Cyclin-dependent kinase 9 expression and its association with CD8+ T cell infiltration in microsatellite-stable colorectal cancer

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Abstract. Programmed death 1 (PD-1)-targeted therapy has benefited patients with microsatellite instability-high metastatic colorectal cancer (mCRC). However, the efficacy of PD-1-targeted therapy is poor in patients with microsatellite-stable (MSS) mCRC. Therefore, it is imperative to explore additional co-inhibitory molecular signalling pathways to improve the efficacy of immunotherapy in MSS mCRC treatment. In the present study, the association between cyclin-dependent kinase 9 (CDK9) expression and the survival of patients with CRC was analysed using RNA sequencing data from 605 patients, including 121 cases of mortality, from human cancer datasets. Furthermore, 35 clinical MSS stage III-IV CRC specimens were collected to assess CDK9 protein expression by immunohistochemistry, and the frequency of tumor-infiltrating $CD8^+$ T cells was assessed by flow cytometry. The human cancer datasets demonstrated that upregulation *CDK9* significantly shortened the survival of patients with stage II-IV colon cancer. Additionally, *CDK9* mRNA expression was positively correlated with the expression levels of genes associated with immune evasion in the tumor. Notably, CDK9 was expression was upregulated in stage IV CRC compared with para-cancerous tissues and early-stage tumors. Interestingly, CDK9 expression was negatively associated with the infiltration of $CD8^+$ T cells at the tumor site. In addition, the expression levels of T-cell immunoglobulin mucin family member 3 and CD39, proteins associated with exhaustion, on tumor-infiltrating $CD8^+$ T cells were significantly elevated in patients with abnormal CDK9 expression levels. The present study demonstrated that CDK9 expression was negatively associated with $CD8^+$ T cell infiltration and positively associated with $CD8^+$ T cell exhaustion in MSS mCRC. In conclusion, CDK9 may be utilized to evaluate the prognosis and the immune-type of the tumor microenvironment in patients with MSS mCRC.

Introduction

Colorectal cancer (CRC) has become the third most common type of cancer and the second leading cause of cancer-associated mortality worldwide (1). In the United States, the 5-year relative survival rate is 90.1 or 69.2% for patients with CRC with localized (stage I-II) or regional metastasis (stage III), respectively. However, the 5-year relative survival rate is only 11.7% for patients with distant metastasis (stage IV) (2). Therefore, improving the diagnosis and treatment efficacy of patients with stage IV CRC is the key to ameliorating the overall survival of CRC.

The American Society of Clinical Oncology (ASCO) Annual Meeting in 2016 named 'Immunotherapy' as the 'Primary Progress' in Cancer Research in 2015 (3). The Federal Drug Agency and China Food and Drug Administration (CFDA) have approved pembrolizumab, an anti-PD-1 inhibitor, for the treatment of microsatellite instability-high (MSI-H) or mismatch repair-deficient (dMMR) metastatic CRC (mCRC) (4). Numerous studies have reported that 44.8-89% of patients with CRC express PD-L1, thus suggesting that abnormal expression of PD-L1 is an independent poor prognostic indicator in CRC (5,6). However, data from clinical trials revealed that PD-1/PD-L1 blockades are only effective in 2.5-5% of patients with mCRC (MSI-H or dMMR), and the vast majority of patients [microsatellite stable (MSS)] does not benefit from this treatment (7-9). This is a great obstacle to the application of immunotherapy in CRC.

Chen and Mellman (10) analysed data from patients undergoing immunotherapy. According to the clinical efficacy and the immunophenotype of the patients, the tumor immuno-microenvironment was divided into three basic types: The immune-desert phenotype, immune-excluded tumor and inflamed tumor (10).

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Abbreviations: CRC, colorectal cancer; mCRC, metastatic colorectal cancer; CDK9, cyclin-dependent kinase 9; ASCO, American Society of Clinical Oncology; CFDA, China Food and Drug Administration; MSI-H, microsatellite instability-high; MSS, microsatellite stable; dMMR, mismatch repair deficient

Key words: CDK9, immunotherapy, $CD8^+$ T cell exhaustion, microsatellite stable, colorectal cancer

Based on this classification, the effectiveness of PD-L1 inhibitors depends not only on the expression of intra-tumor PD-L1 but also on the existence of sufficient immune effector cells, particularly $CD8^+$ T cells, in the tumor microenvironment (TME). Without the infiltration of $CD8^+$ T cells, even if elevated expression of PD-L1 is detected in tumor tissues, a therapeutic effect could not be achieved; this is the case for patients with the immune-desert phenotype (10). It has previously been reported that the number of tumor-infiltrating $CD4^+$ and $CD8^+$ T cells in patients with MSI-H CRC who benefit from pembrolizumab immunotherapy is significantly higher than that in MSS CRC (11). This may be due to patients with MSI-H CRC producing a large number of mutant proteins in tumor tissues, which can stimulate the immune response and promote the local lymphocyte infiltration of tumors (7,12). Therefore, promoting the infiltration of immune effector cells, particularly $CD8^+$ T cells, into tumor tissues and improving their immune function are effective measures to increase the efficacy of immunotherapy in the majority of patients with CRC (MSS phenotype).

Cyclin-dependent kinase 9 (CDK9) is a key regulator of transcriptional elongation, which is a promising therapeutic target in cancer, particularly for types of cancer driven by transcriptional dysregulation (13,14). However, the mechanism and clinical transformation of CDK9 in CRC have been rarely reported (15). In addition, it has been demonstrated that APC/BRAF/SMAD4 gene mutations lead to upregulation of transcription, which leads to the occurrence and development of CRC (16-18). Positive transcription elongation factor (P-TEFb)/CDK9 triggers the release and nuclear export of β-catenin by the α-catenin:APC complex (19). CDK8 and CDK9 provide the coordinated regulation of SMAD transcriptional activators in the bone morphogenetic protein and transforming growth factor β1 signalling pathways (20). Therefore, CDK9 serves an important role in the development of CRC. In addition, studies have demonstrated that CDK9 promotes the proliferation and differentiation of immune cells, including neutrophils, macrophages and lymphocytes, alters the ratio of $CD4^+CD25^+$ forkhead box $P3^+$ Tregs, and regulates the expression of chemokines, thereby affecting the infiltration of effector T cells in an inflammatory environment (21-23). The present study utilized human cancer datasets and clinically resected CRC specimens to assess CDK9 expression and $CD8^+$ T cell infiltration, and examined their alterations in mCRC. The results of the present study suggested that CDK9 may be a promising therapeutic target for patients with MSS mCRC.

Materials and methods

Analysis of public datasets. RNA sequencing-based gene expression data in CRC was obtained from the Gene Expression Profiling Interactive Analysis (GEPIA) database for cancer genomics (24). The association between CDK9 expression and the survival of 605 patients with CRC, including 121 patients who died, was analysed using the Tumor Immune Estimation Resource (TIMER) database (25). The co-expression of proteins was analysed by the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (26). For gene expression, $P<0.01$ was used as the cut-off. The Kaplan-Meier method with a log-rank test was used for estimating patient survival rates. $P<0.05$ was considered to indicate a statistically significant difference.

Patient tissue samples. A total of 35 patients with CRC who were admitted and treated at Tianjin Medical University Cancer Institute and Hospital between May 2018 and September 2018 were enrolled randomly. The patients comprised 16 men and 19 women, aged 27-79 years. During surgical resection, specimen (both tumor and paired control tissues) were obtained from the treatment-naïve patients with CRC. The gene stability of *BAT-25, BAT-26, NR-21, NR-24* and *Mono-27* in the specimens from all patients was examined using next-generation sequencing (Hongzhong Precision Medicine). All patients had MSS CRC according to the definition of the National Cancer Institute (there was no 'instability' in the results of the five aforementioned loci) (27). All samples and clinical data were collected after ethical approval was granted by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. Written informed consent was obtained from all patients for participation in the present study. The clinical features of the patients are presented in Table I. The records of all of the patients contained basic information, including Tumor-Node-Metastasis (TNM) stage, the degree of differentiation, lymph node metastasis and distant metastasis, according to the 2002 International Cancer Alliance TNM staging criteria (28).

Isolation of tissue-infiltrating cells. Fresh colorectal tumor tissues and paired control tissues from patients with CRC were prepared by mechanical disruption, followed by digestion with 0.5 mg/ml collagenase type IV (cat. no. C5138; Sigma-Aldrich; Merck KGaA) in 10% FBS with 10 U/ml DNase I in RPMI-1640 medium (both from Gibco; Thermo Fisher Scientific, Inc.) at 37˚C for 30 min. Digested tissues were incubated for 5 min at 37˚C with EDTA (0.5 M) to prevent dendritic cell/T-cell aggregation and filtered through a 100-μm and a 40-μm filter. The isolation and culture of tissue/tumor-infiltrating cells were performed as previously described (29).

Immunohistochemistry (IHC). Colon and rectal tumor tissues, and paired normal tissues were were fixed with 4% formaldehyde for 24 h at room temperature (RT), embedded in paraffin and cut into 4-μm sections. The sections were dewaxed with xylene then hydrated through a graded series of ethanol (100, 95, 90, 80 and 70%) for 5 min in each percentage at RT. Slides were boiled in 10 mM sodium citrate buffer pH 6.0 at 95˚C for 10 min and subsequently incubated in 3% hydrogen peroxide for 10 min. Sections were blocked with 100-400 μl 5% normal goat serum in TBS-Tween (cat. no. 5425; Cell Signaling Technology, Inc.) for 1 h at RT. To assess CDK9 expression in the MSS phenotype mCRC specimens, sections were incubated with a CDK9 antibody (dilution, 1:100; cat. no. 2316; Cell Signaling Technology, Inc.) overnight at 4˚C, and were then incubated with a horseradish peroxidase-conjugated secondary antibody (dilution, 1:1,000; cat. no. ab6721; Abcam) at room temperature for 1 h. Following washing with PBS, the sections were incubated for 30 min at RT with streptavidin-biotin conjugated with horseradish peroxidase [dilution, 1:100; cat. no. KIT-0305; UltraSensitive™ SP (Mouse/Rabbit) IHC kit]. Subsequently, the slides were stained with 3,3'-diaminobenzidine (Fuzhou Maixin Biotech Co., Ltd.), and the nuclei were counterstained with haematoxylin for 5 min at RT. Morphometric analyses of the tumor and paired normal tissues

Table I. Clinical and pathological characteristics of the patients with colorectal cancer.

Patient no.	Sex	Age, years	Primary tumor location	TNM stage	Tumor stage (28)
P#1	Male	61	Ascending colon	T4aN1bM0	IIIB
P#2	Female	77	Rectum	T3N1bM0	IIIB
P#3	Female	64	Descending colon	T4bN2bM0	IIIC
P#4	Male	79	Ascending colon	T3N2aM0	IIIB
P#5	Female	60	Rectum	T3N0M1c	IVC
P#6	Female	60	Ascending colon	T3N2bM1a	IVA
P#7	Female	49	Descending colon	T3N2bM0	IIIB
P#8	Female	41	Rectum	T3N2bM0	IIIB
P#9	Male	67	Sigmoid	T3N2bM1b	IVB
P#10	Male	65	Ascending colon	T2N1bM0	IIIA
P#11	Female	27	Rectum	T4aN1bM0	IIIB
P#12	Female	54	Rectum	T2N1cM0	IIIA
P#13	Female	70	Rectum	T3N1cM0	IIIB
P#14	Male	56	Descending colon	T3N2aM0	IIIB
P#15	Female	68	Sigmoid	T3N1bM0	IIIB
P#16	Male	73	Rectum	T3N2aM1a	IVA
P#17	Male	50	Descending colon	T3N2aM1a	IVA
P#18	Female	65	Ascending colon	T4bN0M1a	IVA
P#19	Female	53	Ascending colon	T4aN2aM1a	IVA
P#20	Male	76	Ascending colon	T4bN2bM1a	IVA
P#21	Female	48	Ileocecus	T4bN0M1b	IVB
P#22	Male	48	Ascending colon	T4bN2aM1b	IVB
P#23	Male	70	Descending colon	T4aN2M1a	IVA
P#24	Female	65	Ascending colon	T3N2M1a	IVA
P#25	Male	65	Ascending colon	T4aN0M1b	IVB
P#26	Female	60	Transverse colon	T4bN0M1b	IVB
P#27	Male	77	Transverse colon	T3N1aM1b	IVB
P#28	Female	29	Ascending colon	T3N1M1b	IVB
P#29	Male	59	Ascending colon	T3N1bM0	IIIB
P#30	Female	79	Rectum	T2N2bM0	IIIB
P#31	Male	60	Sigmoid	T4aN2bM0	IIIC
P#32	Male	52	Ascending colon	T4aN2bM0	IIIC
P#33	Female	68	Ascending colon	T3N1cM0	IIIB
P#34	Male	62	Sigmoid	T4aN1bM0	IIIB
P#35	Female	65	Ascending colon	T3N1aM0	IIIB

were performed using an Olympus BX51 light microscope (magnification, x20; Olympus Corporation). Images were obtained from 10 randomly selected areas.

Staining intensity was scored as follows: 0, Negative; 1, weakly positive (<25% of cells stained); and 2, positive (>25% of cells stained). Samples were subsequently grouped into 2 categories: Low expression (0 and 1) and high expression (2).

Flow cytometric analysis. Tumor/paired control tissue-infiltrating cells ($1x10^6$ cells) were incubated with human FITC anti-CD3 monoclonal antibody (mAb) (1:20; cat. no. 300452; BioLegend, Inc.), APC anti-CD8 mAb (1:20; cat. no. 344722; BioLegend, Inc.), phycoerythrin (PE) anti-CD39 mAb (1:20; cat. no. 328208; BioLegend, Inc.), APC/Cy7 anti-PD-1 mAb (1:20; cat. no. 329921; BioLegend, Inc.), and PE/Cy7 anti-T-cell immunoglobulin mucin family member 3 (Tim-3) mAb (1:20; cat. no. 345013; BioLegend, Inc.) in cell staining buffer (cat. no. 420201; BioLegend, Inc.) for 15 min at RT. After washing with PBS and centrifugation at 400 x g for 5 min at 4˚C, $1x10^6$ cells were suspended in 300 μl cell staining buffer (cat. no. 420201; BioLegend, Inc.) and analysed on a BD FACSCalibur (BD Biosciences) flow cytometer. The data were analysed using FlowJo v10 software (FlowJo, LLC).

Statistical analysis. Statistical analysis was performed using GraphPad Prism v5 software (GraphPad Software, Inc.). Data are presented as the mean ± standard error of the mean of three repeats. A χ^2 test was used to evaluate the association between CDK9 expression and the clinicopathological parameters. A log-rank test was used to compare the survival curves

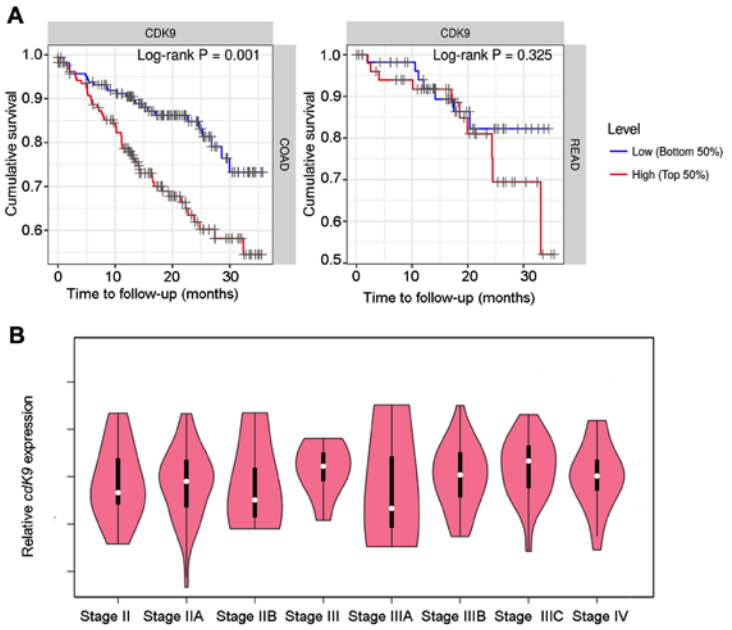

Figure 1. CDK9 significantly shortens the survival of patients with colon cancer. (A) Association between CDK9 expression and the survival of patients with colon and rectal cancer. Kaplan-Meier plots were used to visualize the survival differences. Levels were divided into low and high levels (cut-off, 50%). P-values calculated using a log-rank test are shown in each plot. (B) CDK9 mRNA expression in different stages of colorectal cancer based on data btained from the GEPIA database. CDK9, cyclin-dependent kinase 9; COAD, colon adenocarcinoma; READ, rectum adenocarcinoma.

Table II. Multivariate survival analyses of stage and CDK9 with overall survival of patients with COAD using the Cox proportional hazards model.

Factor	Coefficient	HR	95% CI_l	95% CI_u	P-value
Stage II	0.646	1.908	0.737	4.941	0.183
Stage III	1.166	3.208	1.243	8.277	0.016[a]
Stage IV	2.241	9.406	3.629	24.381	0.000[b]
CDK9	0.625	1.868	1.151	3.033	0.011[a]

Model: Survival (COAD) ~ Stage + CDK9. Data from a total of 445 patients were analysed, including 98 cases of mortality. Data was obtained from the TIMER database. The coefficient reads as a regression coefficient. HR, and its lower and upper 95% CI are shown. [a]P<0.05; [b]P<0.001. 95% CI-l, lower 95% CI; 95% CI-u, upper 95% CI; CDK9, cyclin dependent kinase 9; COAD, colon adenocarcinoma; HR, hazard ratio.

Table III. Multivariate survival analyses of stage and CDK9 with overall survival of patients with READ using the Cox proportional hazards model.

Factor	Coefficient	HR	95% CI_l	95% CI_u	P-value
Stage II	0.156	1.169	0.225	6.090	0.853
Stage III	0.726	2.066	0.426	10.026	0.368
Stage IV	1.657	5.244	1.104	24.907	0.037[a]
CDK9	0.823	2.277	0.775	6.696	0.135

Model: Survival (READ) ~ Stage + CDK9. Data from a total of 160 patients were analysed, including 23 cases of mortality. Data was obtained from TIMER database. The coefficient reads as a regression coefficient. HR, and its lower and upper 95% confidential interval are shown. [a]P<0.05. 95% CI_l, lower 95% CI; 95% CI_u, upper 95% CI; CDK9, cyclin dependent kinase 9; HR, hazard ratio; READ, rectum adenocarcinoma.

of two groups. Cox regression analysis was performed for multivariate analysis of prognostic variables. A correlation between transcripts per million (TPM) of *CDK9* and other genes was calculated for statistical significance, correlation co-efficient, and is represented using a scatter plot. One-way ANOVA followed by Tukey's multiple comparison test was used for multiple-group analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

CDK9 significantly shortens the survival of patients with colon cancer. To examine the association between CDK9 and prognosis in patients with colon and rectal cancer, the present study analysed the TIMER database, and found that high CDK9 expression was significantly associated with a shortened survival of patients with colon cancer (P=0.001; 445 cases total; 98 cases of mortality). The same trend was observed in patients with rectal cancer; however, this was not statistically significant (P=0.325; 160 cases total; 23 cases of mortality; Fig. 1A). As shown in Table II, CDK9 was a risk factor for survival in patients with colon cancer based on a Cox proportional hazard model analysis. However, Cox model analysis demonstrated that CDK9 did not affect rectal cancer progression (Table III). In addition, CDK9 expression had no significant effect on prognosis when the survival time was >3 years (data not shown). Therefore, CDK9 may serve an important role in the progression of advanced colon cancer.

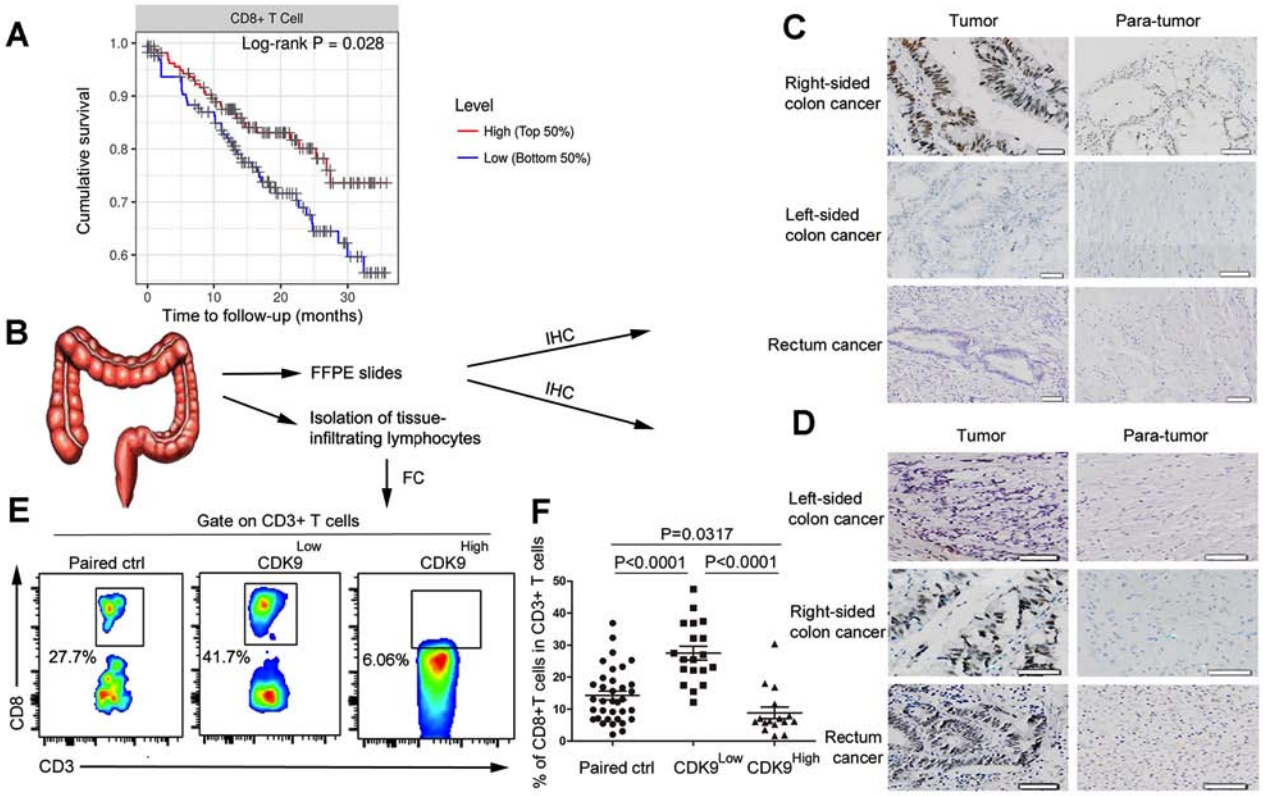

Figure 2. CDK9 is negatively associated with the infiltration of CD8+ T cells in colorectal cancer. (A) Kaplan-Meier curves for CD8+ T cell infiltration in colorectal cancer. Levels were divided into low and high levels (cut-off, 50%). The P-value was calculated using a log-rank test. (B) Experimental scheme. (C) Immunohistochemistry of CDK9 expression in stage III colorectal cancer tissues and adjacent non-cancerous tissues. Scale bar, 50 μm. (D) Immunohistochemistry of CDK9 expression in stage IV colorectal cancer tissues and adjacent non-cancerous tissues. Scale bar, 50 nm. (E) Flow cytometric analysis of tumor-infiltrating CD8+ T cells in stage III-IV colorectal cancer tissues and paired normal tissues. (F) Frequency of tumor-infiltrating CD8+ T cells in stage III-IV colorectal cancer tissues and paired normal tissues. Each dot represents data generated from one patient (n=35). CDK9, cyclin-dependent kinase 9; IHC, immunohistochemistry; FFPE, formalin-fixed and paraffin-embedded; FC, flow cytometry; Paired ctrl, paired normal control tissues; CDK9^{Low} T, tumor tissues with low expression of cyclin-dependent kinase 9; CDK9^{High} T, tumor tissues with high expression of cyclin-dependent kinase 9.

The GEPIA database was searched and *CDK9* mRNA expression in stage III-IV patients was upregulated compared with that in stage II patients, and the expression level was positively associated with the clinical tumor stage in stage IIIA-IIIC patients although the association was not significant (Fig. 1B). These data suggested that CDK9 may promote lymph node metastasis in colon cancer.

CDK9 is negatively associated with the infiltration of CD8+ T cells in CRC. By analysing RNA sequencing (RNA-seq) data obtained from GEPIA, and the pathological estimation data from human CRC tumors, the present study revealed that patients with high expression levels of CDK9 and fewer CD8+ T cells in the TME exhibited poorer prognoses (Figs. 1A and 2A). Next it was determined whether CDK9 affected infiltration of CD8+ T cells in CRC. Specimens were collected from 35 patients with stage III-IV MSS CRC, and the clinical and pathological characteristics of the patients are shown in Table III. CDK9 expression in these patients was detected by immunohistochemical staining. The results demonstrated that the CDK9-positive expression rate (a score of 2) in stage III right-sided colon cancer was 85.7% (6/7), which was significantly higher than that in paracancerous tissues, with a positivity rate of 28.6% (2/7; χ^2=4.667; P=0.03; Fig. 2B and C). However, there was no significant difference identified between CDK9 expression in all cases of left-sided colon cancer and rectal cancer (3/12) compared with the corresponding right-sided cancer (4/12; Fig. 2C). CDK9 expression in stage IV colon cancer was much higher than that in the paracancerous region, regardless of the primary tumor site (Fig. 2D). Clinically, the survival of patients with stage III-IV right-sided colon cancer was significantly shorter than that of patients with left-sided colon cancer (30,31). Therefore, these results suggested that abnormally high CDK9 expression predicted poor prognosis, and this may be a significant factor in the prognosis of patients with left/right-sided colon cancer.

Additionally, cells were isolated from the fresh tumor samples and matched normal tissues of these patients. Flow cytometry was utilized to detect tumor infiltration by CD8+ T cells. According to the immunohistochemical staining results, patients with the MSS-phenotype CRC were divided into the CDK9 high- and low-expression groups, and the results of flow cytometry were analysed. The frequency of tumor-infiltrating CD8+ T cells in the CDK9 low-expression group was markedly higher than that in the CDK9 high-expression group (Fig. 2E and F). This result demonstrated that CDK9 inhibited the recruitment and infiltration of CD8+ T cells in the TME.

CDK9 is associated with genes responsible for immune cell migration and exhaustion in CRC. Subsequently, it was

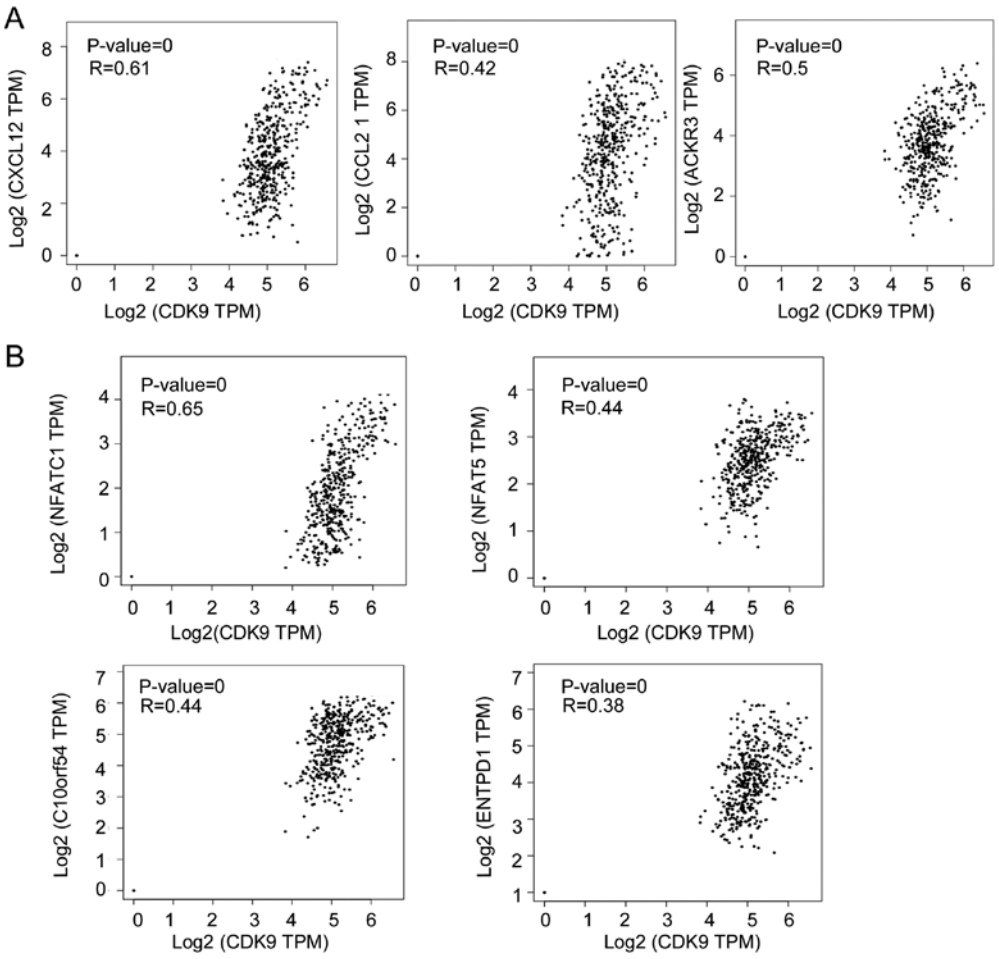

Figure 3. *CDK9* is correlated with genes responsible for immune cell migration and exhaustion in colorectal cancer. (A) Correlations of *CDK9* expression with *CXCL12*, *CCL21* and *ACKR3* mRNA expression in CRC. (B) Correlations of *CDK9* with *NFATC1*, *NFAT5*, *C10orf54* and *ENTPD1* mRNA expression in CRC. CRC, colorectal cancer; *CDK9*, cyclin-dependent kinase 9; *CXCL12*, C-X-C motif chemokine ligand 12; *CCL21*, C-C motif chemokine ligand 21; *ACKR3*, atypical chemokine receptor 3; *NFATC1*, nuclear factor of activated T cells 1; *NFAT5*, nuclear factor of activated T cells 5; *C10orf54* V-set immunoregulatory receptor; *ENTPD1*, ectonucleoside triphosphate diphosphohydrolase 1; TPM, transcripts per million.

explored how CDK9 inhibited the recruitment and migration of $CD8^+$ T cells to the TME by analysing RNA-seq data from CRC samples. As shown in Fig. 3A, *CDK9* mRNA expression was positively correlated with the expression of C-X-C motif chemokine ligand 12 (*CXCL12*), C-C motif chemokine ligand 21 (*CCL21*) and atypical chemokine receptor 3 (*ACKR3*; also referred to as *CXCR7*), which are associated with lymphocyte migration. It has been reported that CXCL12 decreases the number of tumor-infiltrating natural killer (NK) cells and $CD8^+$ T cells (32,33). These data indicated that CDK9 may be involved in CXCL12/CCL21/CXCR7-axis-mediated negative immune regulation in the TME. Since CDK9 was associated with the expression of numerous chemokines in the TME and affected the migration of immune cells, the present study investigated whether CDK9 mediated 'editing' of the TME, and the function and phenotype of immune cells. To explore this, the present study analysed the association between CDK9 and $CD8^+$ T cell exhaustion-associated genes by analysing RNA-seq data from CRC. Notably, there was a significant positive correlation between the transcription of *CDK9* and several genes that induce T lymphocyte exhaustion, including nuclear factor of activated T cells 1 (*NFATC1*), V-set immunoregulatory receptor, nuclear factor of activated T cells 5 (*NFAT5*) and ectonucleoside triphosphate diphosphohydrolase 1 (*ENTPD1*; also referred to as *CD39*; Fig. 3B). Additionally, the anti-inflammatory molecules transforming growth factor β1 (*TGFB1*) and *SMAD4* were correlated with CDK9 expression in CRC tumors (Fig. 4A). Furthermore, *CDK9* was co-expressed with *TGFB1* and *SMAD4* according to STRING database analysis (Fig. 4B). Interestingly, *CDK9* was significantly positively correlated with *BRAF* mRNA expression, which serves an important role in the development of CRC (Fig. 4C). It was revealed that 26.2% (96/367) of patients with CRC had *BRAF* mutations, and their antitumor immunity was more inhibited than that of wild-type patients, based on analysis of the TIMER database. The decrease in tumor-infiltrating immune cells caused by the *BRAF* mutation was significantly different between colon and rectal cancer (Fig. 4D and E). Compared with wild-type rectal cancer (diploid/normal), the arm-level gain (affecting ≥50% of the chromosome) of *BRAF* led to decreased tumor-infiltrating $CD8^+$ T cells ($P<0.001$), B cells ($P<0.05$) and dendritic cells ($P<0.05$) in colon cancer (Fig. 4D-F), whereas only tumor-infiltrating dendritic cells ($P<0.01$) were decreased in rectal cancer (Fig. 4G). These data indicated that CDK9 was involved in BRAF-mediated immunosuppression, which was affected by the site of the primary tumor. Overall, these data

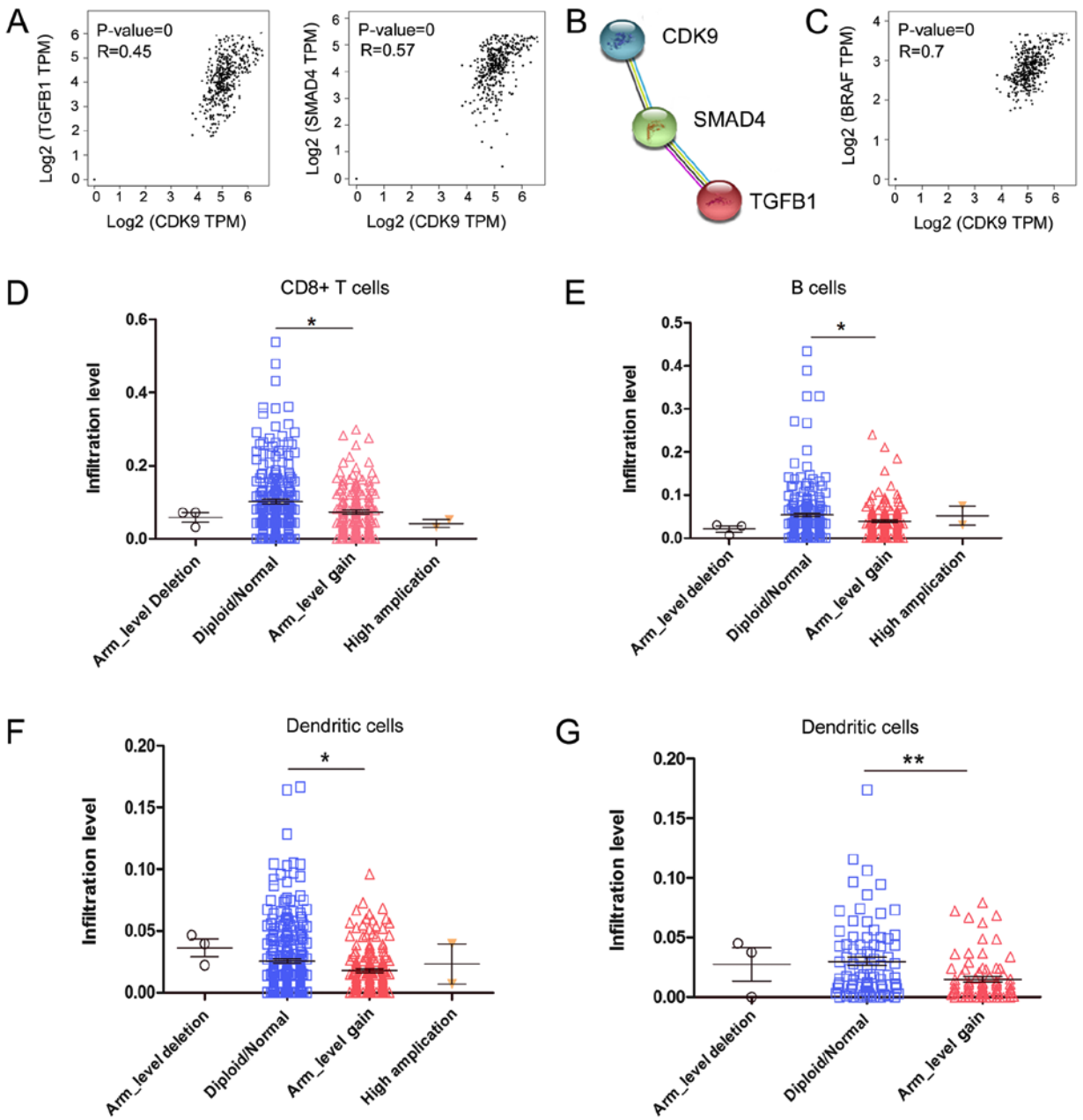

Figure 4. CDK9 is associated with anti-inflammatory molecules in CRC. (A) Correlations of *CDK9* with *TGFB1* and *SMAD4* mRNA expression in CRC. (B) Co-expression analysis of CDK9, TGFB1 and SMAD4 proteins. (C) Correlation of *CDK9* with *BRAF* mRNA expression in CRC. (D) Influence of the *BRAF* mutation on $CD8^+$ T cell infiltration in colon cancer. (E) Influence of the *BRAF* mutation on B cell infiltration in colon cancer. (F) Influence of the *BRAF* mutation on dentritic cell infiltration in colon cancer. (G) Influence of the *BRAF* mutation on dendritic cells infiltration in rectal cancer. Scatter plots are presented to show the distributions of each immune subset at each somatic copy number status for *BRAF* in colorectal cancer. The infiltration level for each category was compared with the diploid/normal group using one-way ANOVA followed by Tukey's multiple comparison test. $^*P<0.05$, $^{**}P<0.001$ vs. normal. CRC, colorectal cancer; CDK9, cyclin-dependent kinase 9; TGFB1, transforming growth factor ß1; SMAD4, SMAD family member 4; TPM, transcripts per million; COAD, colon adenocarcinoma; READ, rectum adenocarcinoma.

implied that CDK9 may contribute to CRC immune escape by promoting $CD8^+$ T cell exhaustion and inhibiting the infiltration of numerous immune cell populations.

Association of CDK9 expression with tumor-infiltrating $CD8^+$ T cell exhaustion in CRC. To confirm the association between CDK9 and tumor-infiltrating $CD8^+$ T cell exhaustion, tumor-infiltrating cells were isolated from fresh CRC tissues and paired normal tissues from advanced CRC tumors, and the cells were co-cultured with antibodies and analysed by flow cytometry. According to the aforementioned results of the CDK9 immunohistochemistry, patients with MSS phenotype CRC were divided into two groups, a CDK9 high- and a CDK9 low-expression group, for statistical analysis. The results revealed that there were no significant differences identified in the $CD8^+PD\text{-}1^+$ T cell frequency between the $CDK9^{high}$ group and the $CDK9^{low}$ group (Fig. 5). Compared with the $CDK9^{low}$ group, the frequency of $CD8^+Tim\text{-}3^+$ T cells was increased by 37.5% in the $CDK9^{high}$ group (Fig. 5). Notably, the frequency of infiltrating $CD8^+CD39^+$ T cells in $CDK9^{high}$ CRC tumors was increased 1.12-fold compared with that in $CDK9^{low}$ tumors (Fig. 5). These data indicated that

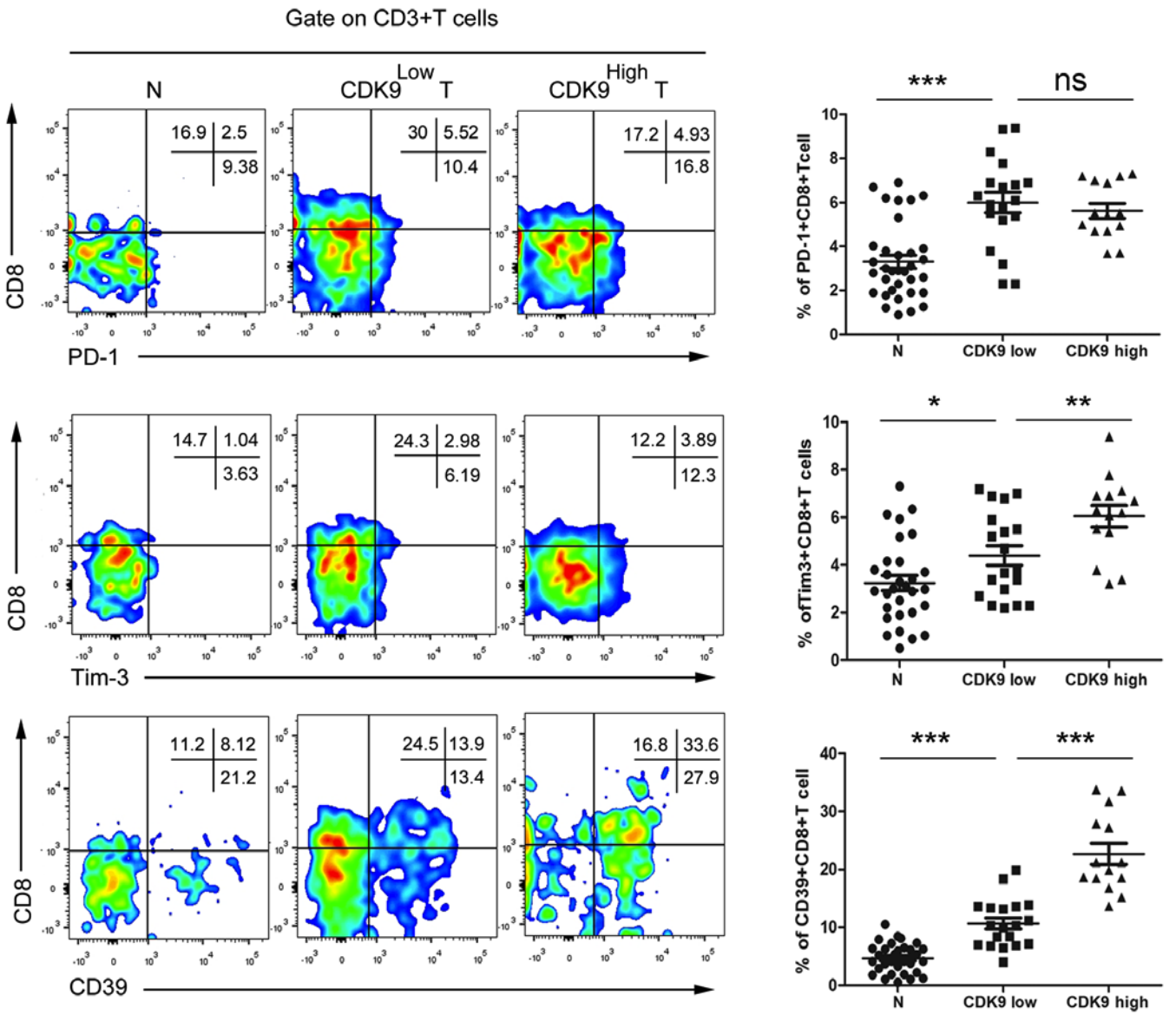

Figure 5. Association of CDK9 expression with tumor-infiltrating CD8+ T cell exhaustion in colorectal cancer. The frequencies of PD-1+CD8+ T cells, Tim-3+CD8+ T cells and CD39+CD8+ T cells were determined by flow cytometry. Each dot presents data generated from one patient (n=35). *P<0.05; **P<0.001; ***P<0.0001. CDK9, cyclin-dependent kinase 9; PD-1, programmed cell death 1; Tim-3, T-cell immunoglobulin mucin family member 3; N, paired normal tissue; $CDK9^{Low}$, low expression of cyclin-dependent kinase 9; $CDK9^{High}$, high expression of cyclin-dependent kinase 9; ns, not significant; T, tumor tissue.

CDK9 was positively associated with tumor-infiltrating CD8+ T cell exhaustion, independent of the PD-1/PD-L1 signal, and may be used to evaluate the immune-type of the TME in patients with MSS mCRC.

Discussion

CDK9 has been widely used in the research and development of antitumor drugs due to its key regulatory role in transcription, and its contribution to the progression and maintenance of several types of cancer (34-37). A preclinical *in vivo* study demonstrated that treatment with a small-molecule CDK9 inhibitor impairs the growth of human melanoma xenografts (38). BAY 1143572, a novel and highly selective CDK9/P-TEFb inhibitor that is currently being investigated in phase I studies, decreases c-Myc and MCL1 apoptosis regulator levels in ATL-derived or HTLV-1-transformed lines, which inhibits their growth (39). It has been reported that CDK9 functions in immune responses and that its inhibition effectively suppresses the inflammatory response in chondrocytes (40). However, the regulatory effect of CDK9 on the antitumor immune response in CRC remains to be elucidated. The present study investigated CDK9 expression in different primary tumor sites in CRC and provided evidence that CDK9 expression was negatively associated with CD8+ T cell antitumor function in MSS mCRC.

With the development of precision medicine, it has become gradually understood that the prognoses of left- and right-sided CRC are different. Venook's CALGB/SWOG 80405 clinical data were published in ASCO in 2016 and revealed that the median survival of KRas wild-type right-sided and left-sided mCRCs was 19.4 and 34.2 months, respectively, and that of KRas mutant right-sided and left-sided mCRCs was 23.1 and 30.3 months, respectively (3). Therefore, the difference

in primary tumor location has become a novel focus in CRC research. The results of the present study demonstrated that CDK9 expression in advanced left-sided CRC was significantly higher than that in early-stage left-sided CRC, and CDK9 expression in stage III-IV right-sided CRC was significantly higher than that in stage III-IV left-sided CRC. Notably, the prognostic difference between left-sided and right-sided CRC is only seen in tumors that are at least at stage III (30). Therefore, CDK9 may be a prognostic factor for left/right-sided colon cancer, which broadens the knowledge for future research.

Since the 5-year relative survival of patients with stage IV CRC is only 11.7%, regardless of primary tumor location, improving the diagnosis and treatment efficacy of stage IV patients is the key to ameliorating the survival of CRC (41). Anti-PD-1 immunotherapy has been demonstrated to be effective only in patients with MSI-H phenotype stage IV CRC, since they have more tumor-infiltrating immune cells (11). However, only 3.5-5% of all stage IV CRC cases are MSI-H (9). The present study determined CDK9 expression and the frequency of $CD8^+$ T cells in tumor and paired normal control specimens of 35 patients with MSS-phenotype CRC, which accounts for >95% of all advanced CRC (9), and identified that CDK9 was negatively associated with tumor-infiltrating $CD8^+$ T cells. This result suggested that CDK9 inhibition may benefit patients with MSS phenotype CRC in combination with anti-PD-1 immunotherapy.

The recruitment and migration of immune cells upon stimulation are regulated by numerous factors, of which the chemokine family serves an important role. It has been reported that fibroblasts help pancreatic cancer to develop chemotherapy resistance, and the chemokine CXCL12 is secreted by fibroblasts and prevents the infiltration of $CD8^+$ T cells into the tumor (42). Zboralski *et al* (33) demonstrated that inhibition of CXCL12 by 'NOX-A12' can promote the infiltration of T cells and NK cells, thereby increasing the efficacy of anti-PD-1 drugs in colon cancer models. CXCR7/C-X-C motif chemokine receptor 4 heterodimer-induced histone demethylation promotes colorectal tumorigenesis (43). In addition, CCL21 is involved in T-cell migration and trafficking to secondary lymphoid organs (44). The present study revealed that *CDK9* expression was positively correlated with *CXCL12*, *ACKR3* (also referred to as *CXCR7*) and *CCL21* expression, suggesting that CDK9 may inhibit $CD8^+$ T cell infiltration via the CXCL12/CXCR7 axis.

The present study investigated how the TME 'edits' T cells in CRC and whether CDK9 serves a role in this process. tumor antigens are weakly immunogenic self-molecules, and the majority of tumor-specific T cells have a low T-cell receptor (TCR) affinity, since tumor-specific T cells with high avidity are cleared during the thymic selection process (45). Therefore, the process of antigen presentation is impaired in the TME, leading to insufficient priming and boosting of T cells (45). Consequently, the complex components in the TME drive T cells to terminally differentiate into 'exhausted' T cells (46). 'Exhausted' $CD8^+$ T cells overexpress a large number of cell surface inhibitory receptors, including PD-1 and Tim-3. NFAT activates the nuclear factor protein of T cells, which results in inability of the *NFATC1* gene to synergize with AP-1 during gene modification, resulting in loss of the TCR signal and upregulation of the expression of inhibitory receptors on the cell surface, thereby weakening the antitumor ability of $CD8^+$ T cells (47). In addition, CD39 may be a functional surface marker for identifying exhausted $CD8^+$ T cell subsets and regulating purine signalling pathways, and the combined blockade of the Tim-3 and PD-1 signalling pathways reverses the exhaustion of T cells that is induced by rectal cancer (48). The present study revealed that *CDK9* expression was positively correlated with *NFATC1*, *ENTPD1* (also referred to as *CD39*) and *NFAT5* expression, suggesting that CDK9 may be involved in the 'cancer immunoediting' of T cells by the TME. Furthermore, CDK9 promoted CD39 and Tim-3 expression in tumor-infiltrating $CD8^+$ T cells in MSS CRC tumors, whereas CDK9 did not significantly affect PD-1 expression.

To the best of our knowledge, the present study was the first to evaluate the association of CDK9 and tumor-infiltrating $CD8^+$ T cells in MSS CRC. A number of studies have demonstrated that CDK9/P-TEFb is involved in the cell growth and survival of several types of cancer, including CRC (15,49,50). However, these studies have focused on the cancer cells themselves, ignoring the effect of CDK9 on the TME. The present study explored the association between CDK9 expression and $CD8^+$ T cells in the TME of MSS CRC. Our previous study demonstrated that PHA767491, a selective CDK9 inhibitor, could impair the activation and proliferation of effector T cells but preserve the function of Treg cells in a mouse inflammatory model (51). To the best of our knowledge, no study has reported that a CDK9 inhibitor could alter anti-inflammatory molecules or T cells in CRC. The present study focused on the immune microenvironment of MSS CRC. In future studies, the effect of CDK9 inhibitors on tumor-infiltrating lymphocytes in CRC will be explored.

In conclusion, the present study demonstrated that CDK9 expression was positively associated with poor survival among patients with colon cancer and was negatively associated with $CD8^+$ T cell antitumor function in MSS mCRC. These findings suggested that CDK9 may be utilized to evaluate the prognosis and the immune-type of the TME in patients with MSS mCRC.

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Availability of data and materials

The datasets generated and analyzed during the current study are available in the GEPIA repository, http://gepia.cancer-pku.cn/detail.php?gene=CDK9.

Authors' contributions

YZ and JL performed the majority of the experiments. FT collected clinical specimens. JW performed data analysis and interpretation. YZ and DK designed and supervised the study.

DK wrote the manuscript. YZ revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Patient samples were collected at the Tianjin Medical University Cancer Institute and Hospital, and ethical approval was granted by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. Written informed consent was obtained from all patients for participation in the present study.

Patient consent for publication

Written consent was obtained from all participants for the publication of data.

Competing interests

The authors declare that they have no competing interests.

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