Research Article

Identification of Biomarkers in Colorectal Cancer Using a Multiplex Immunohistochemistry Technology

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Abstract

Colorectal Cancer (CRC) is a common malignant tumor with high mortality arising from adenomatous polyps of the large intestine. The rapid development of multiple immunofluorescence has led to the widespread application of a newly advanced technology called multiplex immunohistochemistry (mIHC), which enables the detection of multiple fluorescent proteins on a tumor tissue microarray (TMA) within the same temporal and spatial organization. Using this mIHC technology, we detected six tumor-associated proteins, including cluster of differentiation 4 (CD4), cluster of differentiation 8 (CD8), Pan-cytokeratin (P-CK), forkhead box P3 (FOXP3), programmed cell death 1 (PD1) as well as programmed death ligand-1 (PDL1) in cancer tissues and para-carcinomatous normal tissues from a cohort of 79 colorectal cancer patients. Results showed that, in CRC tissues, expression levels of P-CK and FOXP3 were upregulated while CD4 expression decreased significantly in comparison with adjacent normal tissues. What's more, no significantly differential expression of CD8, PD1 or PDL1 was observed between cancer and normal tissues. FOXP3 expression was found to be correlated with tumor size (FOXP3 expression in tumor with volume >10 cm3 was significantly lower than that in tumor with volume ≤ 10 cm3), and reduced FOXP3 expression was associated with worse prognosis. P-CK expression in low-grade (Grade I-II) CRC patients was higher than that in advanced grade (Grade III-IV) patients, while association of P-CK expression with CRC prognosis was of no significance. In conclusion, FOXP3 and P-CK could be utilized as biopredictors of CRC (FOXP3 as a diagnostic and prognostic biomarker; P-CK as a diagnostic biomarker) for their differential expression patterns and clinicopathological correlation, while CD4, CD8, PD1 and PDL1 are more suitable for combined use.

Keywords: Colorectal cancer (CRC), Multiplex immunohistochemistry (mIHC), Tumor tissue microarray (TMA), Diagnosis, Prognosis, Biomarker

Introduction

Colorectal Cancer (CRC), one of the major causes of morbidity and mortality worldwide, is the second most common type of cancer in women and the third most common type of cancer in men, accounting for over 9% of all cancer incidence and causing death for more than 600,000 cases all over the world per year [1-4]. CRC is widely believed to develop in a multi-step process from Aberrant Crypt Foci (ACF), through benign and precancerous lesions (adenomas), to malignant tumors (adenocarcinomas) over an extended period of time [5]. Treatment of CRC usually comprises surgical resection of the primary tumors in patients followed by chemotherapy, radiotherapy and/or immunotherapy for advanced stages (stage III and IV) [6]. Despite advances in detection and available therapeutic strategies, the clinical outcomes for CRC remain poor due to tumor recurrence, metastasis, and resistance to radio-/chemo-therapy [7,8].

Early diagnosis of CRC is of importance for its significant impacts on cancer management, prognosis, recurrence and survival [9-11]. The 5-year survival rate could rise up to 90% in CRC patients who were diagnosed in the early stage, but unfortunately, the great majority of CRC cases had developed to an advanced stage at the time of diagnosis with a low survival rate around 8-9% [12,13]. Invasive techniques used for CRC diagnosis including endoscopic and radiological imaging suffered from poor patient compliance [14]. In addition, tumor markers such as carbohydrate antigen 19-9 (CA 19-9) and Carcinoembryonic Antigen (CEA) commonly used in clinical circumstance have the problems of unsatisfactory sensitivity and specificity, resulting in limited clinical application in CRC diagnosis, prognosis and survival [15]. Thus, the development of noninvasive and accurate screening tools for early detection and precise staging of CRC are of great importance and significance.

Conventional Immunohistochemistry (IHC) is a diagnostic technique widely used in the field of tissue pathology. However, IHC suffers from a number of limitations such as relatively high interobserver variability and limited labelling of a single marker per tissue section, resulting in missed opportunities of important diagnostic and prognostic information [16-18]. By contrast, multiplex Immunohistochemistry (mIHC), allowing simultaneous detection of multiple markers on a single tissue section, has emerged as a promising technology for its capability of provision of high throughput multiplex immunohistochemical staining and standardized quantitative analysis of highly reproducible and efficient tissue studies, as well as comprehensive study of cellular component, marker expression patterns, relative spatial distribution of multiple cell types and cell-cell interactions, which are of benefit to diagnostic accuracy [19-21].

In light of this, we analyzed the expression levels and potential clinicopathological prognosis values of six tumor-associated proteins including cluster of differentiation 4 (CD4), cluster of differentiation 8 (CD8), Pan-cytokeratin (P-CK), forkhead box P3 (FOXP3),

programmed cell death 1 (PD1) and programmed death ligand-1 (PDL1) in colorectal cancer, relying on 7-color fluorescent multiplex immunostaining of tumor tissue microarray (TMA) from a cohort of 79 cancer patients.

Materials and Methods

Information for Patients

The HColA180Su17 tumor Tissue Microarray (TMA) (Outdo, Shanghai, China) consisted of paired colorectal adenocarcinoma tissues and adjacent normal tissues derived from 79 colorectal cancer patients. These patients underwent surgery from Jun. 2006 to Apr. 2007, and the follow-up information was available from Sep. 2007 to Jul. 2015. The study was conducted under the approval of the Institutional Ethics Committee and all procedures were performed according to relevant guidelines and regulations for research. The clinicopathological characteristics of 79 cancer patients were summarized in Table 1.

Preparation of Tissue Microarray (TMA)

Tissue Microarray (TMA) was made on basis of pathological diagnosis of each tissue. Briefly, formalin-fixed and paraffin-embedded samples were identified as well as the specimens were reviewed with hematoxylin and eosin stain by an independent surgical pathologist in order to confirm the presence of colorectal cancer and adjacent normal tissues [22]. For the formation of TMA, core cylinders (1 mm) were punched from each of circled areas and stored in a recipient paraffin block after circling of at least two representative tumor areas from each block by the pathologist. At last, consecutive TMA sections (6 mm thick) were cut and placed onto poly-L-lysinecoated slides for subsequent analysis [23].

Fluorescent mIHC of TMA

For multiplex Immunohistochemistry (mIHC) staining, antibodies for CD4, CD8, PCK, FOXP3, PD1 and PDL1were optimized by concentration and application order, meanwhile, a spectral library was built based on the single-stained slides [24]. The multiplex immunofluorescence staining and multispectral imaging of these six proteins were obtained on a TMA slide using Opal Polaris 7 Color Manual IHC Detection Kit (cat NEL861001KT, Akoya, US). In brief, the slide was deparaffinized by xylene for 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 3 min, slide was pretreated with 100 ml citric acid solution (pH6.0/pH9.0) for antigen retrieval with microwaving (15 min on 20% power after 45 s on 100% power) and transferred to a slide jar containing 1xTBST to mix well. Afterwards, the slide was blocked in 10% blocking solution for 10 min, stained respectively with primary antibody against CD4, CD8, P-CK, FOXP3, PD1 or PDL1 for 1 h at room temperature, washed with 1xTBST for 3 min twice and incubated with polymer HRPanti-mouse/rabbit IgG secondary antibody for 10 min at room temperature. The slide was covered by Tyramide (TSA)-conjugated fluorophore (TSA Fluorescence Kits, Panovue, Beijing, China) at 1:100 dilution and incubated for 10 min at room temperature, washed with 1xTBST for 3 min twice for next staining procedure. Furthermore, the process was repeated by microwave heat-treating the slide for antigen

Table 1: Clinicopathological characteristics of a cohort of 79 colorectal cancer patients.

Clinicopathological characteristics (N=79)	Number	Proportion (%)
Gender		
Male	38	48.10%
Female	41	51.90%
Age (years)		
≤65	36	45.57%
>65	43	54.43%
T stage		
T1	1	1.27%
T2	5	6.33%
T3	58	73.42%
T4	15	18.98%
Lymph node (N stage)		
Negative (N0)	48	60.76%
Positive (N1a, b-N2a, b)	31	39.24%
Metastasis (M stage)		
Negative (M0)	78	98.73%
Positive (M1a, b)	1	1.27%
TNM stage		
I	5	6.33%
II A	34	43.04%
II B	6	7.59%
II C	3	3.80%
III A	0	0.00%
III B	28	35.44%
III C	3	3.80%
IV A	0	0.00%
IV B	0	0.00%
Pathological grade		
I	16	20.25%
II	50	63.29%
III	12	15.19%
IV	1	1.27%
Histology		
Adenocarcinoma	31	39.24%
Canalicular adenoma	41	51.90%
Mucinous adenocarcinoma	6	7.59%
Signet-ring cell carcinoma	1	1.27%
Disease status at last follow-up		
Survival	42	53.16%
Death	37	46.84%

retrieval for every additional marker in mIHC assay, followed by one primary antibody staining during each cycle ordered as CD4, CD8, P-CK, FOXP3, PD1 and PDL1, respectively, and then downstream procedures as mentioned above. After labelling of all these human antigens, cell nucleus were counterstained with 4',6diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, US). Detailed information about primary antibodies was summarized in Table 2.

Table 2: Primary antibodies used for mIHC staining.

Antibodies	Dilution	Antibody Type	Catalogue#	Vender
CD4	1:200	Rabbit monoclonal	ab133616	Abcam
CD8	1:100	Mouse monoclonal	NBP2-34039	NOVUS
P-CK	1:100	Mouse monoclonal	GM351529	Gene Tech
FOXP3	1:200	Mouse monoclonal	14-4777-83	Thermo
PD1	1:200	Mouse monoclonal	GT228129	Gene Tech
PDL1	1:200	Rabbit monoclonal	ab213524	Abcam

Multispectral Imaging

The stained slide was scanned using the Vectra Polaris (Akoya, US) to obtain multispectral images, which precisely captures the fluorescent spectra from 420 to 720 nm (at 20-nm wavelength intervals) with identical exposure time. Next, the scans were combined into a single stack image with high contrast and accuracy

Scoring Multispectral Images

InForm Tissue Analysis Software (Akoya, US) was used in batch analysis of experimental multispectral images [25]. Firstly, images of single-stained and unstained sections were used to respectively extract the fluorescent spectrum of each fluorescein and autofluorescence of tissues. Secondly, the extracted images were used in establishment of a spectral library for multispectral unmixing by InForm image analysis software. Finally, using this established spectral library, gain of reconstructed images of sections with removed autofluorescence was fulfilled. In order to score multispectral images, three to six representative regions of interest for imaging (200×) from each case were selected. A few representative multispectral images were then loaded into analysis software to build an algorithm for segmenting tissues and cells. Next, two tissue categories of STROMA and TUMOR were trained in accordance with intensity of DAPI signals, these detected tissue compartments were selected and quantified for each stained target proteins, and corresponding number of positive and total cells were counted as well. 4-bin (0, 1+, 2+, 3+) scoring system was used for quantification of expression levels of target proteins by calculating H-score (a score which was calculated using the percentage in each bin and ranges from 0 to 300) with cell stains. Results of H-score were shown by the positive rate of cells in each bin, including four levels (0~1, 1~2, 2~3, 3~) so as to measure and categorize protein expression levels into negative, low, medium and high levels, respectively. Generally, H-score with 0~1 and 1~2 (0, 1+) were considered as low expression level, while score with 2~3 and 3~ (2+, 3+) were considered as high expression level.

Statistical Analysis

The significance of experimental data from patient specimens was determined by the Mann-Whitney U test. The Kaplan-Meier test was used to assess overall survival (OS) rates, and survival curves were plotted by the log-rank test. *$P<0.05$ was considered as statistically significant, **$P<0.01$ and ***$P<0.0001$ were considered as strongly significant. Statistics software GraphPad Prism version 8 was used for all statistical analyses.

Results

Demographics

A following-up for the cohort of 79 CRC patients was performed from 2008 to 2015 for the evaluation of a seven-year survival. Among these eight clinicopathological characteristics including gender, age, tumor size, T stage, N stage, M stage, TNM stage and pathological grade, the survival was associated with three of them, namely N stage, TNM stage as well as pathological grade. The results showed that prognosis of patients with negative lymph nodes (N0), early TNM stage (TNM I-II) and low pathological grade (Grade I-II) were significantly better than those with positive lymph nodes (N1-2), late TNM stage (TNM 3-4) and advanced pathological grade (Grade III-IV) ($P<0.05$, Figure 1 and Table 3).

Table 3: Prognostic clinicopathological characteristics of a cohort of 79 colorecta cancer patients.

Clinicopathological characteristics	HR (95%CI)	*P* Value
Gender (male vs. female)	0.806 (0.410-1.584)	0.532
Age (yeas≤65 vs. yeas>65)	0.755 (0.384-1.484)	0.415
Tumor size (V≤10 cm³ vs. V>10 cm³)	0.783 (0.327-1.876)	0.584
T stage (T1-3 vs. T4)	0.812 (0.333-1.978)	0.646
N stage (Negative vs. Positive)	0.3709 (0.179-0.768)	<0.01
M stage (Negative vs. Positive)	2.787 (0.179-43.36)	0.464
TNM (TNM I-II vs. TNM III-IV)	0.413 (0.201-0.852)	<0.01
Pathological grade (I-II vs. III-IV)	0.209 (0.071-0.611)	<0.01
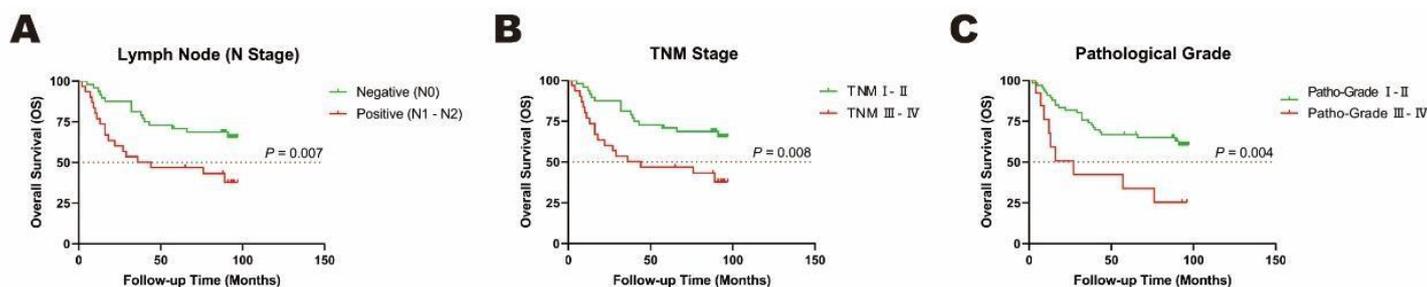
Figure 1: Overall survival (OS) rates of clinicopathological characteristics analyzed by Kaplan-Meier test. **A.** Lymph Node (N Stage), **B.** TNM Stage, **C.** Pathological Grade as clinical prognostic factors in cancer tissues in a cohort of 79 CRC patients. Orange dotted line: Overall survival (OS) rates as 50%.

Fluorescent mIHC Profile on TMA Slides Derived from Colorectal Cancer Patients

In order to obtain multiple fluorescent images, the TMA slides were trained according to intensity of DAPI signals before the selection of detected tissue compartments for each stained target proteins on slides. All six antibodies of CD4, CD8, P-CK, FOXP3, PD1 and PDL1 were then performed ahead of the quantification of protein expression level by scoring system to calculate H-score based on cell fluorescence. In detected tissue compartments and cells, images of monochromatic proteins were shown in the upper row ordered as DAPI, CD4, CD8, P-CK, FOXP3, PD1 as well as PDL1 (Figure 2). In addition, merged images of the multispectral fluorescence of these target proteins and DAPI were displayed at the bottom of the figure. The selected images displayed tumor (Figure 2A) and adjacent normal (Figure 2B) tissues, respectively.

Determination of Significant Markers by Fluorescent mIHC in Colorectal Cancer Patients

In a cohort of 79 colorectal patients, comparison of the expression levels of CD4, CD8, P-CK, FOXP3, PD1 and PDL1 were performed between tumor and paracarcinomatous normal tissues for the exploration of cancer associated potential biomarker. As shown in Figure 3 for monochromatic proteins, expressions of P-CK and FOXP3 were upregulated while CD4 expression decreased significantly in cancer tissues compared with adjacent normal tissues (Figure 3A, $P<0.05$; Figure 3C, $P<0.001$; Figure 3D, $P<0.01$). As shown in Figure 4 for bi- as well as multi-chromatic combinations, except that the expression levels of bichromatic CD4/P-CK, CD8/P-CK and P-CK/FOXP3, trichromatic CD4/CD8/P-CK, multichromatic CD4/CD8/P-CK/FOXP3 and CD4/CD8/P-CK/FOXP3/PD1/PDL1 were of significant differences (Figure 4A to 4F, $P<0.001$), differential expressions were not observed in other combinations in cancer tissues. As to the compared expression levels of single, double or multiple stained combinations of these six target proteins, all data were analyzed by Mann-Whitney U test and the P values were shown in Table 4.
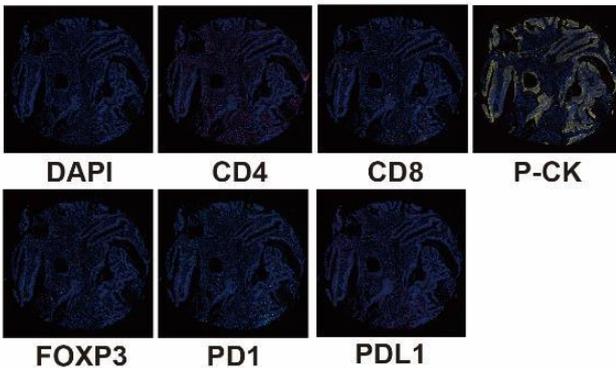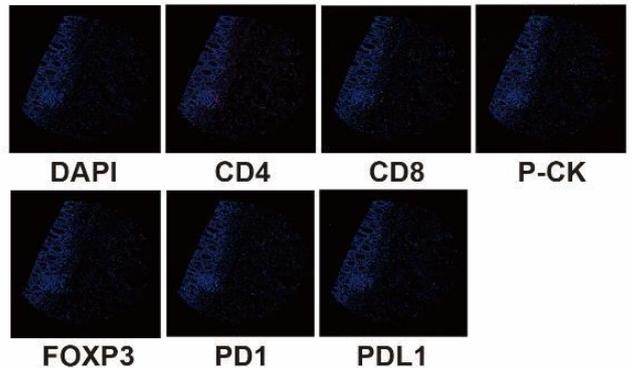

Figure 2: Mono- and multi-chromatic mIHC profile of colorectal cancer and adjacent normal tissues. **A, B.** Representative images of monochromatic and multispectral fluorescence in tissues from colorectal cancer and adjacent normal areas. Small images in the upper row displayed selected tissue compartments stained by DAPI, CD4, CD8, P-CK, FOXP3, PD1 and PDL1. Large images at the bottom showed a merged multispectral fluorescence from DAPI, CD4, CD8, P-CK, FOXP3, PD1 and PDL1.
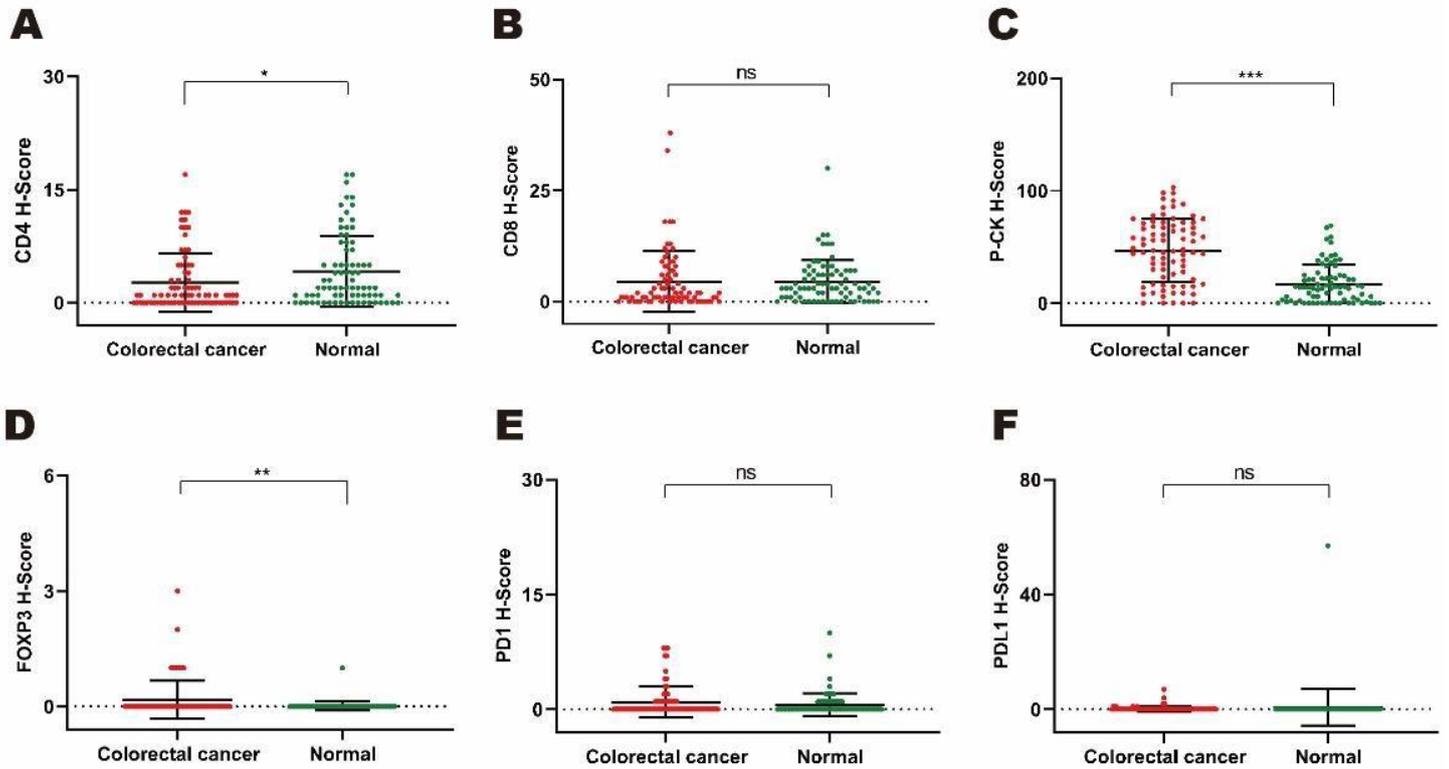
Figure 3: Comparing expression levels of monochromatic target proteins based on Hscores by mIHC from tumor versus normal tissues in a cohort of 79 colorectal cancer patients. **A to F.** Differential expression patterns of single stained proteins including CD4 (3A. $P < 0.05$), CD8 (3B, $P = 0.915$), P-CK (3C, $P < 0.001$), FOXP3 (3D, $P < 0.01$), PD1 (3E, ns. $P = 0.231$) and PDL1 (3F, $P = 0.511$).
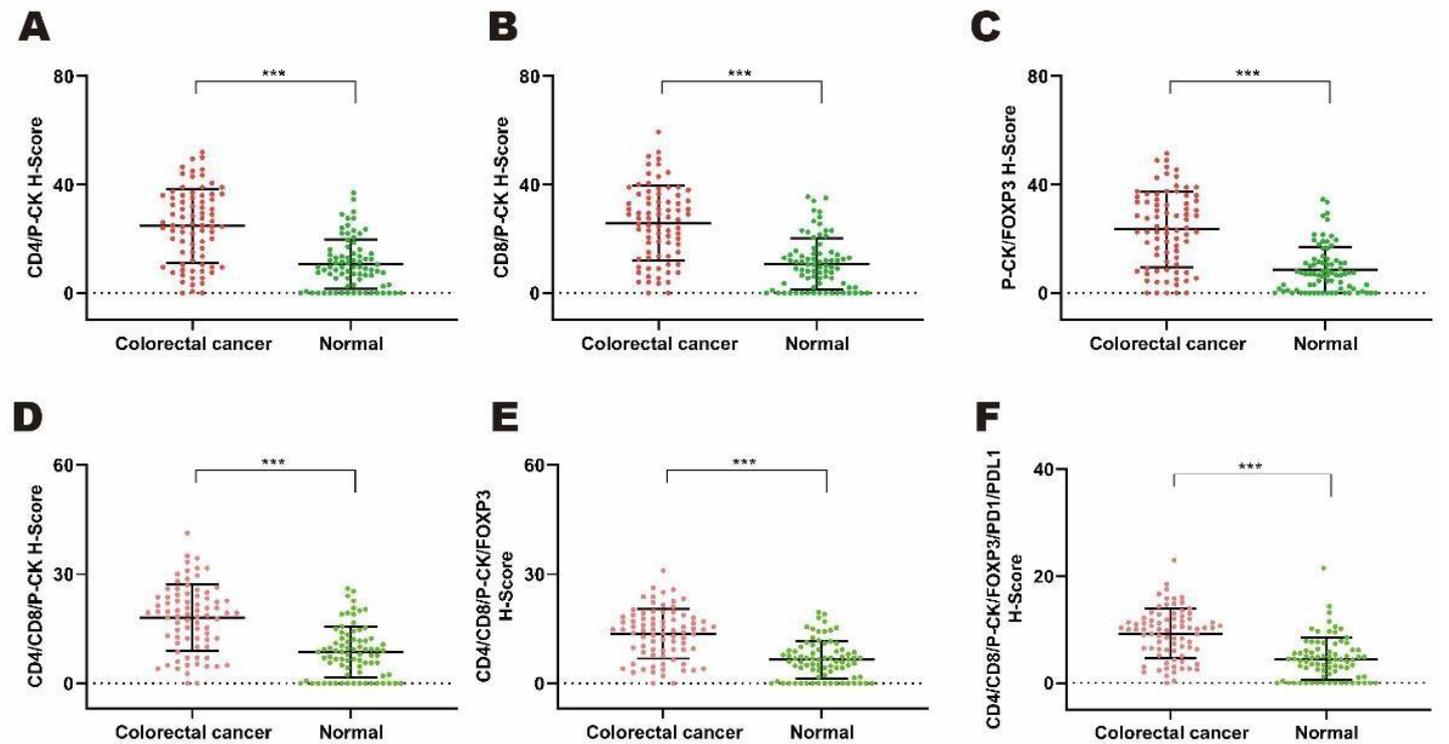
Figure 4: Comparing expression levels of di- and multi-chromatic target proteins based on H-scores by mIHC from tumor versus normal tissues in a cohort of 79 colorectal cancer patients. **A to F.** Comparing expression patterns of combination of double and multiple stained proteins including CD4/P-CK (3A. $P < 0.001$), CD8/PCK (3B, $P < 0.001$), P-CK/FOXP3 (3C, $P < 0.05$), CD4/CD8/P-CK (3D, $P < 0.001$), CD4/CD8/P-CK/FOXP3 (3E, $P < 0.001$), and CD4/CD8/P-CK/FOXP3/PD1/PDL1 (3F, $P < 0.001$).

Table 4: Differential expression of mIHC markers in cancer vs. normal tissues.

mIHC target proteins	Cancer vs. Normal (N=79)
CD4	$P < 0.05$
CD8	$P=0.915$
P-CK	$P < 0.001$
FOXP3	$P < 0.01$
PD1	$P=0.231$
PDL1	$P=0.511$
CD4/CD8	$P=0.340$
CD4/P-CK	$P < 0.001$
CD8/P-CK	$P < 0.001$
CD4/FOXP3	$P=0.058$
CD8/FOXP3	$P=0.781$
P-CK/FOXP3	$P < 0.001$
PD1/PDL1	$P=0.859$
CD4/CD8/P-CK	$P < 0.001$
CD4/CD8/FOXP3	$P=0.402$
FOXP3/PD1/PDL1	$P=0.975$
CD4/CD8/P-CK/FOXP3	$P < 0.001$
CD4/CD8/PD1/PDL1	$P=0.392$
CD4/CD8/FOXP3/PD1/PDL1	$P=0.481$
CD4/CD8/P-CK/FOXP3/PD1/PDL1	$P < 0.001$

Table 5: Correlation between mIHC target proteins and clinicopathological characteristics.

Clinicopathological characteristics	P value					
	CD4	CD8	P-CK	FOXP3	PD1	PDL1
Gender (male vs. female)	0.901	0.232	0.540	0.351	0.375	0.656
Age (yeas ≤65 vs. yeas >65)	0.805	0.419	0.503	0.491	0.768	0.730
Tumor size (V≤10 cm³ vs. V>10 cm³)	0.843	0.673	0.817	<0.01	0.695	0.912
T stage (T1-3 vs. T4)	0.587	0.780	0.995	0.527	0.350	0.231
N stage (negative vs. positive)	0.778	0.432	0.877	0.253	0.784	0.194
M stage (negative vs. positive)	-	-	-	-	-	-
TNM (TNM I-II vs. TNM III-IV)	0.775	0.424	0.866	0.533	0.835	0.939
Pathological grade (I-II vs. III-IV)	0.417	0.423	<0.05	0.924	0.526	0.537

Correlation between Six Proteins and Clinicopathological Characteristics

Statistic analyses were performed by Mann-Whitney U test to explore the correlation between six proteins (CD4, CD8, P-CK, FOXP3, PD1 and PDL1) and eight cancer related clinicopathological factors (gender, age, tumor size, T stage, lymph node, metastasis, TNM stage, pathological grade). FOXP3 and P-CK were found to be correlated with tumor size and pathological grade, respectively , even though most of the correlations were of no significance (Table 5). Among which, expression of FOXP3 in tumor with volume>10 cm³ (N=65) was significantly lower than that in tumor with volume≤10 cm³ (N=14) (Figure 5A, $P<0.01$), and expression of P-CK in low pathological grade (Grade I-II) (N=66) was higher than that in advanced grade (Grade III-IV) (N=13) (Figure 5B, $P<0.05$).

## Association of Prognosis Markers with Clinical Outcomes

For purpose of prognosis potential of these six proteins in CRC, expression of each protein was divided into low-expressed group (H-score 0 to 1+) and high-expressed group (H-score 2+ to 3+) on the basis of H-score representation calculated by the fluorescence intensity from three to six representative regions of each sample. Association of low-/high-level protein expression with the seven-year overall survival (OS) status of 79 CRC patients were analyzed by Kaplan-Meier test. Compared with CD4 (Figure 6A, $P=0.122$), CD8 (Figure 6B, $P=0.905$), P-CK (Figure 6C, $P=0.406$), PD1 (Figure 6E, $P=0.582$) and PDL1 (Figure 6F, $P=0.156$), FOXP3 was the only one with statistically significant association with CRC prognosis (Figure 6D, $P<0.05$). CRC patients with high-level FOXP3 expression (N=6) seemed to have a longer OS time than those with low-level expression (N=73) (Figure 6D, $P<0.05$), which supported the tumor-growth potential of low-expressed FOXP3 observed in our study (Figure 5A, $P<0.01$).

Discussion

In this study, we performed multiplex immunohistochemistry analysis on six target proteins to explore the correlation of these molecules with colorectal cancer. FOXP3 (forkhead box P3, also named IPEX, PIDX) is a member of the forkhead box (FOX) family of
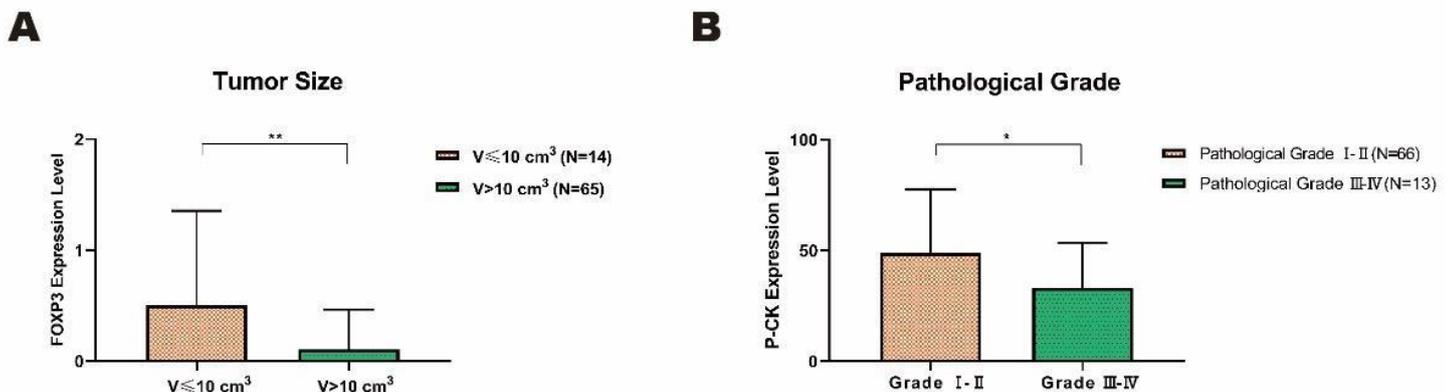
Figure 5: Significant correlations between two target proteins and clinicopathological characteristics in colorectal cancer tissues. **A.** The expression level of FOXP3 significantly declined in larger tumor (V>10 cm³) in comparison with smaller tumor (V≤10 cm³). **B.** The expression level of P-CK in pathological grade I and II was significantly higher than that in advanced grade III and IV. Data on the graph was displayed as mean ± SD (*$P < 0.05$, **$P < 0.01$).
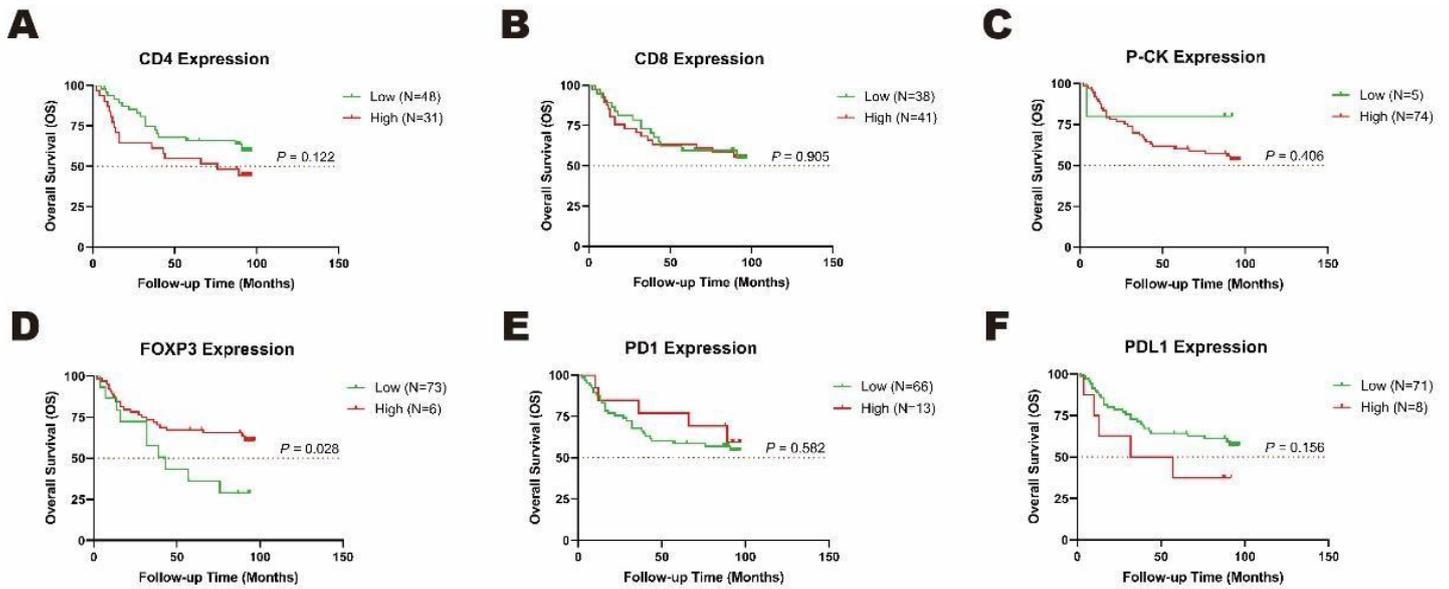
Figure 6: Overall survival (OS) rates with differential expression levels of CD4 and FOXP3 analyzed by Kaplan-Meier test. The low- and high-expression of **A.** CD4 and **B.** FOXP3 were associated with a status of seven-year survival in cancer tissues in a cohort of 79 CRC patients. Orange dotted line: half of OS rates as 50%.

transcription factors consisting of an evolutionarily conserved group of transcriptional regulators whose dysfunction has been associated with human malignant neoplasias [26-35]. FOXP3 is mainly expressed in regulatory T (Treg) cells, and it has also been found in other cells such as B lymphocytes [36-42]. FOXP3 was described as an important molecular actor involved in the development and function of Treg cells playing essential roles in the regulation of autoimmunity, infection and tumor environment [36-38]. FOXP3 is considered as a molecule at the crossroads of tumorigenesis and immunity for its bilateral role of cancer promotor or suppressor [42-46]. Furthermore, the role of FOXP3 in the biogenesis, development as well as clinical prognosis of colorectal cancer is still not completely understood, thereinto, infiltration of FOXP3+ Treg cells indicated favorable prognosis in some but not all studies [47-57]. Our results showed that the expression level of FOXP3 was not only significantly upregulated in tumor tissues (Figure 3D, $P<0.01$), but also associated with tumor size (Table 5, $P<0.01$). Additionally, FOXP3 expression in CRC patients with tumor volume>10 cm³ was significantly lower than that in patients with tumor volume≤10 cm³ (Figure 5A, $P<0.01$), indicating a tumor growth potential of FOXP3 with low expression in CRC, consistently, reduced expression of FOXP3 was associated with worse prognosis (Figure 6D, $P<0.05$). All these results showed that FOXP3 could be applied as a potential biomarker for CRC diagnosis and prognosis.

CD4, a membrane glycoprotein of T lymphocytes, is expressed not only in T lymphocytes, but also in B cells, macrophages as well as granulocytes. CD4 acts as a coreceptor with the T-cell receptor on T lymphocytes in recognition of antigens displayed by antigen-presenting cells in the context of class II major histocompatibility complex (MHC) molecules, and functions to initiate or augment the early phase of T-cell activation. Similarly, CD8, a cell surface glycoprotein found on most cytotoxic T lymphocytes mediating immune cell-cell interactions, acts as a coreceptor with the T-cell receptor on T lymphocytes to recognize antigens displayed by antigen-presenting cells in the context of class I

MHC molecules. In general, CD4+ and CD8+ T cells identify antigens related to cancer cells and play significant roles in cancer immunology and immunotherapy [58,59]. Additionally, various studies have demonstrated that CD4+ and CD8+ T cells may also control tumor growth [60,61]. Increased levels of CD4+ and CD8+ T cells in colorectal tumor microenvironment were shown to correlate with improved response to chemoradiotherapy [62]. Results of prevenient studies suggested that levels of tumor infiltration by CD4+ and CD8+ T cells may be good predictive factors for patient clinical prognosis of various tumors including colorectal cancer, melanoma, oesophageal squamous cell carcinoma, ovarian cancer, pancreatic cancer and renal cancer [63-71]. Differently, compared with adjacent normal tissues, the expression levels of CD4 and CD8 in colorectal cancer tissues here decreased significantly and had no difference, respectively (Figure 3A and 3B), suggesting reduced immune infiltration of CD4+ and CD8+ T cells. What's more, association of differential expression levels of CD4 or CD8 with clinical outcomes of CRC patients was of no significance (Figure 6A and 6B), and they had no significant association with tumor size or other clinicopathological characteristics (Table 5). Malignant tumors like CRC can cause the functional loss of antigen recognition, cell proliferation and activation of effector T cells, which is known as T cell exhaustion accompanied by the activation of multiple inhibitory receptors such as CTLA4 and PD1/PDL1 [72-74]. The decreased infiltration and effects on prognosis of CD4+ and CD8+ T cells observed here may be related to T cell exhaustion. Generally, the biological behaviors of cancers are influenced by the functional status of tumor-infiltrating immune cells whose roles in response to cancer are component- and stage-dependent. The CD4+ T cells consist of multiple morphologically and functionally distinctive subpopulations such as T regulatory (Treg) cells, T helper 1 (Th1), Th2, Th9, Th17 and follicular helper T (Tfh) cells, whose roles related to proinflammation and/or antiinflammation, activation and infiltration of CD8+ T cells in tumor microenvironment display tumor-promoting or tumor-suppressing effects in a case-dependent manner [75-87]. The limited

tumor infiltration and functional potential of CD8$^+$ T cells over served in this study might be partially due to a lack of CD4$^+$ Th cell-mediated function.

PD1 (programmed cell death 1, also named PDCD1, CD279), an immunoinhibitory receptor belonging to the CD28 family that is expressed on activated T cells, is involved in T cell proliferation and functional regulation including those of effector CD8$^+$ T cells, and also able to promote the differentiation of CD4$^+$ T cells into T regulatory cells [88-90]. PD1 is expressed in many types of tumors and has demonstrated to play roles in anti-tumor immunity, safeguarding against autoimmunity as well as the inhibition of effective anti-tumor and anti-microbial immunity. Furthermore, PD1 interacts with ligand PDL1 (also named B7-H1, CD274) to form the PD1/PDL1 axis, an immune checkpoint which is usually up-regulated to help tumor cells avoid immune destruction in an immunosuppressive tumor microenvironment [91]. Overexpressed PDL1 can protect tumor cells by inhibiting the activity of PD1 expressing adjoining tumor-infiltrating effector CD4$^+$/CD8$^+$ T cells [92]. PDL1 is mainly expressed on the surface of antigen-representing and tumor cells in various types of cancer such as carcinomas of the adrenal cortex, bladder, brain, breast, colorectum, esophagus, gastrointestinal tract, kidney, liver, lung, ovary, pancreas, thymus, thyroid and urothelium [93]. Contradictory correlations of expression of PD1 and/or PDL1 with prognosis results and clinicopathological characteristics of colorectal cancer were observed (some investigations showed that overexpression of PD1 and/or PDL1 forecasted better prognosis, while others presented opposite results), even if PD1 and PDL1 may play oncogenic roles in colon cancer carcinogenesis [94-98]. Again, in our study, no significant difference was observed between expression of either PD1 or PDL1 in colorectal cancer tissues and those in normal tissues (Figure 3E and 3F), they also had no significant association with pathological grade or other characteristics (Table 5). Differential expression of PD1 or PDL1 was not statistically associated with CRC prognosis (Figure 6E and 6F). All these results indicated that PD1 and PDL1, in comparison to using alone, are more suitable for combination with other proteins for the application as potential biopredictors of CRC diagnosis.

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