that the elevation of CD68 expression in CRC was partially due to cancer progression of cancer patients, meanwhile partially benefited prognosis of cancer patients at a late stage.

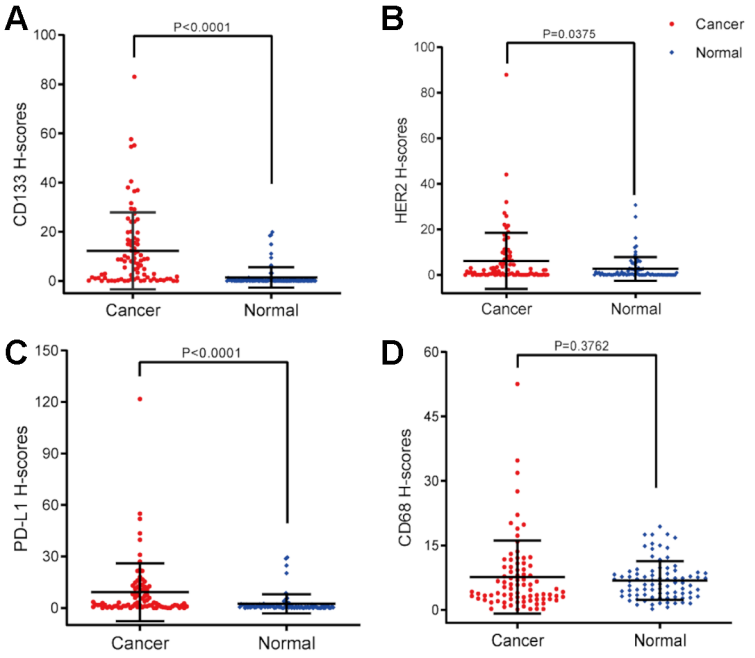

Figure 2. Comparing levels of single stained proteins in total cells based on H-scores by mIHC in cancer versus normal tissues in a cohort of 84 CRC patients. Comparing expression of monochromatic marker A) CD133 (p<0.001), B) HER2 (p=0.0375), C) PD-L1 (p<0.001), and D) CD68 (ns. p=0.3762).

Table 2. Differential expressions of mIHC markers in cancer versus normal tissues in a cohort of 84 CRC patients.

mIHC markers	cancer vs. normal (n=84)		
	total cells	tumor cells	stromal cells
HER2	**=0.0375**	**=0.0207**	=0.1064
CD133	**<0.0001**	**<0.0001**	**<0.0001**
PD-L1	**<0.0001**	**<0.0001**	**<0.0001**
CD68	=0.3762	=0.6407	=0.2062
CD133/PD-L1	**<0.0001**	**<0.0001**	**<0.0001**
CD133/HER2	**<0.0001**	**<0.0001**	**=0.0165**
CD133/CD68	**<0.0001**	**<0.0001**	**=0.0486**
HER2/PD-L1	**=0.0003**	**=0.0003**	=0.2455
HER2/CD68	**=0.0061**	**=0.0018**	>0.9999
PD-L1/CD68	**<0.0001**	**<0.0001**	**<0.0001**
CD133/HER2/PD-L1	**=0.0031**	**=0.0031**	>0.9999
CD133/HER2/CD68	=0.5933	=0.2568	>0.9999
CD133/PD-L1/CD68	**<0.0001**	**<0.0001**	>0.9999
HER2/PD-L1/CD68	=0.1205	=0.1205	>0.9999

Notes: In detected total, tumor and stromal cells of cancer versus normal tissue in a cohort of 84 CRC patients, the protein expression levels of monochromatic CD133, PD-L1, HER2, or CD68; multiple stained combinations as bichromatic CD133/PD-L1, CD133/HER2, CD133/CD68, HER2/PD-L1, HER2/CD68, and PD-L1/CD68; trichromatic CD133/HER2/PD-L1, CD133/PD-L1/CD68, CD133/HER2/CD68, and HER2/PD-L1/CD68 were analyzed by Mann-Whitney U test, respectively. The p-value was displayed in the table above, with boldface word meaning significant difference as p<0.05.

Discussion

Advances in mIHC techniques and digital pathology platforms allow quantification of multiple proteins at the same tissue section and produce continuous data [10]. They significantly enrich the data extracted from tumor tissue and facilitate the analysis of the relationship between multiple proteins retaining spatial connection [11]. In this study, we performed mIHC on four-star molecules, including CD133, PD-L1, HER2, and CD68 to explore the correlation of these well-established biomarkers with CRC. In displayed total cells of 84 paired cancer-versus-normal tissues from TMA slides of CRC patients, all of these four proteins showed consistent higher expression levels in cancer

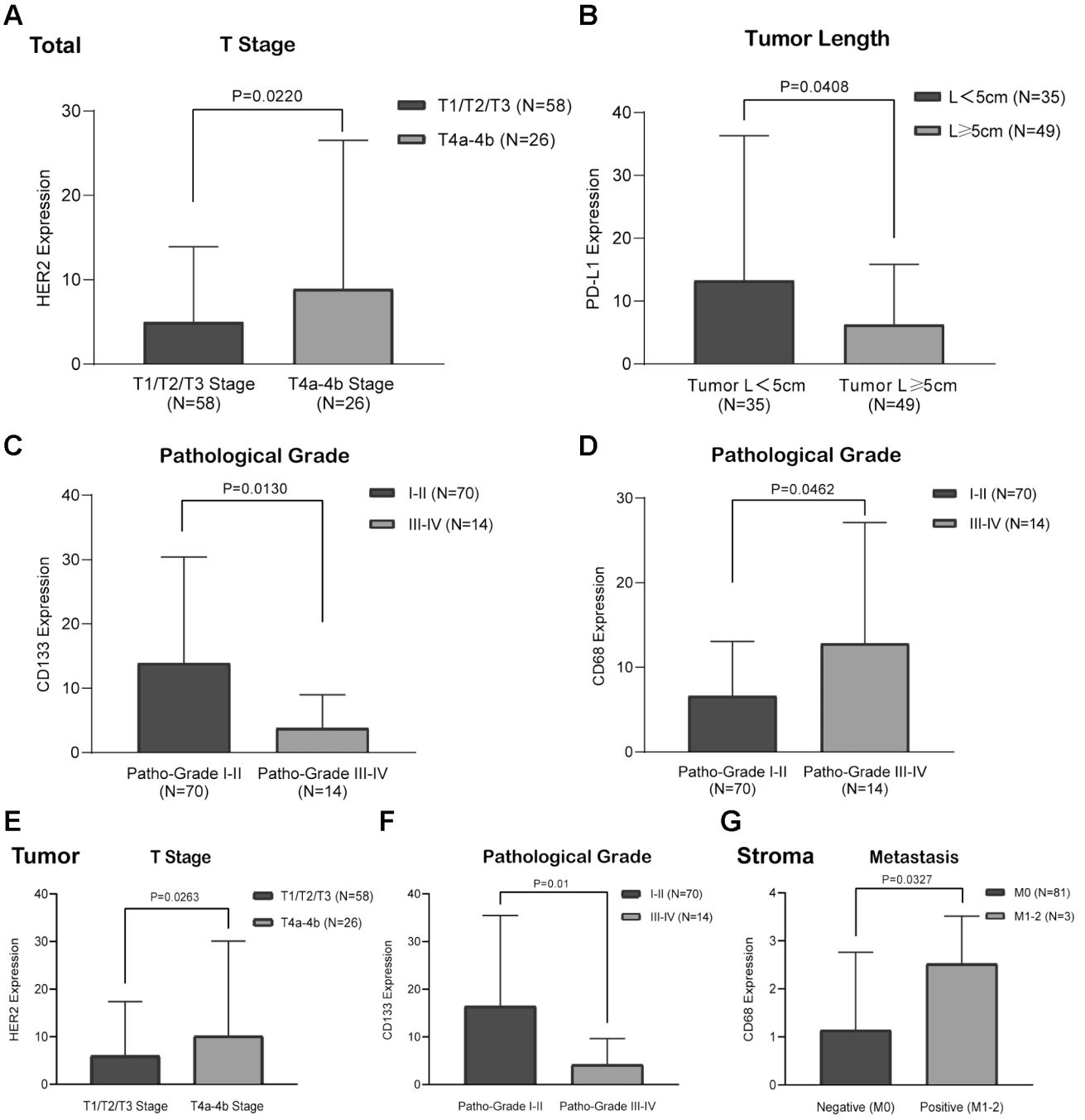

Figure 3. Significant correlations between four markers and clinicopathological characteristics in three cell classifications of CRC cancer tissues. A–D) In total cells of cancer tissue; E, F) In tumor cells of cancer tissue; G) In stromal cells of cancer tissue. A. E) The expression of HER2 was increased in CRC patients at late T4 stage than those at early stages as T1, T2, and T3. B) The expression of PD-L1 was decreased in small tumor with a maximum length ≥5 cm compared in large tumor L<5 cm the levels of C) CD133 in total similar to F) CD133 in a tumor, were reversely expressed in advanced pathological grading III, IV compared with Grade I, II to D) CD68 in total cells. G) CD68 expression was increased in CRC patients with distant metastasis than negative patients.

tissues than in para-carcinomatous normal tissues (Table 2; Figure 2, Supplementary Figures S2, S3).

Increasing research studies have shown that cancer stem cells (CSCs) are involved in tissue regeneration and carcinogenesis in sporadic CRC, and a variety of molecules have been investigated as putative markers of CSCs in CRC [12]. Among them, CD133 (also named AC133 or prominin-1), being a transmembrane glycoprotein mainly expressed in

Table 3. χ^2-test for the correlation of four protein expressions with clinical subtypes in a cohort of 84 CRC patients.

Markers vs. Clinicopathological Characteristics (n = 84)	CD133			HER2			PD-L1			CD68		
	Low (0~1+)	High (2+~3+)	p-value	Low (0~1+)	High (2+~3+)	p-value	Low (0~1+)	High (2+~3+)	p-value	Low (0~1+)	High (2+~3+)	p-value
Gender												
Male	7	38	0.554	27	18	0.699	12	33	0.115	1	44	0.24
Female	8	31		25	14		5	34		3	36	
Age (years)												
≤65	11	33	0.073	23	21	0.057	9	35	0.959	3	41	0.353
>65	4	36		29	11		8	32		1	39	
Tumor size (cm)												
V≤5 cm^3	1	10	0.415	9	2	0.145	2	9	0.856	0	11	0.426
V>5 cm^3	14	59		43	30		15	58		4	69	
L≤5 cm	6	29	0.885	20	15	0.448	3	32	**0.024**	0	35	0.083
L>5 cm	9	40		32	17		14	35		4	45	
T stage												
T1, T2, T3	9	49	0.403	36	22	0.963	9	49	0.108	3	55	0.792
T4	6	20		16	10		8	18		1	25	
Lymph Node (N stage)												
Negative (N0)	12	45	0.267	36	21	0.731	9	48	0.14	2	55	0.433
Positive (N1a, b-N2a, b)	3	24		16	11		8	19		2	25	
Metastasis (M stage)												
Negative (M0)	14	67	0.476	49	32	0.166	17	64	0.374	4	77	0.693
Positive (M1a, b)	1	2		3	0		0	3		0	3	
TNM stage												
I–II	11	44	0.48	34	21	0.982	9	46	0.224	2	53	0.505
III–IV	4	25		18	11		8	21		2	27	
Pathological Grade												
I–II	11	58	0.326	39	30	**0.029**	14	55	0.98	4	65	0.339
III–IV	4	11		13	2		3	12		0	15	
Differentiation												
Well	2	17	0.343	14	5	0.229	2	17	0.231	1	18	0.907
Moderate/Poor	13	52		38	27		15	50		3	62	
Survival status												
Survival	9	42	0.95	36	15	**0.042**	9	42	0.462	1	50	0.134
Death	6	27		16	17		8	25		3	30	

Notes: Four markers were divided into low- and high-expression by H-score with 0~1+ and 2+~3+, respectively. The correlation between clinical subtypes and protein expressions for 84 CRC patients in total cells of cancer tissue were analyzed by χ^2-test. The p-value in boldface represents significant difference as $p<0.05$.

Table 4. Spearman analysis for the correlation of clinical single variables with each protein expression.

Clinical Variables (n=84)	CD133 expression		HER2 expression		PD-L1 expression		CD68 expression	
	Spearman correlation	p-value	Spearman correlation	p-value	Spearman correlation	p-value	Spearman correlation	p-value
Gender	–0.065	0.56	–0.042	0.704	0.172	0.118	–0.128	0.246
Age (years)	0.196	0.075	–0.208	0.058	0.006	0.959	0.101	0.359
Tumor length (cm)	–0.016	0.887	–0.083	0.454	–0.245	**0.024**	–0.189	0.085
Tumor volume (cm^3)	–0.089	0.422	0.159	0.148	–0.02	0.858	–0.087	0.432
T stage	–0.091	0.409	0.005	0.964	–0.175	0.110	0.029	0.795
Lymph node (N stage)	0.121	0.272	0.037	0.735	–0.161	0.144	–0.085	0.439
Metastasis (M stage)	–0.078	0.482	–0.151	0.170	0.097	0.380	0.043	0.698
TNM stage	0.077	0.486	–0.002	0.982	–0.133	0.228	–0.073	0.511
Pathological grade	–0.107	0.332	–0.238	**0.029**	0.003	0.980	0.104	0.345
Differentiation	–0.103	0.349	0.131	0.234	–0.131	0.236	0.013	0.909
Survival status	–0.007	0.951	0.222	**0.042**	–0.08	0.468	–0.164	0.137

Notes: The p-value in boldface represents significant difference as $p<0.05$.

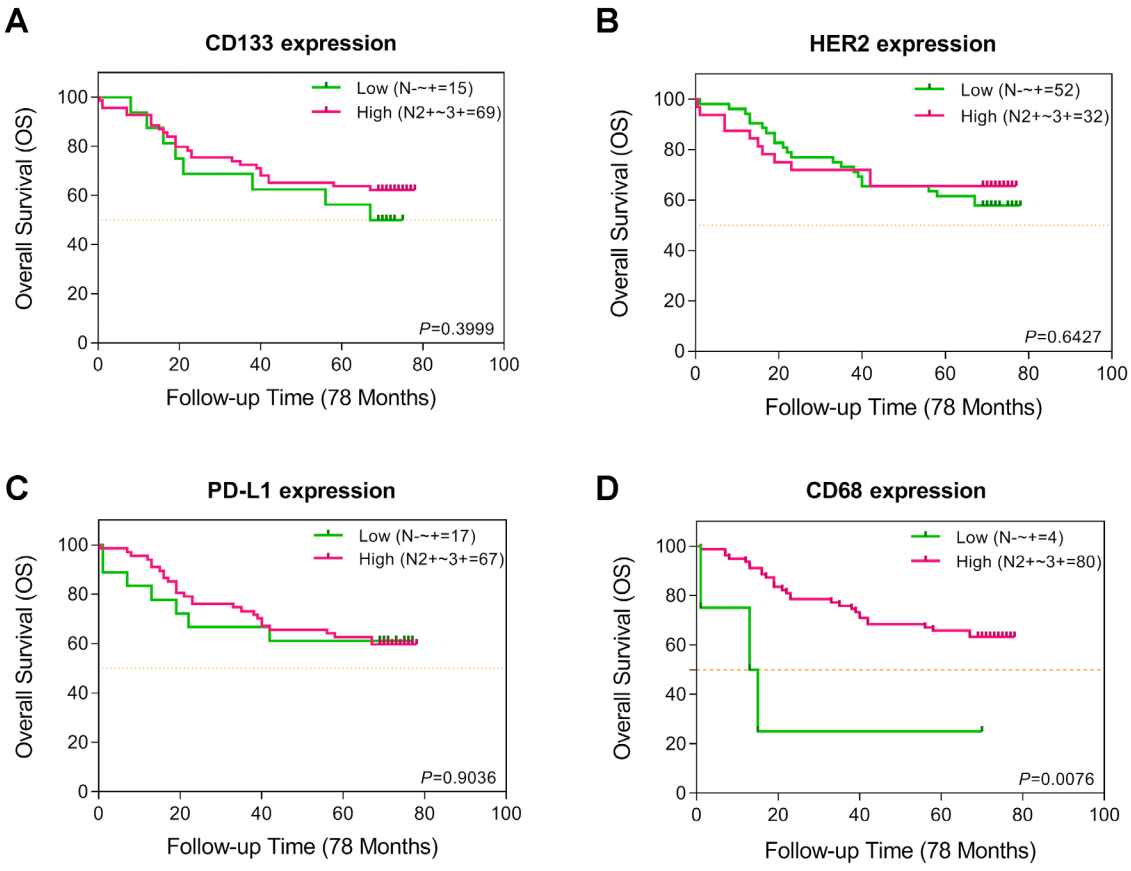

Figure 4. Kaplan-Meier analysis of OS rates with four marker expressions in CRC patients. The high- and low-expressions of A) CD133, B) HER2, C) PD-L1, and D) CD68, were associated with seven-year survival status in total cells of cancer tissues in 84 CRC patients. Dotted line: half of OS rates as 50%.

hematopoietic cells, endothelial cells, and neuroepithelial cells [13], has been reported as one of the most robust surface markers of CSCs in CRC [14]. In 2007, O'Brien et al. [15] found that CD133+ cells in CRC had the ability to initiate tumor growth. Then in 2008, there was a controversial finding reporting that both $CD133^{+}$ and $CD133^{-}$ metastatic colon cancer cells initiate tumors [16]. As for the clinical status of CRC patients, both Kashihara et al. [17] and Wang's team [18] demonstrated that high CD133 expression in CRC correlated with poor clinical outcomes. Huang's group [19] also confirmed from existing 37 studies that CD133 overexpression would serve as a poor predictive indicator for lower 5-year overall survival/disease-free survival (OS/DFS) rate and higher HR of OS/DFS in CRC patients. Furthermore, Akbari et al. [20] confirmed that CD133 has been argued to have prognostic and therapeutic values in CRC along with its related pathways such as Wnt, Notch, and Hedgehog. Nevertheless, Hong et al. and other studies showed that low expression of CD133 patients was associated with advanced tumor stage and exhibited a poor prognosis [21]. And our study was consistent with this report, that the CD133 expression was significantly low in advanced CRC patients with pathological Grade III and IV in both total and tumor cells of cancer tissue, indicating a poor prognosis potential of low CD133 in CRC (Figures 3C, 3F).

PD-L1 (also named CD274, B7-H1) interacting with the programmed cell death 1 (PDCD1, PD-1) is to form the PD-1/PD-L1 axis, which is an immune checkpoint, and usually upregulated to create an immunosuppressive tumor microenvironment and help cancer cells escape immune-mediated destruction [22]. In 2013, Shi [23] has demonstrated that PD-L1 may have an oncogenic function during colon cancer carcinogenesis. As for the correlation between PD-L1 expression on tumor cells and discrepant clinical outcomes, both Liang [24] and Yang [25] demonstrated that PD-L1 expression in a tumor is an independent predictor of poor CRC prognosis. Whereas some other studies presented negative results suggesting that higher expressions of either PD-1 or PD-L1 forecast a better prognosis of CRC patients [26, 27]. In our study, PD-L1 expression was also high in a smaller tumor, but unusually low in tumor length >5 cm (Figure 3B), which owned clinical negative correlation (Tables 3, 4). In addition, the expressions of both CD133 and PD-L1 were obviously significantly higher in cancer versus

normal tissues in all kinds of detected cells (total, tumor, and stromal cells) ($p<0.0001$; Table 2), it indicated that CD133 and PD-L1 could serve as diagnostic biomarkers for CRC, and especially to be applied as a potential biomarker for early screening in CRC patients.

HER2 (also known as HER2/neu, C-erbB2, and p185) is a member of the EGFR family of receptor tyrosine kinases [28]. Over the past two decades, HER2 has been shown to have an important role in the development and progression of approximately 30% of breast cancer [29] cases and 10% of gastric cancer cases [30]. Recently, novel studies have indicated that HER2 is an emerging therapeutic target in CRC [31]. Even the frequency of HER2 overexpression or amplification was low in CRC in the Chinese population, the evaluated HER2 status showed a clinicopathological association and survival impact on CRC by the HERACLES criteria [32]. Our study verified that HER2 expressed significantly in both total and tumor cells ($p<0.05$; Table 2), but probably because HER2 not only expresses on the cell membrane, but also could be secreted into the cytoplasm, the differential expression of HER2 was not significant in stromal cells ($p=0.1064$). Besides, HER2 expressed significantly in the advanced T stage in both total and tumor cells of cancer tissue (Figures 3A, 3E), and displayed a positive correlation with pathological grade (Tables 3, 4). These results indicated that the spreading distribution made HER2 a clinical indicator in the more aggressive cancer status of CRC.

On the contrary, the performance of CD68 is thought provoking.

As a marker of macrophage, CD68 might exert immunological effect against tumorigenesis, and all the differential expressions of CD68 in cancer versus normal CRC tissues were of no significance ($p>0.05$; Table 2). However, the elevation of CD68 expression was correlated with the advanced pathological grading (Figure 3D), which could possibly be explained by that as the tumor malignancy gets advanced, there are more inflammatory immune cells, like macrophages, infiltrating within the tumor microenvironment (TME). What's more, as the only one marker with significant expression related to clinical features in stromal cells, CD68 correlated its high expression to CRC patients with positive tumor metastasis (Figure 3G). Since stromal cells just indicate the para-cancerous immune infiltration areas, CD68 might exert its tumor-infiltration effect to promote tumor cells to achieve distant metastasis through stromal cells in the extracellular matrix (ECM). Accordingly, CD68 expression could be accumulated in both advanced cancer and metastatic stromal cells, which brought CD68 to be a potential diagnostic indicator in the late period of CRC patients, as well as a metastatic promoter in CRC-related TME.

In terms of survival correlation, the study of Pinto et al. [33] indicated that higher CD68 expression in stage III colorectal tumors is associated with decreased overall survival. However, being the only prognostic marker in our analysis, these CRC patients whose tumors featured high CD68, had a longer survival period than those whose tumors featured low CD68 (Figure 4D). As the most accepted model of macrophage classification currently describes several polarization statuses between two extreme populations: the M1-like or pro-inflammatory, being the typical activated macrophage to exert immune effect; and the M2-like or anti-inflammatory, mainly consisting of tumor-associated macrophages (TAMs), which have important roles in the tumor invasive, angiogenic, and metastatic processes [34]; our finding was in line with the Zhao's study [35]. Their investigation of pan-macrophages indicated that unlike other solid tumors, high-density CD68+ macrophage infiltration could be a good prognostic marker for CRC. Hence, we could speculate that in advanced CRC patients, more M1-macrophages were transformed to improve the prognosis of CRC patients. Anyhow, since that the sample size in our study for CD68 prognostic analysis was quite small, together with the specific markers CD80 for M1-macrophage or CD136 for M2-macrophage were not involved in the detection, the result turned out to be not so convincing and needs further solid confirmation.

In conclusion, by applying the newly advanced mIHC technology to detect four typical cancer-associated biomarkers in CRC patients' tissue microarray, we could achieve a better understanding of their specific diagnostic and prognostic significance in CRC progression. In brief, CD133 and PD-L1 could be applied as the early, while HER2 and CD68 as the late diagnostic biomarkers for CRC patients. Being consistent in clinical correlation, positively related HER2 and negatively related PD-L1 were two significantly correlative biomarkers with clinicopathological features. Moreover, the different expression of CD68 could bring CD68 to be a potential diagnostic indicator in the late period of CRC patients, as well as a metastatic promoter in CRC-related TME. Since that there are some limitations for this study, for instance, the analytical deviation might be caused by the heterogeneity of tumor, specificity of antibodies, and small size of the sample, subsequently abundant functional assays performed for confirmation of these tumor-related mIHC proteins are of great need. Moreover, in view that most of the significant results were obtained from the total cells of CRC cancer tissue, the importance of TME should be taken into full consideration in cancer research in further studies. Only correlating tumor-associated antigens with the occurrence and progression of the tumor within the tumor microenvironment could faithfully reflect the tumor status of cancer patients, and thus provide effective diagnostic and therapeutic treatment for them.

Supplementary information is available in the online version of the paper.

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topics; SZW instructed selection of tissue chip; ZW was in charge of data analyses and manuscript writing; ZBY was mainly in charge of connecting with companies, WJL purchased TMA and antibodies. All of the authors engaged much in the analyzing and writing work of this study. And the authors declare that they have no conflicts of interest. The funds were applied for researching and publishing as following: National Natural Science Foundation of China [Grants No. 81460463]; Joint Yunnan Provincial Foundation of Science and Technology Department & Kunming Medical University [2019FE001(-173)]; Foundation of Kunming Key Laboratory of Tumor Molecular and Immune Prevention [2018-1-A-17334]; and Yunnan Digestive Endoscopy Clinical Medical Center Foundation for Health Commission of Yunnan Province [2019LCZXKF-XH03, 011; 2020LCZXKF-XH06; 2021LCZXXF-XH07] of [2X2019-01-02]. All the support and effort are to be appreciated.

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Multiplex immunohistochemistry indicates biomarkers in colorectal cancer

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Supplementary Information

Supplementary Table S1. Primary antibodies used for mIHC staining.

Antibodies	Dilution	Antibody Type	Clone#	Catalogue#	Vendor
CD133	1:1000	Rabbit monoclonal	D4W4N	CST86781	Cell Signaling Technology
HER2	1:200	Rabbit monoclonal	BP6020	BX50015	Biolynx
PD-L1	1:500	Rabbit monoclonal	BP6141	BX00005	Biolynx
CD68	1:800	Rabbit monoclonal	BP6036	BX50031	Biolynx

Supplementary Table S2. Prognostic clinicopathological features of a cohort of 84 CRC patients.

Clinicopathological Features	HR (95 CI%)	p-value
Gender (Male vs. Female)	1.386 (0.6997–2.744)	0.3546
Age (years, ≤65 vs. >65)	1.13 (0.5709–2.236)	0.7251
Tumor size (V≤5 cm^3 vs. V>5 cm^3)	0.1746 (0.06747–0.4519)	0.0501
(L≤5 cm vs. L>5 cm)	0.5012 (0.2529–0.9933)	0.0609
T stage (T1–2–3 vs. T4)	0.4644 (0.2170–0.9937)	**0.0233**
N stage (N0 vs. N1–2)	0.2912 (0.1336–0.6345)	**0.0001**
M stage (M0 vs. M1)	0.1849 (0.01436–2.381)	**0.0015**
TNM (TNM I–II vs. III–IV)	0.243 (0.1129–0.5230)	**<0.0001**
Pathological Grade (I–II vs. III–IV)	0.3381 (0.1148–0.9952)	**0.0033**
Differentiation (W vs. M+P)	0.2766 (0.1277–0.5989)	**0.0222**

Supplementary Table S3. Correlation between potential markers and clinicopathological features in a cohort of 84 CRC patients.

Clinicopathological features	p-value of prognostic markers											
in cancer tissue	Total cells				Tumor cells				Stroma cells			
Marker expression	HER2	CD133	PD-L1	CD68	HER2	CD133	PD-L1	CD68	HER2	CD133	PD-L1	CD68
Gender (Male vs. Female)	0.4266	0.1347	0.5678	0.8374	0.4585	0.1766	0.4737	0.7959	0.5955	0.1072	0.5978	0.9644
Age(years, ≤65 vs. >65)	0.8202	0.9893	0.1389	0.3796	0.8202	0.8342	0.1233	0.3241	0.6765	0.7237	0.148	0.8478
Tumor size (V≤5 cm^3 vs. V>5 cm^3)	0.2012	0.8548	0.4315	0.2013	0.1701	0.9791	0.3235	0.2061	0.8528	0.572	0.8034	0.4463
(L≤5 cm vs. L>5 cm)	0.5694	0.9568	0.0408*	0.3113	0.5451	0.964	0.0567	0.3907	0.5844	0.5865	0.0584	0.8002
T stage (T1–2–3 vs. T4)	0.022*	0.1382	0.6002	0.6069	0.0263*	0.1096	0.6392	0.7256	0.1654	0.7391	0.8135	0.9885
N stage (Negative vs. Positive)	0.0829	0.4809	0.9091	0.5234	0.0642	0.3551	0.6222	0.7972	0.3501	0.389	0.567	0.9014
M stage (Negative vs. Positive)	0.7851	0.8918	0.6646	0.2819	0.9098	>0.9999	0.7093	0.1508	0.6014	0.5038	0.8382	0.0327*
TNM (TNM 1–2I–II vs. III–IV)	0.1454	0.5618	0.7862	0.6944	0.1058	0.41	0.9199	0.9627	0.3322	0.3468	0.844	0.5299
Pathological Grade (I–II vs. III–IV)	0.4941	0.013*	0.5248	0.0462*	0.4866	0.01*	0.5547	0.0581	0.5178	0.2193	0.3679	0.0593
Differentiation (W vs. H+L)	0.5451	0.4902	0.6032	0.9831	0.5451	0.5037	0.7325	0.9072	0.9164	0.4363	0.7824	0.8781

*p<0.05; Statistical analysis were performed by Mann-Whitney U test to correlate positive expression of four proteins to related clinical characteristics in Total, Tumor and Stroma cells of cancer tissue, respectively.

Supplementary Table S4. Association of four proteins with clinical prognosis in a cohort of 84 CRC patients.

Detected proteins in CRC Cancer (N=84)	Total cells		Tumor cells		Stroma cells	
	HR (95 CI%)	p-value	HR (95 CI%)	p-value	HR (95 CI%)	p-value
CD133 (Low vs. High)	0.7144 (0.2979–1.713)	0.3999	1.274 (0.5296–3.066)	0.6144	0.8221 (0.4068–1.661)	0.5929
HER2 (Low vs. High)	0.8436 (0.4165–1.709)	0.6427	0.7909 (0.3926–1.593)	0.5218	1.751 (0.1301–23.58)	0.5738
PD-L1 (Low vs. High)	0.9503 (0.4086–2.210)	0.9036	1.34 (0.5951–3.016)	0.5129	1.007 (0.5074–1.999)	0.9839
CD68 (Low vs. High)	4.343 (0.4361–43.25)	**0.0076**	1.911 (0.4352–8.391)	0.5138	1.172 (0.3855–3.560)	0.7649

HR (95 CI%): Hazard Ratio (95% Confidence interval)

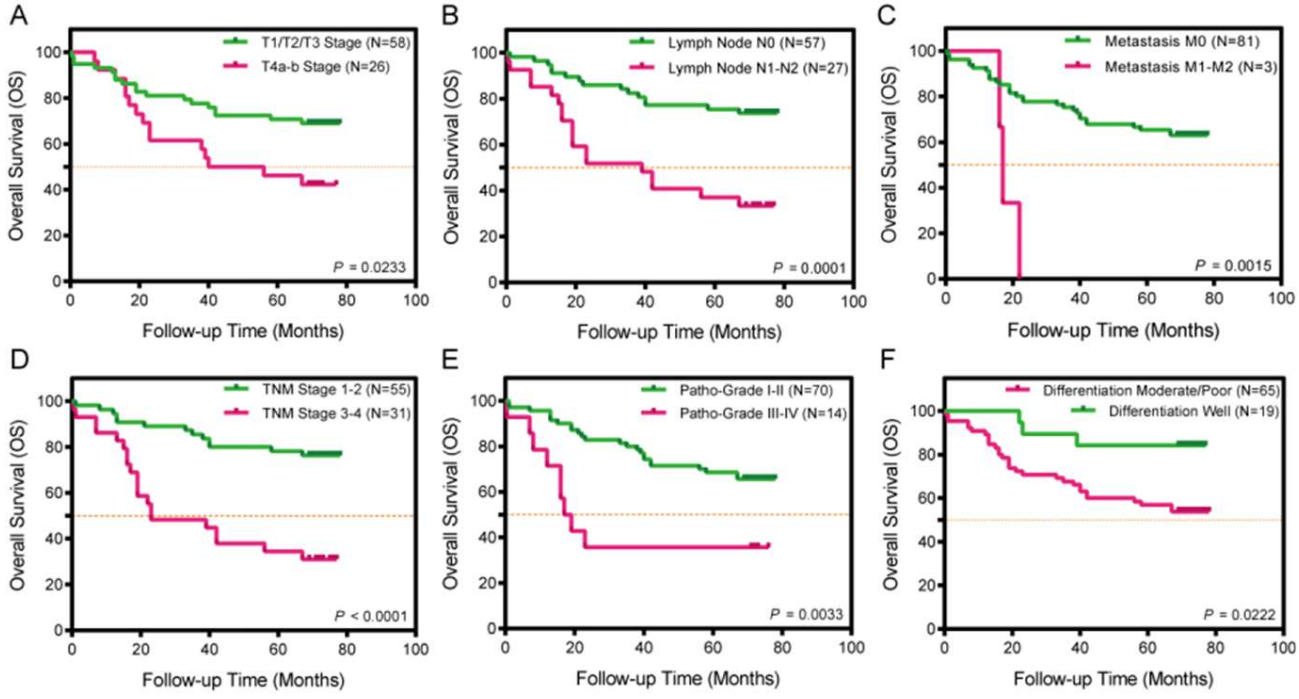

Figure S1. Kaplan-Meier analyzed seven-year overall survival (OS) rates with clinicopathological characteristics. A. T Stage, B. Lymph Node, C. Metastasis, D. TNM Stage, E and F. Pathological Grade as clinical prognostic factors in CRC cancer tissues. Orange line: half of overall survival rates as 50%.

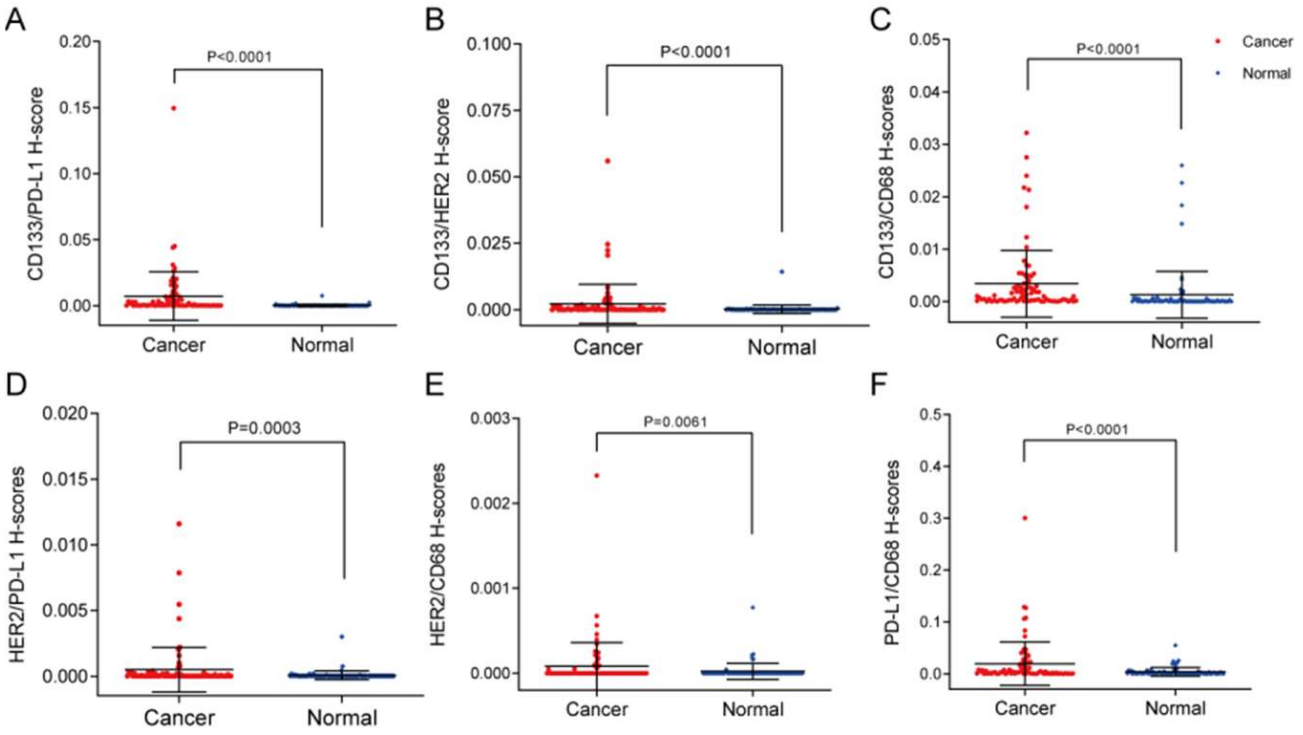

Figure S2. Comparing levels of double stained proteins in Total cells based on H-Scores by mIHC in cancer versus normal tissues in a cohort of 84 CRC patients. Comparing expression of bichromatic combination A. CD133/PD-L1, B. CD133/HER2, C. CD133/CD68, D. HER2/PD-L1, E. HER2/CD68 and F. PD-L1/CD68. p<0.01.

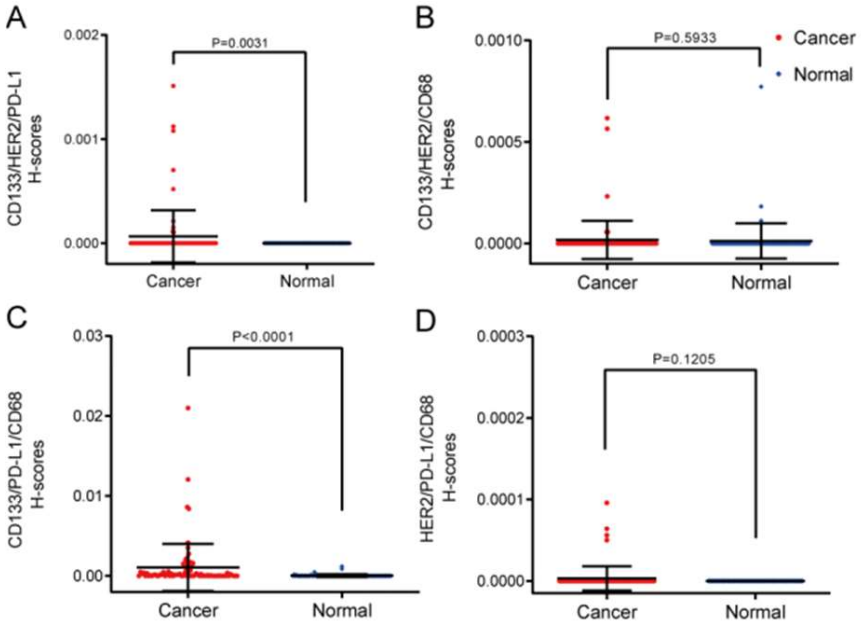

Figure S3. Comparing levels of triple stained proteins in Total cells based on H-Scores by mIHC in cancer versus normal tissues in a cohort of 84 CRC patients. Comparing expression of trichromatic combination A. CD133/HER2/PD-L1 (p=0.0031), B. CD133/PD-L1/CD68 (ns. p=0.5933), C. CD133/HER2/CD68 (p<0.0001) and D. HER2/PD-L1/CD68 (ns. p=0.1205).