

ORIGINAL RESEARCH ARTICLE

Effects of Wnt signaling pathway on tertiary lymphoid structure in the immune microenvironment of colorectal cancer with different MMR subtypes

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Abstract

Tertiary lymphoid structure (TLS) and tumor-resident memory T cells (T_{RM}), as the important components of tumor microenvironment, play key roles in the anti-tumor immunity and therapy functions in cancer patients. However, the T_{RM} and TLS distribution in colorectal cancer (CRC) patients with different mismatch repair (MMR) subtypes and Wnt pathway activation, as well as their relationship, remain underexplored. In this study, we evaluated the MMR subtypes and Wnt/ β -catenin using immunohistochemical staining and analyzed their relationship in a sample of 117 cases. We detected TLS distribution, quantity, and maturity in CRC samples of different MMR subtypes by multiple immunofluorescence staining and analyzed its correlation with Wnt/ β -catenin pathway. We then detected T_{RM} expression inside and outside the TLS and tumor tissues in 34 CRC samples by means of multiple immunofluorescence staining and analyzed its correlation with Wnt/ β -catenin pathway. Our study showed that the proportion of β -catenin in MMR-proficient (pMMR) CRC was significantly higher than that in MMR-deficient (dMMR) CRC. The peritumoral and intratumoral TLS quantity in dMMR group was obviously higher than that in high and low pMMR- β -catenin expression groups. The intratumoral TLS quantity negatively correlated with β -catenin expression in dMMR group and low pMMR- β -catenin group, respectively. In addition, the positive rate and density of $CD8^+T_{RM}$ in the TLS were significantly higher than that outside. The positive rate and density of $CD8^+T_{RM}$ of tumor tissues in dMMR CRC were higher than those in pMMR CRC. Furthermore, the positive rate of $CD8^+T_{RM}$ in tumor tissues of CRC patients in the low β -catenin expression group was higher than that in the high β -catenin expression group. Taken together, TLS and T_{RM} cells in CRC with different Wnt classical pathway activation states and MMR subtypes could serve as a potential biomarker for the prediction of the efficacy of immunotherapy in CRC patients.

Keywords: Colorectal cancer; Mismatch repair; Wnt signaling pathway; Tertiary lymphatic structure; Tissue-resident memory T cells

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1. Introduction

Colorectal cancer (CRC) is a malignant tumor occurring in the colon or rectum, with key features of poor prognosis and strong metastasis.¹ According to the global cancer statistics in 2020, the incidence and mortality of CRC ranked third and second, respectively.² Early CRC can be treated by surgical resection, whereas in the case of advanced CRC, there is no effective therapy.¹ Despite significant advances in novel immune checkpoint inhibitors (ICIs) in recent years, the effect is still unsatisfactory due to the lack of effective biomarkers. Mismatch repair-deficient (dMMR) state, a type of MMR (MMR), has been identified as a predictive biomarker for late-stage CRC in patients using PD-1 ICIs, which has an objective response rate ranging from 30% to 50% only.³ Therefore, it is essential to explore efficacious biomarkers to predict the efficacy of immunotherapy, so as to provide scientific basis for precise treatment of CRC patients with different MMR status.

Tertiary lymphoid structures (TLSs) are transient ectopic lymphoid aggregates that exhibit a structural organization and function similar to that of secondary lymphoid organs.⁴ TLSs consist of regions enriched with B cells and T cells, serving as sites for the differentiation of effector and memory T cells, as well as B cells.⁵ TLS can induce the infiltration of cytotoxic T lymphocytes into the tumor, thereby enhancing the anti-tumor immune response.⁶ Extensive research suggests that TLSs can facilitate a good prognosis and serve an effective predictive biomarker in cancer patients.⁷⁻¹¹ TLSs also may be targets of PD-1 blockade and serve as a valuable predictive immune indicator for ICIs therapy in cancer patients.⁷⁻¹¹ Our previous study showed the peritumoral and intratumoral TLS quantity were higher in dMMR than in MMR-proficient (pMMR) CRC, and patients' scores based on the intratumor TLS evaluation were positively correlated with the clinical efficacy of immunotherapy.¹¹

Tumor-resident memory T cells (T_{RM}) are a newly identified subset of long-lived memory T cells that reside

permanently in peripheral tissues without recycling.¹² Due to its unique functions, including retention in tissues and rapid response to re-stimulation, T_{RM} can actively participate in local tumor surveillance and exhibit anti-tumor immunity actions. Notably, T_{RM} is correlated with improved prognosis in cancers.¹²⁻¹⁴ In addition, $CD8^+T_{RM}$ can predict a better clinical response to PD-1/PD-L1 ICIs therapy, in diseases including CRC.¹²⁻¹⁴ Workel *et al.*¹⁵ found that the T_{RM} cells were associated with the formation of TLS. In analysis of differentially expressed genes of $CD8^+CD103^-$ and $CD8^+CD103^+$ cytotoxic T lymphocytes in ovarian, uterine, lung, and breast cancers from TCGA databases, the data showed that TLS gene was associated with $CD8^+CD103^+T_{RM}$. A study in lung cancer showed that $CD8^+CD103^+T_{RM}$ within TLSs were correlated with the TLS maturity as well as improved prognosis.¹⁶ Another study reported that $CD103^+T_{RM}$ cells resided around the TLSs, and were associated with good prognosis in gastric cancer patients.¹⁷ These results suggest that T_{RM} may have a close relationship with TLS. However, there have been no reports on the T_{RM} distribution and density in CRC patients with different MMR status and their correlation with TLS.

Most CRCs are driven by adenomatous polyposis coli (APC) mutation-induced deregulated Wnt signaling.¹⁸ Wnt/ β -catenin pathway, as Wnt classical pathway, is a growth regulation pathway that participates in regulating embryonic development, cell proliferation, differentiation, and apoptosis, as well as cancers associated with inflammation.¹⁸ Accumulating evidence suggests that Wnt/ β -catenin pathway plays important roles in the occurrence, development, metastasis, and chemotherapy resistance in CRC.¹⁹⁻²¹ More importantly, a clinical research showed that CRC patients with abnormal Wnt pathway activation had a lower response rate to ICIs.²² *In vitro* study further demonstrated that the activation of Wnt/ β -catenin pathway inhibited the T cells infiltrating into tumor cells to reduce anti-tumor immune responses.²³ However, the correlation of Wnt/ β -catenin pathway with TLSs and T_{RM} remains unclear.

In this study, we assessed the distribution, quantity, and maturity of TLS and the positive rate and density of T_{RM} cells in CRC patients with different Wnt/ β -catenin pathway activation status and MMR subtypes, and analyzed their relationship, with the aim to establish a biomarker for prognosis and prediction of immunotherapy efficacy in CRC patients.

2. Data and methods

2.1. Patients and tumor specimens

A total of 117 patients with Stage II – III CRC who underwent surgery from 2019 to 2021 at the Tianjin Medical University Cancer Hospital were enrolled in this study. Pathological TNM staging adopted in this study was based on the 8th edition of the Union for International Cancer Control TNM classification. Paraffin-embedded tumor tissue specimens of these patients were collected for subsequent immunohistochemistry and multiple immunofluorescence staining, in which 39 patients were dMMR negatively expressing one or more MMR proteins, such as MLH1, PMS2, MSH2, and MSH6, and the 78 patients were pMMR positively expressing above four MMR proteins.

Detailed sample clinical information and their associations with MMR subtypes are summarized in [Table 1](#). None of the patients had ever undergone any treatments

before surgery. Thirty-four cases, comprising 18 dMMR patients and 16 pMMR patients, were selected for multiplex immunohistochemical staining. The study protocol was approved by the Ethics Committee of the Tianjin Medical University Cancer Institute and Hospital (Ek2020214), and all enrolled patients provided written informed consent.

2.2. Multiple immunofluorescence staining

Multiple immunofluorescence staining was conducted using a PerkinElmer Opal 7-color Technology Kit (NEL81001KT; Akoya, USA) following the manufacturer's guidelines. Sections of tumor tissue embedded in paraffin were incubated with antibodies against CD20 (1:800; ab9475, Abcam), CD21 (1:800; ab75985, Abcam), CK (1:800; ab215838, Abcam), BCL-6 (1:100; NBP3-07540, NOVUS), GP2 (1:400; D277-3, MBL), CD4 (1:1000; ab133616, Abcam), CD8 (1:1500; ab217344, Abcam), CD103 (1:1500; ab129202, Abcam), and CD69 (1:1000; ab233396, Abcam). Then, the nuclei were counterstained with DAPI (1:100; Akoya, USA) after completing all the staining cycles. Finally, the slides were covered with an anti-fluorescence attenuation sealant and a cover glass.

2.3. Multispectral imaging and TLS evaluation

Tumor slides were scanned and visualized using a PerkinElmer Scanner (Massachusetts, USA). Multispectral

Table 1. Baseline characteristics of patients (n=117)

Feature	pMMR CRC patients (n=78) (%)	dMMR CRC patients (n=39) (%)	χ^2	P-value
Sex				
Male	39 (68.4)	18 (31.6)	0.154	0.695
Female	39 (65.0)	21 (35.0)		
Age				
<65 years	35 (64.8)	19 (35.2)	0.155	0.694
≥65 years	43 (68.3)	20 (31.7)		
Tumor site				
Left semicolon	44 (68.8)	20 (31.2)	0.494	0.781
Right semicolon	23 (62.2)	14 (37.8)		
Rectum	11 (68.8)	5 (31.2)		
TNM stage				
Stage II	45 (67.2)	22 (32.8)	0.017	0.895
Stage III	33 (66.0)	17 (34.0)		
Differentiation degree				
Poorly differentiated	33 (61.1)	21 (38.9)	0.280	0.597
Moderately differentiated	45 (71.4)	18 (28.6)		
Mucinous adenocarcinoma				
Yes	15 (46.9)	17 (53.1)	7.996	0.005
No	63 (74.1)	22 (25.9)		

Abbreviations: CRC: Colorectal cancer; dMMR: Mismatch repair-deficient; pMMR: Mismatch repair-proficient.

images were obtained and quantified using the inform image analysis software (v2.4.4; PerkinElmer). Spectral libraries were constructed using images of single-stained tissues labeled with each antibody. The TLSs were then manually identified. All TLSs from each tumor section were collected; and three to five fields from areas outside the TLSs were randomly selected.

The number of TLSs per mm² of the tumor area in the sections was counted as the density of TLS. Immune subsets were identified based on antibody expression, including CD4⁺T cells, CD8⁺T cells, B cells (CD20⁺), CD4⁺T_{RM} (CD4⁺CD69⁺), CD8⁺T_{RM} (CD8⁺CD103⁺CD69⁺). The proportion of the immune subsets in each TLS (or field) was counted as the percentage of the subpopulation to all nucleated cells in the TLS (or field). The average proportion in all fields (within the TLS and outside the TLS) across the entire section was used to count the proportion of the immune subsets in each patient.

2.4. Statistical analysis

Statistical analyses were performed using the SPSS software (version 23.0; IBM, USA) and GraphPad Prism (v.9.0; GraphPad Software, USA). The Shapiro–Wilk test was used to examine the normality of continuous variables. Normally distributed data are expressed as mean ± standard deviation, while data not conforming to normal distribution are presented as median and quartiles. Categorical variables are described by frequencies and percentages. Student's *t*-test or Mann–Whitney test was used for comparisons of unpaired numerical variables between the two groups. Analysis of variance (ANOVA) was used in comparisons between multiple groups. Chi-squared test or Fisher's exact test was used for comparisons of categorical variables between groups. Statistical difference was considered significant at $P \leq 0.05$.

3. Results

3.1. Correlation between different Wnt/ β -catenin pathway activation and clinicopathological features in CRC

According to the scoring standard from immunohistochemical staining performed in CRC tumor samples, the patients were divided into a high β -catenin expression group (β -catenin staining score >4 , $n = 34$) and low β -catenin expression group (β -catenin staining score ≤ 4 , $n = 83$). As shown in Table 2, the high β -catenin expression group had more subjects in TNM Stage II and with tumors located at the right semicolon (all $P < 0.05$).

3.2. Correlation between MMR subtypes and different Wnt classical pathway activation status in CRC

Based on the immunohistochemical scoring standard, dMMR CRC cases showed no high expression of β -catenin (Figure 1A). Besides, pMMR CRC cases ($n = 78$) can be classified into two groups: high β -catenin expression group (43.5%; $n = 34$) and low β -catenin expression group (56.5%; $n = 44$). We found that the proportion of β -catenin in pMMR CRC was significantly increased compared to dMMR CRC ($P < 0.05$, Figure 1B). These results indicate that the Wnt classical pathway activation in pMMR CRC was higher than that in the dMMR CRC.

3.3. Association of TLS distribution, quantity, and maturity with Wnt/ β -catenin pathway activation status in different MMR subtypes CRC patients

We first evaluated TLS distribution, quantity, and maturity in CRC patients of different MMR subtypes and Wnt classical pathway activation status (Figure 2A). We found that there was no significant difference in peritumoral and intratumoral TLS maturity between dMMR group and pMMR groups with high and low β -catenin expression (Figure 2B and 2D). The peritumoral and intratumoral TLS quantity in dMMR group was obviously higher than that in pMMR groups with high and low β -catenin expression (P all < 0.05 , Figure 2C and E). Besides, the peritumoral TLS quantity in the pMMR-high β -catenin expression group was higher than that in the pMMR-low β -catenin expression group ($P < 0.05$, Figure 2C), while the opposite result was observed in intratumoral TLS quantity ($P < 0.05$, Figure 2E).

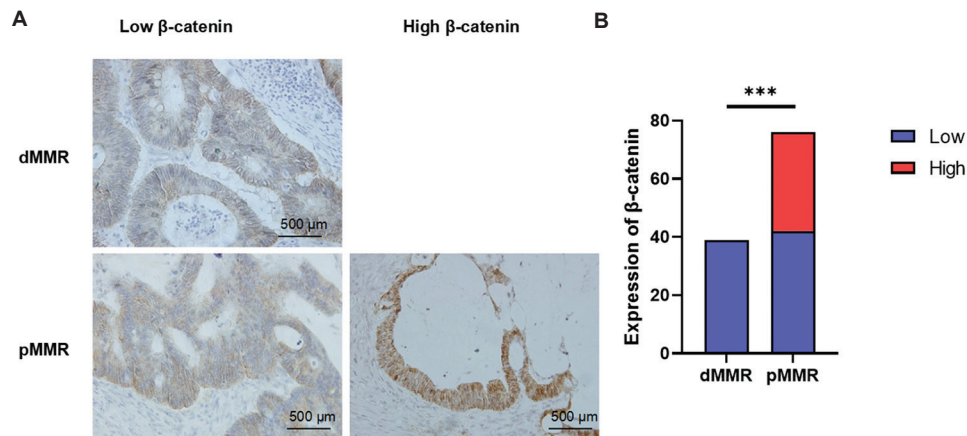
We next examined the association of TLS distribution and quantity in CRC patients of different MMR subtypes with β -catenin expression according to β -catenin staining intensity. While the total TLS number in dMMR group and pMMR group had no correlation with β -catenin expression, the intratumoral TLS number negatively correlated with β -catenin expression in dMMR group ($P < 0.05$) and pMMR-low β -catenin expression group ($P < 0.05$), respectively (Figure 3A–G).

3.4. Expression of CD4⁺T_{RM} and CD8⁺T_{RM} inside and outside the TLS and tumor tissues of CRC with different MMR subtypes

To further explore the distribution of T_{RM} in CRC patients of different MMR subtypes and Wnt classical pathway activation status, samples from 34 patients were selected for multiplex immunohistochemical staining. Representative images of CD8⁺T_{RM} and CD4⁺T_{RM} in the tumor tissues are depicted in Figures 4A and B. The clinicopathological

Table 2. Correlation between β -catenin expression and clinicopathological features in CRC ($n=117$)

Feature	High β -catenin expression ($n=34$) (%)	Low β -catenin expression ($n=83$) (%)	χ^2	P-value
Sex				
Male	15 (26.3)	42 (73.7)	0.406	0.524
Female	19 (31.7)	41 (68.3)		
Age				
<65 years	22 (40.7)	32 (59.3)	0.638	0.075
≥ 65 years	12 (19.0)	51 (81.0)		
Tumor site				
Left semicolon	8 (12.5)	56 (87.5)	13.77	0.002
Right semicolon	24 (64.9)	13 (35.1)		
Rectum	2 (12.5)	14 (87.5)		
TNM stage				
Stage II	26 (38.8)	41 (61.2)	7.224	0.007
Stage III	8 (16.0)	42 (84.0)		
Differentiation degree				
Poorly differentiated	20 (37.0)	34 (63.0)	3.096	0.079
Moderately differentiated	14 (22.2)	49 (77.8)		
Mucinous adenocarcinoma				
Yes	6 (18.8)	26 (81.2)	2.271	0.132
No	28 (32.9)	57 (67.1)		

**Figure 1.** Correlation between MMR subtypes and different Wnt classical pathway activation status in CRC. (A) Representative images of β -catenin expression in dMMR and pMMR patient samples stained by means of immunohistochemistry. Magnification: 200 \times . (B) Comparison of β -catenin expression in pMMR and dMMR CRC samples. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Abbreviations: dMMR: Mismatch repair-deficient; MMR: Mismatch repair; pMMR: Mismatch repair-proficient.

features of the patients are shown in Table 3, and the pathological parameters did not differ significantly between the two groups (all $P > 0.05$).

We first analyzed the positive rate and distribution density of $CD8^+T_{RM}$ and $CD4^+T_{RM}$ in TLS and tumor tissues. The results showed that the positive rate of $CD8^+T_{RM}$ in the TLS was significantly higher than that outside TLS ($P < 0.05$, Figure 4C). Besides, the density of $CD8^+T_{RM}$

cells in the TLS and tumor tissues was significantly higher than that outside TLS and in the stroma (all $P < 0.05$, Figure 4C and E). We also found that the positive rate of $CD4^+T_{RM}$ in the tumor tissues was significantly higher than that in the stroma ($P < 0.05$, Figure 4F). Meanwhile, the density of $CD4^+T_{RM}$ in TLS and tumor tissues was significantly higher than that outside TLS and in the stroma ($P < 0.05$, Figure 4D and F).

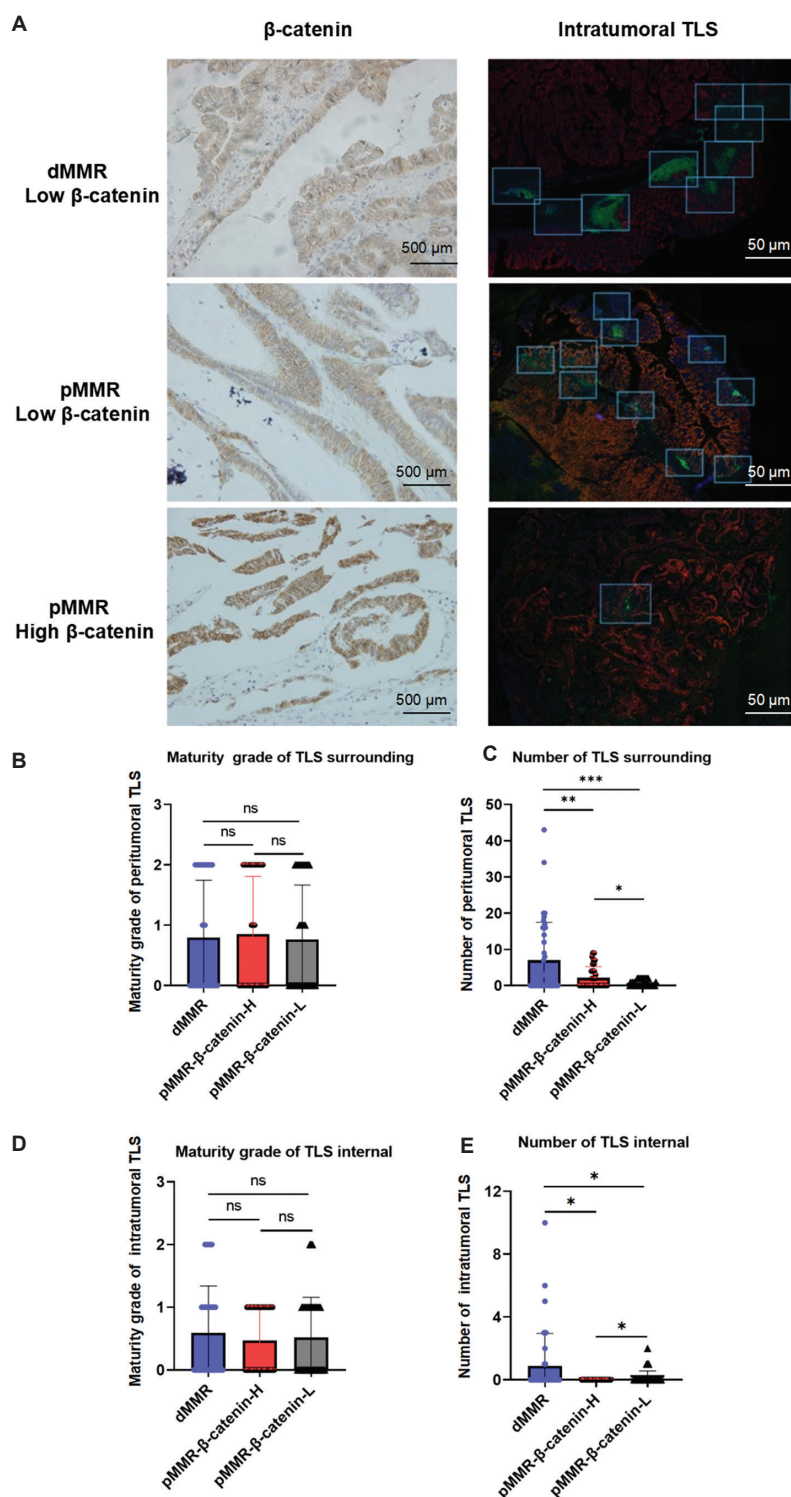


Figure 2. Comparison of TLS maturity and quantity in dMMR and pMMR CRCs with different Wnt classical pathway activation status ($n = 117$). (A) Representative images of intratumoral β -catenin and TLS in dMMR group, pMMR-high β -catenin group, and pMMR-low β -catenin group. Magnification: 200 \times . (B) Comparison of peritumoral TLS maturity in dMMR group, pMMR-high β -catenin group, and pMMR-low β -catenin group. (C) Comparison of peritumoral TLS number in dMMR group, pMMR-high β -catenin group, and pMMR-low β -catenin group. (D) Comparison of intratumoral TLS maturity in dMMR group, pMMR-high β -catenin group, and pMMR-low β -catenin group. (E) Comparison of intratumoral TLS number in dMMR group, pMMR-high β -catenin group, and pMMR-low β -catenin group. "ns" denotes $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Abbreviations: dMMR: Mismatch repair-deficient; pMMR: Mismatch repair-proficient; TLS: Tertiary lymphoid structure.

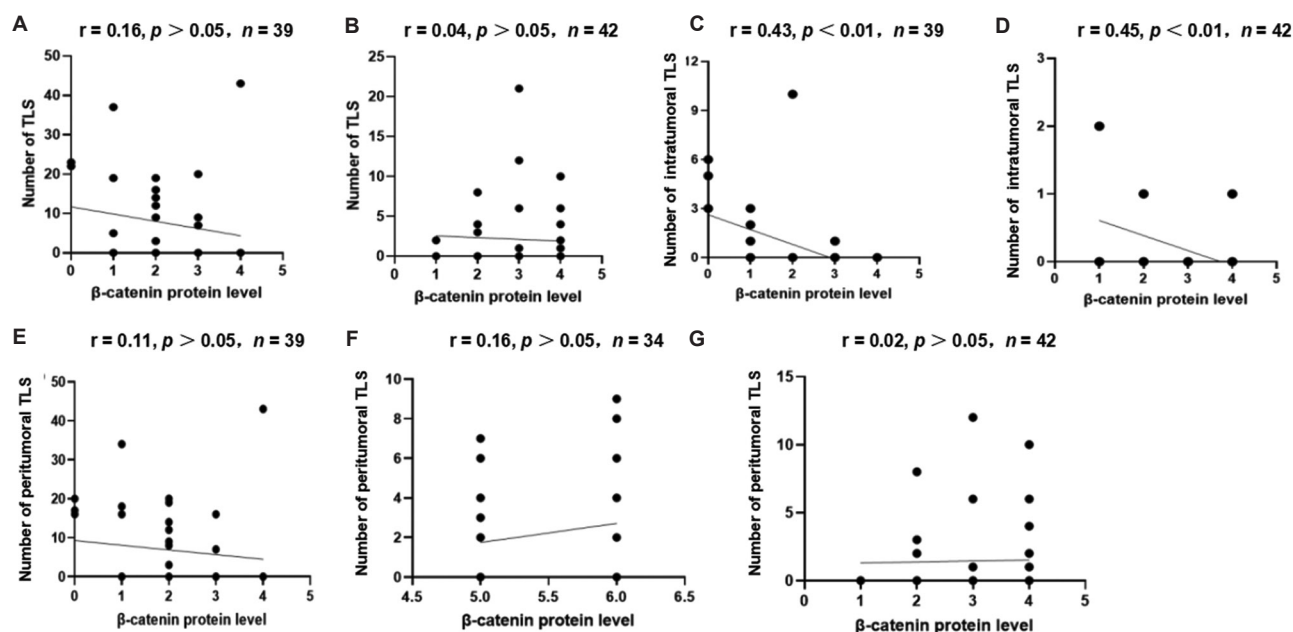


Figure 3. Association of TLS distribution, quantity and maturity with Wnt classical pathway activation status in CRC patients with different MMR subtypes. (A) Association of TLS number in the whole tumor with β -catenin staining intensity in dMMR group ($P > 0.05$). (B) Association of TLS number in the whole tumor with β -catenin staining intensity in pMMR group ($P > 0.05$). (C) Association of intratumoral TLS number with β -catenin staining intensity in dMMR group ($P < 0.01$). (D) Association of intratumoral TLS number with β -catenin staining intensity in pMMR-low β -catenin expression group ($P < 0.01$). (E) Association of peritumoral TLS number with β -catenin staining intensity in pMMR-low β -catenin expression group ($P > 0.05$). (F) Relationship between peritumoral TLS number with β -catenin staining intensity in pMMR-high β -catenin expression group ($P > 0.05$). (G) Association of peritumoral TLS quantity with β -catenin staining intensity in pMMR-low β -catenin expression group ($P > 0.05$).

Abbreviations: CRC: Colorectal cancer; dMMR: Mismatch repair-deficient; pMMR: Mismatch repair-proficient; TLS: Tertiary lymphoid structure.

Table 3. Baseline characteristics of patients ($n=34$)

Feature	pMMR CRC patients ($n=16$) (%)	dMMR CRC patients ($n=18$) (%)	χ^2	P-value
Sex				
Male	12 (50.0)	12 (50.0)	0.283	0.595
Female	4 (40.0)	6 (60.0)		
Age				
<65 years	10 (43.5)	13 (56.5)	0.366	0.545
≥ 65 years	6 (54.5)	5 (45.5)		
Tumor site				
Right semicolon	5 (55.5)	4 (44.5)	1.164	0.559
Left semicolon	11 (45.8)	13 (54.2)		
Rectum	0 (0.0)	1 (100.0)		
TNM stage				
Stage II	10 (43.5)	13 (56.5)	0.366	0.545
Stage III	6 (54.5)	5 (45.5)		
Differentiation degree				
Poorly differentiated	10 (52.6)	9 (47.4)	0.537	0.464
Moderately differentiated	6 (40.0)	9 (60.0)		
Mucinous adenocarcinoma				
Yes	2 (22.2)	7 (77.8)	3.031	0.082
No	14 (56.0)	11 (44.0)		

Abbreviations: CRC: Colorectal cancer; dMMR: Mismatch repair-deficient; pMMR: Mismatch repair-proficient.

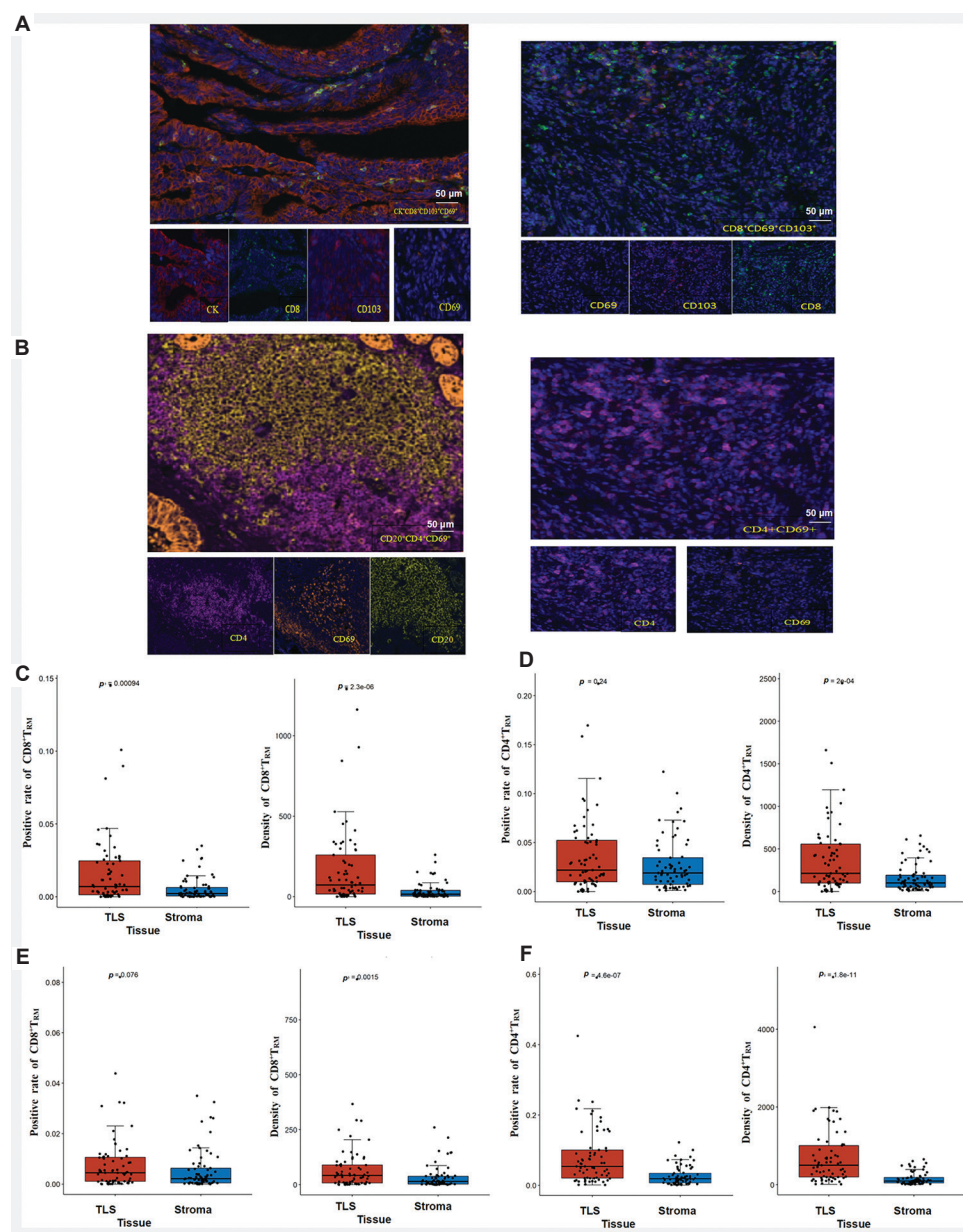


Figure 4. Comparison of positive rate and density of CD8⁺T_{RM} and CD4⁺T_{RM} in TLS and tumor tissues. (A) Representative images of CD8⁺T_{RM} in the tumor tissues (left) and the stroma (right). Magnification: 200×. (B) Representative images of CD4⁺T_{RM} inside (left) and outside (right) TLSs. Magnification: 200×. (C) Comparison of the CD8⁺T_{RM} positive rate (left) and density (right) inside and outside TLSs. (D) Comparison of the CD4⁺T_{RM} positive rate (left) and density (right) inside and outside TLSs. (E) Comparison of the CD8⁺T_{RM} positive rate (left) and density (right) in tumor tissues. (F) Comparison of the CD4⁺T_{RM} positive rate (left) and density (right) in tumor tissues. **P*<0.05, ***P*<0.01, ****P*<0.001. Abbreviations: TLS: Tertiary lymphoid structure; T_{RM}: Tumor-resident memory T cells.

We next compared the difference in the positive rate and density of CD8⁺T_{RM} and CD4⁺T_{RM} in TLS and tumor tissues between dMMR and pMMR CRCs. The results showed that there was no significant difference in the positive rate of CD4⁺T_{RM} in TLS and tumor tissues and CD8⁺T_{RM} cells in TLS between the two groups (Figure 5A-C), while the positive rate of CD8⁺T_{RM} of tumor tissues in dMMR CRC was higher than that in pMMR CRC (*P* < 0.05,

Figure 5D). We also found that there was no significant difference in the density of CD4⁺T_{RM} cells in TLS and tumor tissues and CD8⁺T_{RM} cells in TLS between the two groups (Figure 5E-G), while the density of CD8⁺T_{RM} in tumor tissues in dMMR CRC was higher than that in pMMR CRC (*P* < 0.05, Figure 5H). These results indicate that the positive rate and density of CD8⁺T_{RM} of tumor tissues in dMMR CRC were higher than those in pMMR CRC.

3.5. Association of CD4⁺T_{RM} and CD8⁺T_{RM} expression with Wnt classical pathway activation status in CRC patients

We further explored the positive rate of CD4⁺T_{RM} and CD8⁺T_{RM} in CRC patients with different Wnt classical

pathway activation status. The results showed that there was no significant difference in the positive rate of CD4⁺T_{RM} between the high and low β -catenin expression groups (Figure 6A), while the positive rate of CD8⁺T_{RM} in tumor tissues of CRC patients in the low β -catenin expression group was higher than that in high β -catenin expression group ($P < 0.05$, Figure 6B).

4. Discussion

In the present study, we first evaluated the correlation between different Wnt classical pathway activation status and clinicopathological features in CRC. We found that CRC patients with Stage II had higher β -catenin expression than those with Stage III. Consistent with this finding, Scholtka *et al.*²⁴ found that 80% of early-stage CRC exhibited Wnt pathway activation. Another study from India reported that early-stage CRC is associated with more enhanced Wnt non-canonical pathway activation.²⁵ Besides, patients with CRC on the right colon showed a higher β -catenin expression. This was consistent with a study from China, showing that homeobox C6 (HOXC6), mainly expressed in the right colon, promoted epithelial-mesenchymal transition, migration, and invasion of CRC cells by activating Wnt/ β -catenin pathway and inhibiting DKK1 secretion.²⁶ A large-scale primary CRC patient study indicated that T-cells infiltrating into tumor were reduced when the Wnt pathway was activated in subgroups with high microsatellite instability (MSI-H).²⁷ Another recent investigation evaluated the association among β -catenin, CMTM6, PD-L1 expression, MMR status, and clinicopathological features in 704 patients with CRC, revealing that PD-L1 expression in tumor-infiltrating immune cells was associated with a higher frequency of adenocarcinoma, β -catenin, and CMTM6 expression.²⁸ However, the relationship between Wnt/ β -catenin and

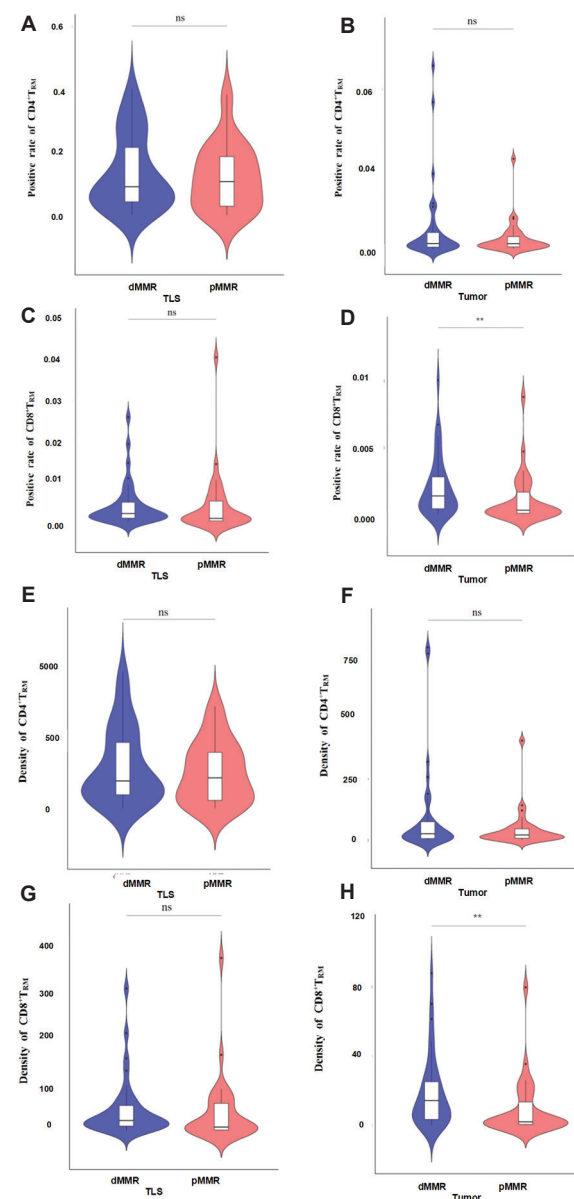


Figure 5. Comparison of positive rate and density of CD8⁺T_{RM} and CD4⁺T_{RM} in TLS and tumor tissues between dMMR and pMMR CRCs. (A-D) Comparison of positive rate of CD8⁺T_{RM} and CD4⁺T_{RM} in TLS and tumor tissues between dMMR and pMMR CRCs. (E-H) Comparison of density of CD8⁺T_{RM} and CD4⁺T_{RM} in TLS and tumor tissues between dMMR and pMMR CRCs. "ns" denotes $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Abbreviations: dMMR: Mismatch repair-deficient; pMMR: Mismatch repair-proficient; TLS: Tertiary lymphoid structure; T_{RM}: Tumor-resident memory T cells.

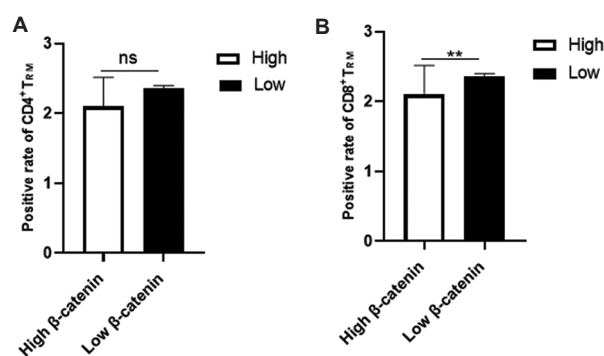


Figure 6. Association of CD4⁺T_{RM} and CD8⁺T_{RM} expression with Wnt classical pathway activation status in CRC patients. (A) Association of the positive rate of CD4⁺T_{RM} with β -catenin expression in CRC patients. (B) Association of the positive rate of CD8⁺T_{RM} with β -catenin expression in CRC patients. "ns" denotes $P > 0.05$, ** $P < 0.01$. Abbreviation: T_{RM}: Tumor-resident memory T cells.

MMR status in CRC remains unclear. Our study found that the expression of β -catenin in pMMR CRC was higher than that in the dMMR CRC. Therefore, we speculate that the activation of Wnt/ β -catenin pathway in pMMR CRC was higher than that in the dMMR CRC.

Our previous study has shown that both peritumoral and intratumoral TLS numbers were higher in dMMR CRC than in pMMR CRC.¹⁶ Further analysis of the association of TLS distribution and number with Wnt classical pathway activation in CRC patients of different MMR subtypes showed that the β -catenin expression was lower and the whole TLS quantity was higher in dMMR CRC, and the peritumoral TLS number was higher and the intratumoral TLS was lower in the pMMR-high β -catenin expression group. We also found that the intratumoral TLS number was negatively correlated with β -catenin expression in dMMR group and pMMR-low β -catenin group, respectively. These results suggest the distribution and quantity of TLS may be associated with the MMR status, as well as Wnt/ β -catenin pathway activation. This is consistent with the findings in hepatoblastoma, in which 11 APC inactivation cases who received cisplatin-based neoadjuvant chemotherapy exhibited massive TLS containing both lymphocytes and antigen-presenting cells and a good prognosis, indicating that Wnt/ β -catenin pathway inactivation can synergize with cisplatin to induce an immunogenic cell death that initiates an anti-tumor immune response.²⁹ Moreover, another study showed that CRC patients with MSI-H had lower T-cells infiltration when Wnt signaling was activated.²⁷ Mechanistically, it could be caused by the activation of Wnt signaling pathway that leads to the dysfunction of dendritic cells maturation, thus affecting the aggregation of T cells and B cells, and resulting in failed formation of TLS.²⁷ These results indicate the Wnt signaling pathway activation may have negative regulatory effects on the distribution and number of TLS in CRC.

Next, we evaluated the distribution of $CD4^+T_{RM}$ cells and $CD8^+T_{RM}$ cells in tumor tissue and found that the positive rate and density of $CD8^+T_{RM}$ within TLS was significantly higher than that outside. Besides, the density of $CD4^+T_{RM}$ in TLS was significantly higher than that outside. Our previous study on the distribution of T_{RM} in patients with lung adenocarcinoma reported similar results.¹⁶ The proportion of $CD4^+T_{RM}$ and $CD8^+T_{RM}$ within TLSs was significantly higher than that outside and was increased with the gradual maturation of TLSs.¹⁶ Another study on gastric cancer also showed that T_{RM} cells were present around the TLSs,¹⁷ which contained 70% $CD8^+T_{RM}$. These results indicate that there is a close relationship between the T_{RM} and TLS. There are few reports on T_{RM}

cells in CRC patients of different MMR subtypes. Only a study showed that dMMR bladder cancer patients with elevated $CD8^+CD103^+T_{RM}$ cell infiltration had improved response rate to neoadjuvant chemotherapy.³⁰ We found that the positive rate and density of $CD8^+T_{RM}$ of tumor tissues in dMMR CRC was higher than that in pMMR CRC. Therefore, we speculate that $CD8^+T_{RM}$ could serve as potential targets for precise treatments in CRC patients of different MMR subtypes.

Finally, the association of the positive rate of $CD8^+T_{RM}$ in CRC patients with different Wnt/ β -catenin pathway activation was analyzed. The data showed that the positive rate of $CD8^+T_{RM}$ of tumor tissues in the low β -catenin expression group was significantly higher than that in the group featuring high expression. This was consistent with the finding that ICCs with low proportions $CD8^+T_{RM}$ exhibited significant enrichment of genes related to the Wnt/ β -catenin and transforming growth factor beta pathways in cholangiocarcinoma. Besides, tumors with high proportions of $CD8^+T_{RM}$ displayed higher levels of parameters associated with response to ICIs, including number of $CD8^+TIL$ infiltrates, PD-L1 expression in the tumor, and T-cell inflammation gene expression.³¹ An *in vitro* study further demonstrated that the activation of Wnt/ β -catenin pathway inhibited $CD8^+T_{RM}$ differentiation and promoted immune memory formation.³² Mechanistically, it is possible that the activation of Wnt signaling pathway affects dysfunction of dendritic cells, which further affects T_{RM} formation.³² All these results indicate that Wnt signaling pathway is possibly related with $CD8^+T_{RM}$ in CRC.

5. Conclusion

The present study showed that the activation of Wnt/ β -catenin pathway in pMMR CRC was more pronounced than that in dMMR CRC. The peritumoral and intratumoral TLS number and the positive rate and density of $CD8^+T_{RM}$ of tumor tissues in dMMR CRC were higher than those in pMMR CRC. The activation of Wnt/ β -catenin pathway has negative regulatory effects on the distribution and quantity of TLS in CRC. Therefore, an in-depth analysis of TLS and T_{RM} cells in CRC with different Wnt classical pathway activation states and MMR subtypes can facilitate the exploration of potential biomarkers for the prediction of immunotherapy efficacy in CRC patients. However, this study has some limitations, and further clinical or experimental investigations will be needed to explore the underlying mechanism.

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Conflict of interest

Huijing Feng is an Editorial Board Member of this journal but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Separately, other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the Tianjin Medical University Cancer Institute and Hospital (Ek2020214), and all enrolled patients provided written informed consent before participation.

Consent for publication

Not applicable.

Availability of data

All data from the study have been presented in the article.

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