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RESEARCH

PRMT5 highly expressed on CD16 + CD56- natural killer cells is correlated with NK cells exhaustion in colorectal cancer mesenchyme

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Abstract

Objective To investigate the relationships between changes in the phenotype of natural killer cells (NK cells) in the microenvironment of colorectal cancer (CRC) and the expression of important immune checkpoints. To assess the expression level of CD16 bright CD56 negative (CD16 + CD56-) NK cell-associated immune checkpoints, including protein arginine methyltransferase 5 (PRMT5) and T-cell immunoreceptor with Ig and ITIM domains (TIGIT), single-immunoglobulin interleukin-1-related receptor (SIGIRR), in CRC mesenchyme.

Methods A total of 194 patients who were diagnosed with CRC were screened. The percentage of NK cells and the expression levels of their surface receptors, including PRMT5, CD56, CD69, TIGIT, CD16, IFN-γ, and SIGIRR, in the tumor microenvironment (TME) of CRC were assessed. Immunohistochemical staining, multiplex immunohistochemistry, and single-cell sequencing were performed.

Results Compared with normal mesenchyme, NK cells were less in CRC mesenchyme. The percentage of CD16 + CD56- NK cells in tumor mesenchyme was significantly higher, the number of CD16 + NK cells was more, and the number of CD56 + NK cells was less in CRC mesenchyme. High expression of TIGIT and PRMT5 expression affected the progression of CRC. The expression of PRMT5 and SIGIRR expression was significantly increased in CD16 + CD56- NK cells, and both genes were identified as important morbidity factors. PRMT5 and SIGIRR may contribute to the phenotype changes of NK cells in CRC.

Conclusion The microenvironment of CRC is in an immunosuppressive state characterized mainly by high expression of TIGIT, CD16, PRMT5, and SIGIRR; low expression of CD56, IFN-γ, and CD69; significantly decreased percentage of CD56 + NK cells; and significantly increased percentage of CD16 + CD56- NK cells with weakened killing ability. PRMT5 and TIGIT may be closely related to the formation of CD16 + CD56- NK cells with weakened killing ability.

Keywords CD16 + CD56- NK cells · Multiplex immunohistochemistry · Colorectal cancer · PRMT5 · SIGIRR · Single-cell sequencing

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Introduction

There were 560,000 new colorectal cancer (CRC) cases and 290,000 deaths caused by CRC in China in 2020 [1]. Approximately 25% of patients with CRC presented distant metastasis at the time of initial diagnosis. Among these patients, 75–90% developed unresectable metastases [2]. The prognosis of advanced CRC is poor, and the 5-year survival rate is low [3]. Studies have shown that T lymphocytes, B lymphocytes, macrophages, dendritic cells, and natural killer (NK) cells play important roles in tumor microenvironment (TME) of CRC [4]. NK cells constitute an important

part of the innate immune system and performed dual functions of cytotoxicity and immunomodulation. Furthermore, NK cells are important components of innate immunity that mediates the anti-tumoral immune response. Positive result of $CD16^+$ immunohistochemical staining is considered a marker of toxic NK cells, which play an important role in the anti-tumor immune response. Zhang et al. reported that blockade of the checkpoint receptor TIGIT prevents NK cell exhaustion and elicits potent anti-tumor immunity [5]. NK cells can be divided into two types, namely, CD56bright and CD56dim, which show different phenotypic characteristics. CD56bright NK cells can produce many cytokines, whereas CD56dim NK cells exhibit more cytotoxic activity and express more immunoglobulin-like receptors and Fc gamma RIII (Fc gamma receptor III, also known as CD16). While the CD16 + CD56 + NK cell subset is best known for its cytotoxic functions, the CD16-CD56 + NK cell subset produces many cytokines comparable to those cells produced by CD4 + T helper cells. CD56dimCD16 + NK cell subsets are highly cytotoxic effector cells that are predominantly found in peripheral blood [6]. Compared with other NK cell subpopulations, CD16 + CD56- NK cells express higher levels of inhibitory NK receptors and lower levels of natural cytotoxic receptors, resulting in the limitation of their role in the antiviral immune response, and may be derived from CD56dim NK cells [7]. Tumor progression or chronic infections usually result in the exhaustion of NK cells, thus limiting the ability of NK cells to combat tumor growth and infection. NK cells occur functional changes with phenotypic changes in CRC, which is also an important reason for the progression of CRC.

Many studies have shown that, compared with the normal colonic epithelial microenvironment, the number and killing function of NK cells decreased significantly in the CRC microenvironment, and CRC patients with many tumor-infiltrating NK cells show a favorable prognosis [8–10], suggesting that NK cells play an important role in anti-tumor immunity in CRC. Activating receptors include NKG2D, CD16, and CD226, among which, CD16 plays a key role in antibody-dependent cell-mediated cytotoxicity. Inhibitory receptors of NK cells include killer cell immunoglobulin-like receptors (KIRs), NKG2A, TIM-3, and TIGIT in humans. Moreover, the important therapeutic targets for CRC tumor cells are RAS and BRAFV600E. Recently, new targets, including NTRK and HER-2, have emerged. PRMT5 has been identified as a new immune checkpoint [11–13]. PRMT5 interacting and methylating SMAD4 was required for TGF-β1-induced epithelial–mesenchymal transition (EMT) and colorectal cancer (CRC) metastasis [14]. The role of PRMT5 in NK cells in colorectal cancer is unknown. Moreover, PRMT5, a member of the arginine methyltransferase family, is involved in the symmetrical methylation of arginine residues in target proteins and regulates many biological processes, including cell growth and development, differentiation, splicing, translation, the DNA damage response, protein transport, and cell signaling [15]. PRMT5 catalyzes the symmetric dimethylation of the N-terminal Arg124 residue of cGAS, preventing cGAS from binding to self-DNA and thus inhibiting the innate immune response [16]. PRMT5 inhibitors disrupt the antigen presentation and cytokine production of tumor cells, thereby weakening the immune escape ability of tumors. Several PRMT5 inhibitors have entered clinical trials.

The NK cell-associated immune checkpoint gene SIGIRR is a member of the TIR superfamily (Toll/IL-1R, TIR), and SIGIRR is widely expressed in tumors of the digestive tract, kidney, lung, liver, and lymphoid organs [17, 18]. PRMT5 is further upregulated in colorectal cancer cells with KRAS mutation (compared with KRAS wild type), and inhibition of PRMT5 expression at this stage or condition can achieve more significant inhibitory effects on cancer cells (such as cell cycle obstruction and promotion of apoptosis), suggesting that PRMT5 can be used as a potential therapeutic target in this colorectal cancer. Hartley's research group found that two new signaling pathways (PRMT5/YBX1/NF-KB and PKCVPRMTS/NF-KB) are closely related to the progression of colorectal cancer, suggesting that targeting PRMT5 to block these signaling pathways is a new anticancer strategy. Among the inhibitory receptors, SIGIRR has recently been identified as a checkpoint molecule that regulates NK cell effector function [19]. An in vitro investigation of NK cell activation revealed that SIGIRR defects led to increased expression and levels of basic anti-tumor molecules such as granzyme B, IFN-γ, and Fas ligand in NK cells and NK cell supernatants [20]. Enhanced NK cell effector function has also been observed in tumor-infiltrating SIGIRR-deficient NK cells [21]. How the NK cell phenotype changes in the TME of CRC patients and how these changes facilitate immune escape and affect the progression of CRC need to be further studied.

Materials and methods

Study participants

A total of 194 patients who were diagnosed with CRC in the Department of Pathology of Xi'an Daxing Hospital between January 2020 and January 2022 were screened. It consisting of surgically resected CRC samples from primary tumors. This study was approved by the Ethics Committee of Xi'an Daxing Hospital. The formalin-fixed, paraffin-embedded (FFPE) tissue samples were reviewed. Clinicopathological information was retrieved from electronic medical records. A total of 194 patients, 108 men (56%) and 86 women (44%), with an average age of 64 years were chosen. The

clinicopathological characteristics of all these patients are as follows: 21 patients had well-defined tumors, 141 had moderately defined tumors, and 32 had poorly defined tumors. A total of 6 (3%) patients had stage I disease, 15 (8%) had stage II disease, 153 (79%) had stage III disease, and 20 (10%) had stage IV disease. The median progression-free survival (PFS) was 31 months.

Materials

All tissues used in this project were provided without patient identification or clinical information. Three-micron-thick sections from formalin-fixed, paraffin-embedded (FFPE) tissue blocks were sequentially prepared for H&E, immunohistochemistry (IHC), and multiplex immunohistochemistry (mIF) staining. Reagents and resources: All reagents, staining devices, and software for the image analyses used in this study were purchased from Ai Bo Kang (Shanghai) Trading Co., Ltd. Antibodies against CD16, CD56, TIGIT, PRMT5, CD69 (**dilution:** 1:250), NKG2D (**dilution:** 1:50), SIGIRR (**dilution:** 1:1500), and IFN-γ (dilution: 1:500) were used. All the antibodies were purchased from Ai Bo Kang (Shanghai) Trading Co., Ltd., and were lyophilized.

IHC and histopathological analysis

Sections from paraffin-embedded specimens were stained with hematoxylin and eosin. The tissue sections were deparaffinized in xylene and alcohol, and antigen retrieval was performed with citrate buffer. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide at room temperature for 30 min in the dark. Nonspecific binding was blocked via incubation with 10% normal goat serum at 37 °C for 1 h. The tissues were incubated overnight with primary antibodies at 4 °C. Then, 194 CRC tissue samples were examined with IHC. A total of seven antibodies against CD56, NKG2D, CD16, TIGIT, PRMT5, SIGGIR, IFN-γ, and CD69 were used. The secondary antibody used in the experiment was from an IHC polymer detection kit purchased from Xiamen Talent Biomedical Technology.

Cell counting (IHC and computer-assisted image analyses)

The positively stained cells in the cancer tissues and adjacent normal tissues were counted by two attending physicians. Five high-power fields were selected, and their average values were calculated. PRMT5: The intestinal mucosal epithelium was positive for cytoplasmic protein, with heterogeneous staining, and staining of cell nuclei was observed. TIGIT: cell membrane; CD16: The membrane and cytoplasm were stained brown and yellow, respectively. CD56, NKG2D, and SIGIRR: The cell membrane was stained brown or yellow for positive expression. IFN-γ: The membrane and cytoplasm were stained brown or yellow for positive expression. A value of PRMT5-positive cells ≥ 20 was defined as high density and PRMT5-positive cells < 20 was defined as low density.

Tissue samples obtained from 20 of 194 patients were subjected to multiplex immunohistochemistry (mIHC)

A total of six antibodies against CD56, CD16, TIGIT, PRMT5, SIGIRR, and AE1/AE3 were used for 10 patients, and antibodies against MPO, CD3, CD68, CD16, CD56, and PRMT5 were used for another 10 patients. The tissue sections were incubated at 60 °C for 1 h. After baking, the tissue sections were deparaffinized with two treatments of dewaxing solution (Abcarta, PS000) at 65 °C for 1 min each, followed by five rinses with 100% ethanol. Antigen retrieval was performed with pH 9.0 EDTA antigen retrieval solution (1X) (Abcarta, PS900) at 100 °C for 20 min. A TSA 7-color multiplex fluorescence IHC kit (Abcarta, PS017) was used, and the contents of the kit included the following: TSA-fluorophore 425, TSA-fluorophore 488, TSA-fluorophore 532, TSA-fluorophore 594, TSA-fluorophore 633, TSA-fluorophore 680, DAPI, antifade mounting medium, HRP-conjugated goat anti-rabbit & mouse polymer, TSA signal amplification solution, and endogenous peroxidase blocking reagent. Sample pretreatment and staining were performed on the FAIP-48 T automatic immunohistochemical platform. The sections were subjected to six sequential rounds of staining with each primary antibody followed by incubation with a secondary HRP-conjugated goat anti-rabbit and mouse polymer. Signal amplification was achieved with TSA-fluorophores. Between two rounds of staining, or after each round of staining, a heat-induced epitope retrieval (HIER) step was performed to remove primary–secondary–HRP complexes before staining with the next primary antibody. After the final round of antibody staining, the slides were counterstained with DAPI and mounted with antifade mounting medium.

Single-cell sequencing

Individual cell suspensions were separated from fresh blood. The single-cell suspensions were counted via a LunaTM cell counter (Logos Biosystems), and appropriate amounts of cells were subsequently loaded onto a microfluidic chip (10 K Genomics 3' single-cell RNA kit v1.1), along with reverse transcription reagents, barcoding gel beads and droplet generation oil. The microfluidic chip was run on a 10 K Genomics-Perseus™ single-cell processing system to generate a droplet emulsion, which was transferred to

a thermal cycler for drop reverse transcription. Barcoded cDNA was then purified from the emulsion via SPRI beads and amplified via PCR. The sequencing libraries were prepared according to the manufacturer's instructions for the 10 K Genomics Library Amp Kit and sequenced on an Illumina NovaSeq 6000TM system in paired-end mode.

Statistical analyses

All analyses were performed via SPSS 25.0 (SPSS, Inc., Chicago, IL, USA). Student's t-test was used to determine the statistical significance of differences in the IHC data, immune cell data, and clinical information. Unless stated otherwise, the data are presented as the means ± standard errors of the means (SEMs). Paired and unpaired single-tailed Student's t-tests were used for comparisons with the indicated groups. Differences with $P < 0.05$ were considered statistically significant. For all figures, P values < 0.05 (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****) indicated significance.

Follow-up

Although 14 of 194 patients were followed up, no information was available from these patients, and the remaining 180 patients were followed up for 8–41 months. The median survival was 31.9 months, and 22 patients died. Patients were followed for survival and disease-free survival (DFS), and medication use.

Results

PRMT5 and SIGIRR were expressed at high levels in immune cells according to IHC

We determined the number of NK cells expressing CD16 in the CRC mesenchyme via immunohistochemistry and the density of PRMT5 in intestinal cancer samples, and the results indicated that the density of CD16, PRMT5, and SIGIRR foci was significantly higher than that in normal samples. The expression levels of IFN-γ, CD69, and TIGIT were significantly decreased in the CRC mesenchyme (Table 1). The distribution of the density of tumor-associated lymphocytes in the surrounding microenvironment was detected. The density of CD16 + NK cells in CRC increased significantly, whereas the density of CD56 + NK cells in CRC decreased significantly. Using IHC, we found that PRMT5 was expressed at higher levels in areas close to the tumor and exhibited more dispersed expression in normal tissues (Figs. 1 and 2).

In addition, we compared the levels of PRMT5 and SIGIRR in the tumor and normal mesenchyme, as well as their ratios and correlations with clinicopathological characteristics, such as sex, age, status of metastasis, location, differentiation, and stage. We found that SIGIRR expression was significantly correlated with moderately differentiation and stage 3 compared with normal tissues ($P < 0.05$) (Table 2).

Table 1 Expression of NK cell-related cytokines in cancer foci and adjacent tissues

$N = 194$	Cancer		Normal		Rank-sum test
	Cells	Mean value	Cells	Mean value	$P < 0.05$
TIGIT	0–86	8.0	0–80	11.9	$P < 0.05$
CD16	1–501	60.3	1–404	30.4	$P < 0.05$
CD56	0–47	0.7	0–40	4.5	$P < 0.05$
PRMT5	0–200	48.4	0–400	22.3	$P < 0.05$
SIGIRR	0–60	18.5	0–40	7.6	$P < 0.05$
CD69	0–33	8	0–69	22	$P < 0.05$
IFN-γ	0–92	16	0–186	65	$P < 0.05$

Multiplex fluorescence staining: Ten types of cells in the TME of CRC were detected

CD16 + cells were composed of MPO-CD3-CD68-CD16 + CD56 + cells and MPO-CD3-CD68-CD16 + CD56- cells. MPO-CD3-CD68-CD16 + CD56- cells accounted for more than 76.9–99.9% of the total CD16 + cells. The numbers of MP0-CD3-CD68-CD16 + cells (9/10) and MPO-CD3-CD68-CD16 + CD56- cells (9/10) were significantly greater than the number of normal cells (Table 3 and Fig. 5). We selected another 10 samples for detection and found that in the intestinal cancer samples, the numbers of CD16, PRMT5, and IL-1R8 foci were significantly greater in the cancer samples than in the normal samples (Supplementary Table 4 and Fig. 5). The distribution of the density of tumor-associated lymphocytes in the surrounding microenvironment was detected. The density of CD16 + NK cells in CRC was significantly increased, whereas the density of CD56 + NK cells in CRC was significantly decreased. The proportions of CD16 + CD56 + NK cells and CD16 + CD56- NK cells in the 10 bowel cancer tissues increased, and the proportions of PRMT5 + NK cells and SIGIRR + NK cells, CD16 + CD56-PRMT5 + NK cells, CD16 + CD56-SIGIRR + NK cells, CD16 + PRMT5 + SIGIRR + NK cells, and CD16 + CD56- TIGIT + NK cells increased in the cancer tissues ($P < 0.05$) (Table 3, Figs. 3 and 4).

scRNA-Seq analysis of CRC samples

In this study, six samples (tumor and normal tissues from three patients) were captured using the 10X Genomics-Perseus™ platform, which is based on microfluidic technology.

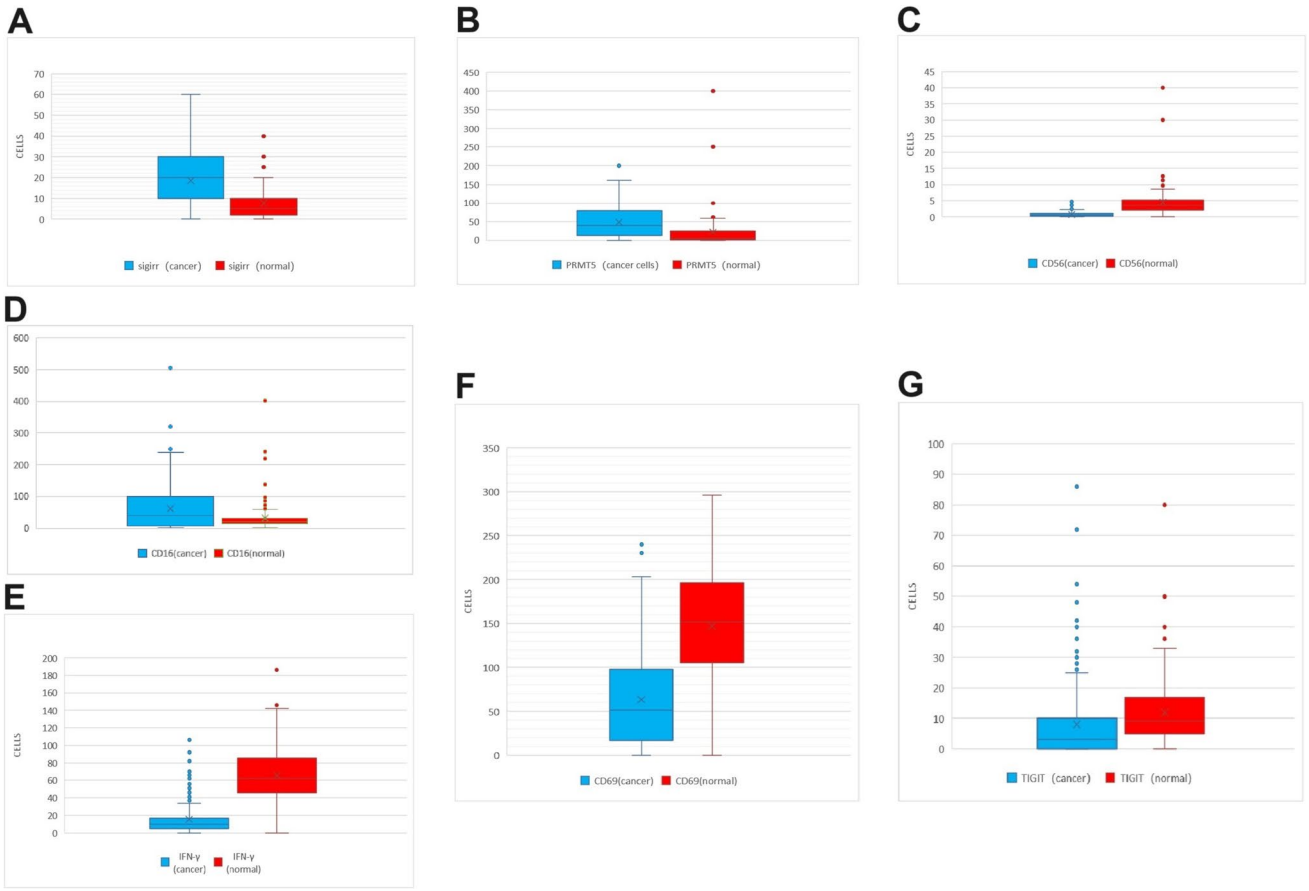

Fig. 1 Immunohistochemical staining and cell counts. The distributions of CD56 (**C**), IFN-γ (**E**), CD69 (**F**), and TIGIT (**G**) in tumor-associated inflammatory cells in tumor tissues were significantly lower, and the distribution of CD16 (**D**) was higher than that in adjacent tissues ($P < 0.05$), and the distributions of SIGIRR (**A**) and PRMT5 (**B**) in tumor-associated inflammatory cells in tumor tissues were significantly higher than those in adjacent tissues ($P < 0.05$)

Sequencing was performed using the Illumina NovaSeq 6000 sequencing platform in PE150 sequencing mode. The original data were filtered, compared, quantified, identified, and recovered by CellCosmo, and 5042, 6127, 5548, 5354, 4537, and 6011 cells were obtained, respectively, and divided into 10 cell groups. The percentages of uniquely mapped reads were 88.85, 89.42, 88.42, 77.14, 89.14, and 93.75%, respectively, which are all greater than 70%. The Q30 values of the RNA barcodes were all greater than 94%, which far exceeded the minimum limit of quality control, indicating that the quality of the obtained single-cell transcriptomic data was at an ideal level, and the datasets could be used for further analysis. Subsequently, the characteristic genes in each cell cluster were used to estimate the differences in the cell populations via the MCP-counter algorithm. The NK cell infiltration subgroups were identified specifically. The proportions of T lymphocytes and B lymphocytes decreased significantly, but markers of these cells were expressed more highly in cancer foci than were markers of NK cells. The expression of epithelial cell markers was upregulated in cancer tissues. Compared with those in adjacent tissues, the levels of NK cell markers were significantly lower in bowel cancer tissues. The heatmap displays the PRMT5, SIGIRR, TIGIT, CD69, IFN-γ, FCGR3A (CD16), and NCAM1 (CD56) expression levels in normal and colorectal cancer (CRC) tissues (Fig. 5). The expression level of CD56 on NK cells decreased significantly in bowel cancer tissues ($P < 0.05$), but the expression levels of CD16, PRMT5, and SIGIRR on NK cells increased significantly in bowel cancer tissues. Moreover, the expression levels of TIGIT on NK cells also increased significantly in CRC tissues ($P < 0.05$). No significant difference in the expression of IFN-γ on NK cells was observed between CRC and normal tissues (Fig. 6).

Follow-up message

A total of 180 patients were followed for 43 months, and the other 14 persons or individuals were lost to follow-up. Among the 180 patients, 22 died, and 158 were disease

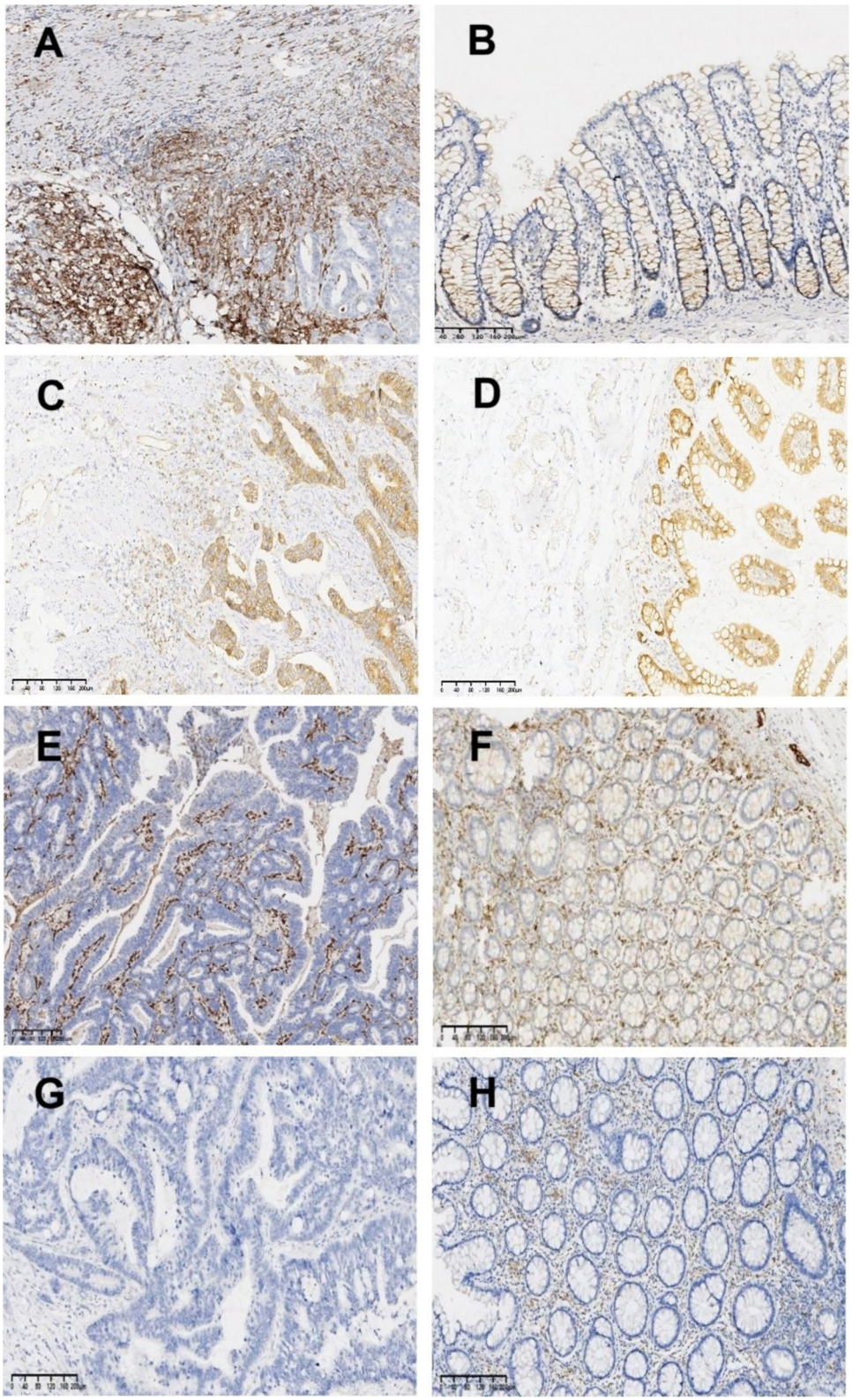

Fig. 2 Immunohistochemical staining. **A**. PRMT5 (HEx100) expression was high in tumor stromal immune cells. **B**. PRMT5 was expressed at lower levels in peritumoral stromal immune cells. **C**. SIGIRR (HEx100) was highly expressed in immune cells in the tumor stroma. **D**. SIGIRR expression (HEx100) revealed a low density of immune cells in the stroma. **E**. CD16 (HEx100) was highly expressed in the tumor microenvironment. **F**. CD16 (HEx100) was expressed at low levels in the paracancerous microenvironment. **G**. CD56 (HEx100) was expressed at low levels in the tumor microenvironment. **H**. CD56 (HEx100) was highly expressed in the paracancerous microenvironment

free. The DFS ranged from 8 to 41 months. The median survival was 39 months. CRC patients with high PRMT5 expression in the TME of tumors had worse prognosis (DFS) than patients with low PRMT5 expression (P = 0.128) (Fig. 7A). And we counted the expression of CD16high-PRMT5high, CD16lowPRMT5high, CD16highPRMT5low, and CD16lowPRMT5high in cancer (Fig. 7B).

Table 2 PRMT5 and SIGIRR in the tumor mesenchyme

	PRMT5 in the tumor microenvironment								SIGIRR in the tumor microenvironment							
	Cancer				Normal				Cancer				Normal			
Gender	≥20	<20	X2	*P*	≥20	<20	X2	*P*	≥20	<20	X2	*P*	≥20	<20	X2	*P*
Man	77	31	0.092	0.762	35	73	0.105	0.746	61	47	1.49	0.41	7	101	3.026	0.082
Woman	63	23			26	60			41	45			12	74		
Age																
>50 years	121	49	0.668	0.414	56	114	0.924	0.336	90	80	0.073	0.478	17	153	0.067	1
≤50 years	19	5			5	19			12	12			2	22		
Status of metastasis																
Metastasis	73	32	0.795	0.373	30	75	0.876	0.349	55	50	0.004	0.534	12	93	0.692	0.279
No metastasis	67	22			31	58			47	42			7	82		
Location																
Left colon	12	3	0.164	0.685	1	14	3.468	0.063	9	6	0.359	0.373	2	13	0.231	0.645
Right colon	128	51			60	119			93	86			17	162		
Differentiation																
High	16	5	0.406	0.816	4	17	2.829	0.243	5	16	7.81	0.02	2	19	0.011	0.994
Middle	100	41			49	92			79	62			14	127		
Low	24	8			8	24			18	14			3	29		
Stage																
1	5	1	1.93	0.587	6	0	14	0.002	4	2	8.1	0.017	0	6	7.85	0.049
2	12	3			4	11			7	8			4	11		
3	107	46			45	108			79	74			11	142		
4	16	4			6	14			12	8			4	16		

Table 3 TME of CRC the cell classification and proportion

		MP0-CD3-CD68-CD16+cells		MP0-CD3-CD68-CD16+CD56+cells	MP0-CD3-CD68-CD16+CD56-cells
1	Cancer	20,607	4752(29.1%)		15,855(76.9%)
	Normal	16,409	2878(17.5%)		13,531(82.5%)
2	Cancer	10,857	300(2.8%)		10,557(97.2%)
	Normal	180	18(10.0%)		162(90.0%)
3	Cancer	876	1(0.1%)		875(99.9%)
	Normal	24	5(20.8%)		19(79.2%)
4	Cancer	55,227	6396(11.6%)		48,831(88.4%)
	Normal	80	53(66.2%)		27(33.8%)
5	Cancer	19,136	144(0.8%)		18,992(99.2%)
	Normal	1345	214(15.1%)		1131(84.1%)
6	Cancer	17,683	146(0.8%)		17,537(99.2%)
	Normal	517	186(36%)		331(64%)
7	Cancer	238	4(1.7%)		234(98.3%)
	Normal	583	312(53.5%)		271(46.5%)
8	Cancer	18,522	1736(9.4%)		16,786(90.6%)
	Normal	0	0		0
9	Cancer	23,114	768(3.3%)		22,346(96.7%)
	Normal	670	85(12.7%)		585(87.3%)
10	Cancer	592	6(1%)		586(99%)
	Normal	107	9(8.4%)		98(91.6%)

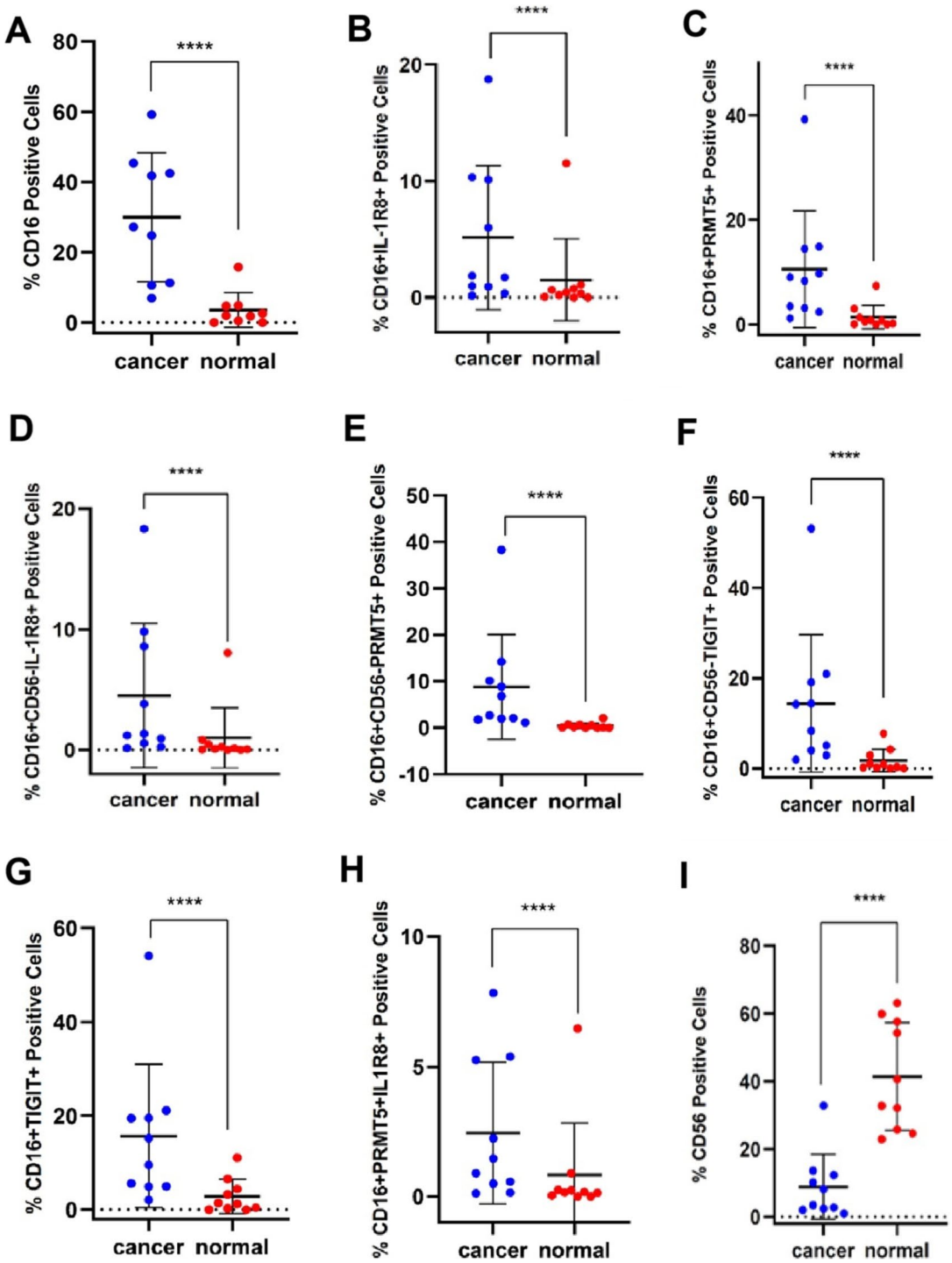

Fig. 3 Cell counts obtained from multiplex immunohistochemical staining. The percentages of CD16 + (**A**), CD16 + IL-1R8 + (**B**), CD16 + PRMT5 + (**C**), CD16 + CD56-IL-1R8 + (**D**), CD16 + CD56-PRMT5 + (**E**), CD16 + CD56-TIGTI + (**F**), CD16 + TIGIT + (**G**), and CD16 + PRMT5 + SIGIRR + (**H**) cells were significantly increased, and the percentage of CD56 + cells was significantly decreased in the TME (* for $P < 0.05$, ** for $P < 0.01$, and **** for $P < 0.001$)

Discussion

In this study, NK cells from patients with colorectal cancer presented significant phenotypic changes, such as higher expression of the NK cell inhibitory receptor CD16 and lower expression of CD69, TIGIT, IFN-γ, and CD56 in tumors than in normal control tissues, as determined by IHC. NK cells and T cells express the activation markers

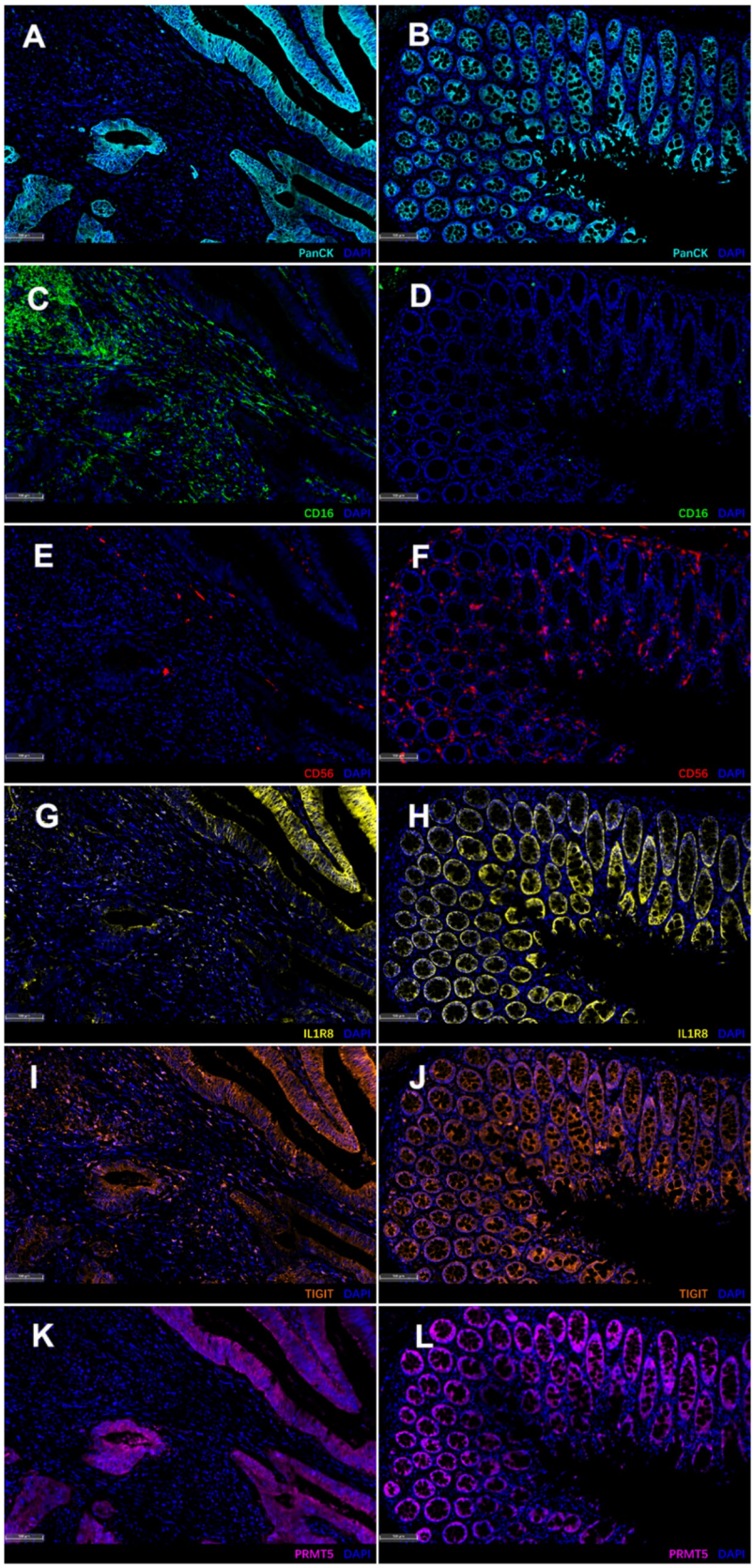

Fig. 4 Multiplex immunohistochemical staining ($n = 20$) shows the distribution of the density of various CD56-, CD16-, IL-1R8-, PRMT5-, and TIGIT-positive lymphocytes in cancer tissues. CD56-positive cells are red, and IL-1R8-positive lymphocytes are green in tumor sections. IL-1R8-positive cells. CD16-positive cells are shown in green. PRMT5-positive cells are shown in purple. TIGIT-positive samples are shown in dark red. Representative images of the multiplex immunofluorescence panel (CD16, CD56, IL1R8, PRMT5, TIGIT, PanCK, and DAPI). A human colorectal carcinoma tissue sample and histologically normal tissue adjacent to the tumor were stained with the mIF panel. Each image is of the same region of the tissue with only the indicated marker channels shown. Merged: CD16 (green), CD56 (red), IL1R8 (yellow), PRMT5 (magenta), TIGIT (orange), PanCK (cyan), and DAPI (blue). Scale bars represent 100 µm

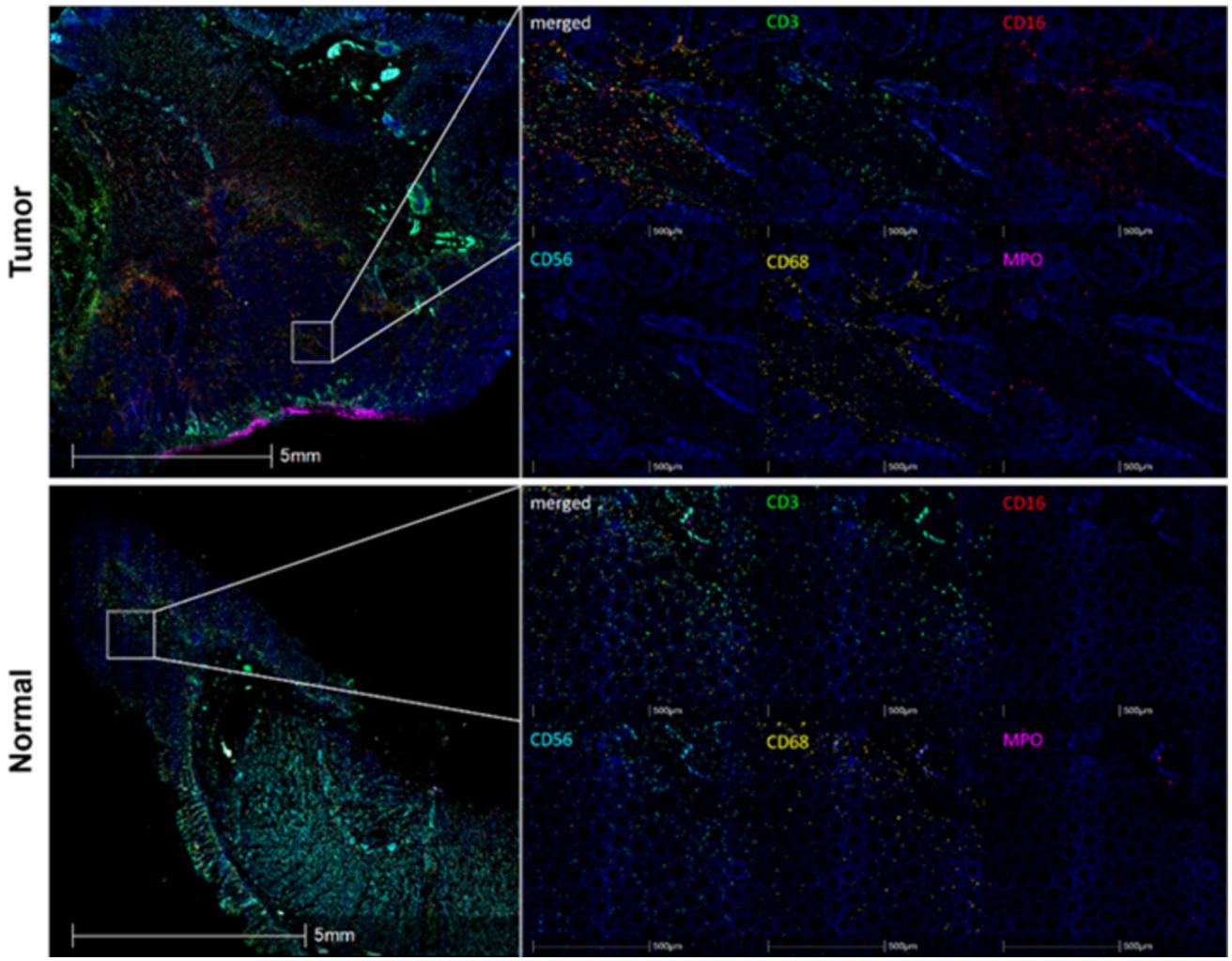

Fig. 5 In 10 cases of CRC, the expression of CD16 (red), MPO (pink), and CD3 (green) in cancer foci was significantly higher than that in normal, while CD56 (blue) was significantly lower in cancer foci, and CD68 (yellow) was not significantly different between bowel cancer and normal

CD16, CD69, and NKG2D [22], and the downregulation of NKG2D expression on tumor-infiltrating lymphocytes represents a potential mechanism of immune evasion or tumor escape [23]. In this study, immunohistochemistry revealed the downregulation of IFN-γ expression in the cancer mesenchyme. The secretion of IFN-γ and CD69 was decreased in cancer mesenchyme, and CD16-positive cells were found throughout the CRC mesenchyme, which suggested that the TME of CRC specifically contains NK cells and their surface receptors [24]. This finding indicated that the number of activated CD16 + CD56- NK cells in the cancer mesenchyme was significantly greater than that in the normal mesenchyme. Similar to ILCs, CD16 + CD56- NK cells also produce interferon-gamma (IFN)-γ and tumor necrosis factor (TNF)-α upon stimulation. IFN-γ is an important secretory factor secreted by CD16 + CD56- NK cells. A reduction in IFN-γ secretion is a marker of NK cell depletion [25]. We found that the INF-γ level in the cancer mesenchyme was significantly lower than that in the normal mesenchyme, suggesting a decrease in the number of activated NK cells in the cancer mesenchyme [26]. NK cells present significantly lower activation and degranulation activity and IFN-γ production. The previous studies have shown that the function of NK cells is enhanced by gene modification and that metabolic disorders in the tumor microenvironment may also affect their long-term survival and function.

These findings suggest that NK cells in colorectal cancer are in an immunosuppressed state. However, immunohistochemistry could not locate the type of CD16 + CD56- NK cells or CD16-CD56 + NK cells in the tumor mesenchyme, and thus, we performed staining with multiple fluorescent antibodies. CD3 is the most common marker of T cells [27], and CD68 is the most common

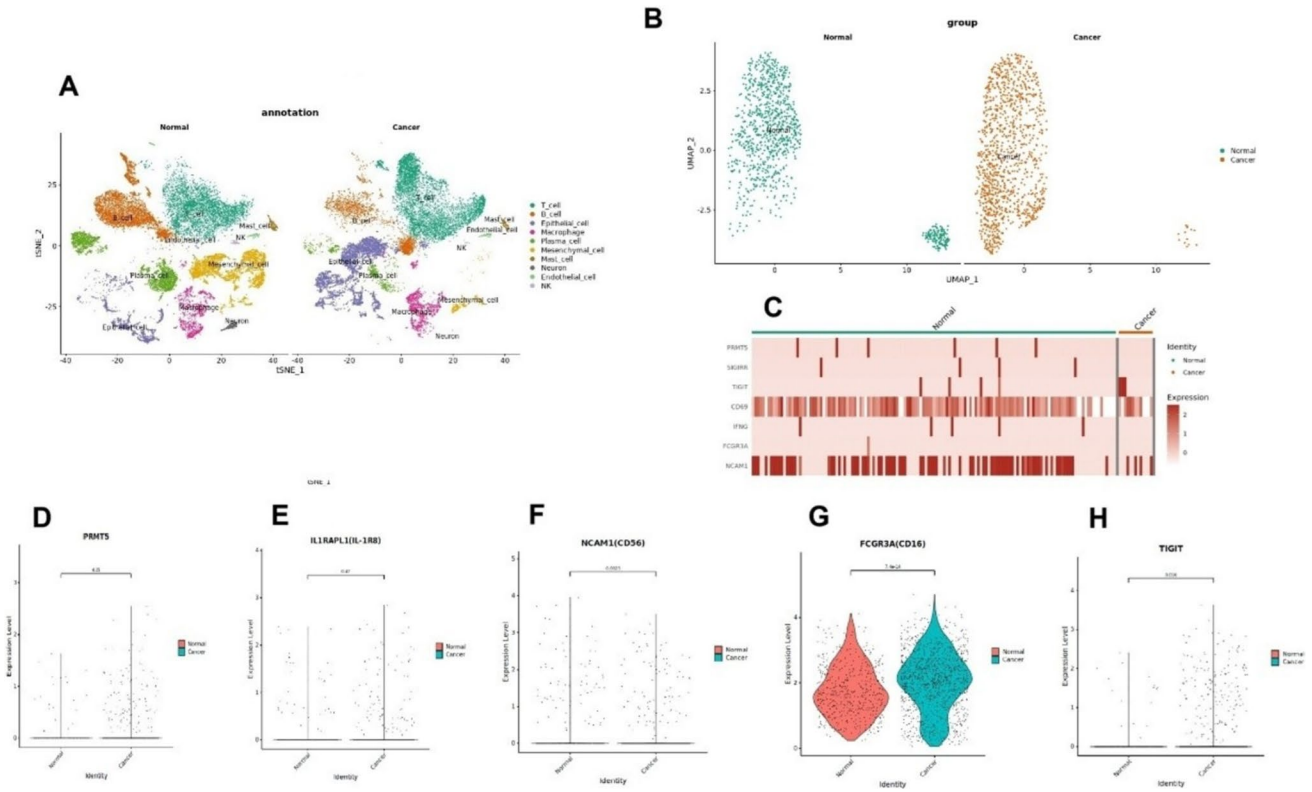

Fig. 6 Single-cell sequencing results ($n=3$). Different types of T-cell clusters in the 50X scRNA-seq data from patients with colorectal carcinoma. **A** t-SNE projection displaying the integrated information six samples of normal ($n=3$) and colorectal cancer ($n=3$) cells. t-SNE projection displaying cells from three patients with normal tissues (left panel) and colorectal cancer (CRC) (right panel). **B** The number of NK cells in the carcinoma foci was higher than that in than normal with CRC mesenchyme. **C** Heatmap displaying the genes associated with the top cell types, heatmap displaying PRMT5, SIGIRR, TIGIT, CD69, IFNGIFN-γ, FCGR3A (CD16), and NCAM1 (CD56) expression in normal tissues and colorectal cancer (CRC). **D** Expression of PRMT5 in different tissues (Wilcoxon test, two-sided) ($P=0.25$). **E** The expression of IL-1RAPL1 (IL-1R8) in different tissues (Wilcoxon test, two-sided) ($P=0.47$). **F** The expression of NCAM1 (CD56) in different tissues (Wilcoxon test, two-sided) ($P=0.0023$). **G** The expression of FCGR3A (CD16) in different tissues (Wilcoxon test, two-sided) ($P<0.05$). **H** TIGIT expression in different tissues (Wilcoxon test, two-sided) ($P=0.016$)

marker of macrophages [28]. Myeloperoxidase (MPO) is a heme-containing peroxidase that is expressed mainly in neutrophils and, to a lesser extent, in monocytes [29]. NK cells, monocytes, macrophages, and neutrophils also express CD16. Therefore, staining with multiple fluorescent antibodies was performed to determine whether NK cells constitute the main cell population that secretes CD16 and to further determine the proportion of CD16 + NK cells in Leydig cells. Among all types of MPO-CD3-CD68-CD16 + cells (representing NK cells), MPO-CD3-CD68-CD16 + CD56- cells constitute the subgroup of NK cells present at the maximum proportion in the mesenchyme of CRC. CD16 + CD56- cells constitute the most numerous group of NK cells in the mesenchyme. NK cells in the mesenchyme of CRC are in an inhibited state, which is characterized mainly by an increase in the CD16 + CD56- NK cell phenotype; the proportion of CD16 + CD56 + NK cells, which have cell killing ability, is decreased. The lower expression of granzyme B in conventional regulatory NK cells and CD3-CD56-CD16 + NK cells in patients than in healthy donors suggests reduced cytotoxic activity of NK cells in breast cancer. These results might suggest the accumulation of NK subsets with a dysfunctional phenotype [30].

The density of CD56 + NK cells in the TME of CRC patients decreased significantly, whereas the number of CD16 + NK cells increased markedly in the mesenchyme. CD16 + CD56- NK cells are a subgroup of NK cells with reduced killing ability. NK cells with a killing function were presumed to have disappeared in the TME of CRC, but this study revealed that NK cells were silenced by changing the cellular phenotype and that the inhibition of this phenotypic transformation restored the killing ability of NK cells and inhibited tumor development. Numerous inhibitory immunoreceptors, including but not limited to PD-L1, CTLA-4, LAG3, TIM3, TIGIT, and BTLA, have been identified in cancer in recent decades [31]. We observed changes in bowel cancer phenotypes across a

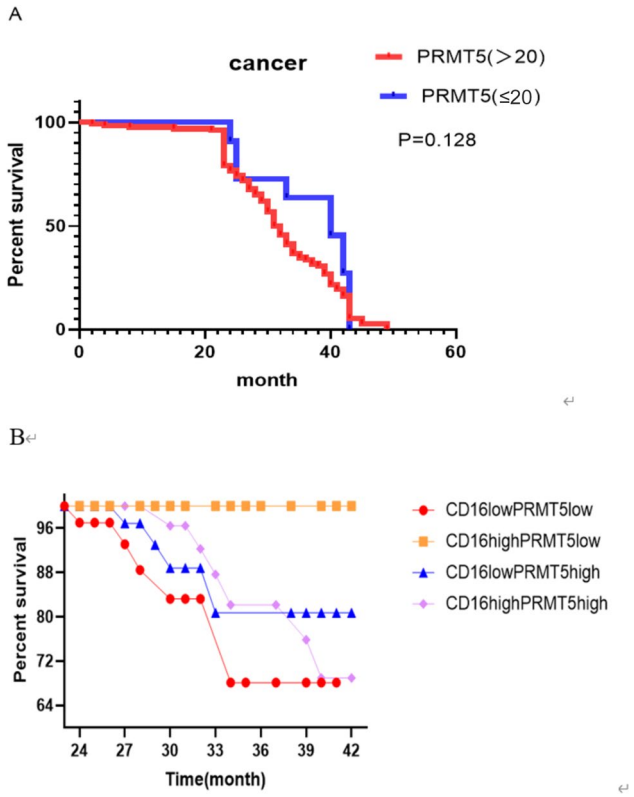

Fig. 7 The CRC patients' DFS. **A**. CRC patients with high PRMT5 expression in the TME of tumor showed worse prognosis (DFS) than those patients with low PRMT5 expression ($P=0.128$). **B**. CRC patients with CD16high PRMT5high (purple) expression in the had a significantly poorer prognosis (DFS) than did those with CD16low PRMT5low (red) expression ($P>0.05$). But the CD16low PRMT5high (blue) and CD16highPRMT5low (orange) no significance ($P>0.05$)

variety of disease states. The expansion of dysfunctional CD56-CD16 + NK cells in chronic hepatitis B patients [32], a highly dysfunctional NK subset that is expanded in HIV-infected viremic individuals, is characteristic of CD56-CD16 + natural killer (NK) cells [33]. The expansion of CD56- NK cells has more recently also been described in patients with chronic HCV infection [34]. The CD56-CD16 + subset is also detected in elderly individuals [35]. However, NK cell phenotypes have not been reported in colorectal cancer. We first proposed that changes in the NK cell phenotype affect CRC.

PRMT5 is an important regulator of CD4 + T follicular helper (Tfh) cell responses [36]. The inhibition of PRMT5 reduces T-cell proliferation and effector function, thereby mitigating disease severity [37]. PRMT5 inhibitors block catalytic activity, affecting the symmetric dimethylation of its substrates and thereby suppressing tumor cell proliferation and survival. Research has shown that PRMT5 in the nucleus is associated with cell growth inhibition, whereas cytoplasmic PRMT5 promotes cell growth. Furthermore, PRMT5 inhibition may affect the tumor microenvironment, such as by increasing PD-L1 expression and immune resistance [38]. The role of PRMT5 in the microenvironment of colorectal cancer has not been studied. Single-cell sequencing revealed that the expression levels of PRMT5 and SIGIRR, which are important therapeutic targets, are significantly increased in CD16 + CD56- NK cells in cancer. In addition, TIGIT and CD69 were expressed at significantly higher levels in CRC samples than in normal control tissues. TIGIT, a protein expressed by T and NK cells, indirectly inhibits the cytotoxicity of NK cells by binding to the poliovirus receptor (PVR) and poliovirus receptor-related 2 (PVRL2). This inhibition may be achieved by regulating the activity of dendritic cells (DCs) in the CRC microenvironment, thereby limiting the ability of NK cells to kill tumor cells [39]. These results suggest that PRMT5 and SIGIRR, TIGIT expression are significantly increased in CD16 + CD56- NK cells, and CRC of TME is a state of exhaustion.

The results of multiplex fluorescence staining revealed that the expression of TIGIT in cancer foci was significantly higher than that in adjacent tissues. A comprehensive analysis of the TME revealed a low number of NK cells in CRC tissues. The Fc receptor CD16 expressed on the surface of CD16 + CD56- NK cells can mediate ADCC; namely, effector cells secrete cytotoxin and dissolve tumor cells, or virus-infected cells coated with IgG antibodies secrete IFN-γ. Although they have lower cytotoxic activity, CD16 + CD56- NK cells can still regulate the immune response by secreting cytokines such as IFN-γ and TNF-α [40]. Therefore, the factors that lead to phenotypic changes in NK cells, which affect the inhibition of NK cell function in bowel cancer and thus enable immune escape in bowel cancer cells, are unknown. This report presents an overview of the epigenetic regulation of NK cell-mediated anti-tumor immunity, including DNA methylation, histone modification, transcription factor changes, and microRNA expression. The activity of NK cells is regulated by a variety of stimulatory and inhibitory signals, and PRMT5 may indirectly affect the function of NK cells through its methylation of arginine residues [41]. Although SIGIRR and PRMT5 play different roles in the immune system, they are both involved in the regulation of inflammation and immune responses. SIGIRR inhibits inflammatory reactions by negatively regulating the IL-1 and TLR signaling pathways, while PRMT5 influences gene expression and cellular functions through the methylation of histones, thereby affecting the function of immune cells and tumor development. However, no studies on the function of PRMT5 and SIGIRR in the microenvironment of colorectal cancer have been conducted.

PRMT5 participates in the symmetrical methylation of arginine residues in proteins and contributes to a wide range of biological processes. PRMT5 deficiency induces

the transcriptional and epigenetic programs of T cells and promotes cancer development. Inhibitors of PRMT5 promote CD8 + T-cell apoptosis by increasing P53 expression and reducing AKT pathway activity [42]. Research has revealed that PRMT5 deficiency in T cells leads to a greater reduction in CD8 + T cells and induces an increase in CD8 + regulatory T (Treg) cells, thus promoting tumor progression in model mice transplanted with a cancer cell line. PRMT5 promotes cholesterol biosynthesis and mediates Th17 responses in an experimental autoimmune encephalomyelitis model [43]. SIGIRR is a molecule that regulates NK cells and is an important "brake" for tumor and viral immune responses. Moreover, SIGIRR negatively regulates the inflammatory signaling pathways of interleukin receptors (ILRs) and Toll-like receptors (TLRs). SIGIRR is a novel immune checkpoint protein expressed by NK cells that negatively regulate their anti-tumor and antiviral activities [44]. TGF-β can be produced by multiple immune cell subsets in the TME, including regulatory T (Treg) cells, myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and cancer cells themselves [45]. These findings reveal new insights that targeting NK cell function may increase cancer immunotherapy response rates across a more diverse range of cancers [46].

The number of CD16 + CD56- NK cells in the TME of CRC patients is positively correlated with the prognosis. In patients with gastric cancer, a high density of NK cells is generally associated with favorable clinical outcomes [43]. This study revealed that the density of CD16-CD56 + NK cells in colorectal cancer tissues was significantly lower than that in adjacent tissues and that the number of activated CD16-CD56 + NK cells was lower in colorectal cancer tissues. High SIGIRR and PRMT5 expression may be important factors affecting the phenotypic transformation of CD16 + CD56- NK cells. The microenvironment of colorectal cancer is an immunosuppressive state characterized mainly by high expression of TIGIT, CD16, PRMT5, and SIGIRR; low expression of IFN-γ and CD69; significantly decreased numbers of CD16-CD56 + NK cells; and significantly increased numbers CD16 + CD56- NK cells with a weakened killing ability. PRMT5 and IL-1R8 levels were positively correlated with the inhibitory phenotype of CD16 + CD56- NK cells. This finding has not been reported in other studies. These molecules may be involved in regulating the expression of inhibitory phenotypes, and the relevant mechanisms need to be further verified. CRC patients with high PRMT5 expression in the TME showed a significantly shorter DFS ($P = 0.128$). CRC patients with CD16highPRMT5high expression in the TME experienced a significantly shorter DFS than patients with CD16lowPRMT5low expression ($P > 0.05$), the prognosis of PRMT5 in colorectal cancer needs to be further explored. At present, PRMT5 is an important potential drug target for the treatment of CRC and for clinical application. In the future, the mechanism underlying the interactions between NK cells and PRMT5 and SIGIRR, or among NK cells, PRMT5 and SIGIRR should be further explored to identify more effective immunotherapeutic strategies and provide additional approaches for the clinical treatment of CRC patients.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Consent for publication Written informed consent was obtained from the patient for the publication of anonymized data and any accompanying images.

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