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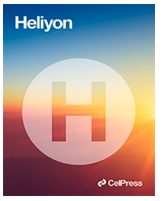

Research article

Diagnostic and prognostic value of double-negative T cells in colorectal cancer

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ABSTRACT

Objective: To evaluate the T-lymphocyte subset distribution and the diagnostic and prognosis value of double-negative T (DNT) cells in colorectal cancer (CRC).
Methods: This retrospective study compared the T-lymphocyte subsets and DNT of 114 patients with CRC with those of 107 healthy controls (HC). The diagnostic potential of DNT and T-lymphocyte subsets was assessed using the receiver operating characteristic (ROC) curve, and prognostic values were evaluated using the Kaplan–Meier curve and the Cox regression model.
Results: The percentages of $CD8^+$ T cells and DNT cells, and value of carcinoembryonic antigen (CEA), were remarkably higher in patients with CRC than in those with HC, but the ratio of $CD4^+/CD8^+$ was decreased. Using ROC curve analysis, DNT cell percentage, CEA, and $CD4^+/CD8^+$ ratio all had good diagnostic efficacy, with areas under the curve (AUCs) of 0.865, 0.786 and 0.624, respectively. The combination of DNT cell percentage and CEA had an AUC of 0.905, which was significantly higher than that of any single biomarker ($p < 0.05$). In univariate analysis, the Tumor Node Metastasis (TNM) clinical stage, $CD4^+/CD8^+$ ratio, and DNT cell percentage were significantly associated with overall survival (OS) ($p < 0.05$). In multivariate analysis, TNM clinical staging (HR = 2.37, 95 % CI: 1.15–4.90), a decreased $CD4^+/CD8^+$ ratio (HR = 0.33, 95 % CI: 0.15–0.74), and an increased DNT cell percentage (HR = 2.29, 95 % CI: 1.11–4.73) were independent prognostic factors for CRC.
Conclusion: The percentage of DNT cells may be useful as an evaluation index for CRC diagnosis and prognosis, which was even better when combined with serum CEA.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer-related mortality worldwide [1]. Over the last 15 years, the incidence of CRC has been rising rapidly in people under age 50 [2], The 5-year overall survival (OS) rate of early-stage CRC can exceed 95 % after treatment and can even be cured completely [3], but current methods for early diagnosis and treatment of CRC are ineffective. Carcinoembryonic antigen (CEA)—a serological marker—has been widely used for diagnosis and efficacy evaluation in CRC, but it is less effective for early CRC diagnosis [4]. The gold standard for the clinical diagnosis of CRC is the

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histopathological examination of a biopsy sample; however, its invasive nature is difficult for some patients to tolerate, and the examination can cause complications, such as intestinal perforation [5].

The immune system plays a vital role in the development and progression of cancer. Double-negative T (DNT) cells, defined by the expression of CD3 in the absence of CD4 and CD8, comprise 3%–5% of the peripheral blood mature T-lymphocyte pool [6,7]. Such DNT cells exist as an essential T-cell subset, which plays an indispensable role in immune system function and DNT cells can have either tumor-promoting or tumor-inhibiting effects depending on the distinct tumor microenvironment and tumor type [8]. DNT cell number has been found to be increased in malignant pleural effusions in lung cancer [9], and the percentage of DNT cells acted as a diagnostic immunogenomic marker of thyroid cancer in thyroid fine-needle aspiration samples [10], suggesting that DNT are a potential diagnostic tool for cancers. However, the diagnostic value of DNT cells in CRC remains unknown.

In this study, we analyzed T-lymphocyte subsets in patients with CRC, especially DNT cells, and explored the diagnostic and prognostic value of peripheral blood $CD4^+/CD8^+$ ratio, DNT percentage, and CEA level in CRC.

2. Materials and methods

2.1. Patient selection

A total of 114 patients initially diagnosed with CRC who were admitted to the 960th Hospital of the PLA Joint Logistics Support Force, China, from June 2014 to December 2016 were retrospectively enrolled in this study. All diagnoses of CRC were confirmed by histological findings. Inclusion criteria: (1) Both imaging and pathological examination met the diagnostic criteria for colorectal cancer; (2) No receiving surgery, radiotherapy, chemotherapy, medication, or other anti-cancer treatments; (3) The patient has complete clinical medical records. Exclusion criteria: (1) Patients with heart, lung, and other significant organ dysfunction; (2) Patients with infectious and immunological diseases; (3) Patients with no preoperative serum CEA measurements. A patient selection flow chart is shown in Fig. 1.

The control group consisted of 107 healthy people. According to their physical examination and screening at the hospital, none of the healthy participants had clinical evidence of any apparent disease. There was no significant difference in gender and age between the two groups ($p > 0.05$). This study was approved by the institutional ethics commission of the 960th Hospital of the PLA Joint Logistics Support Force, China.

2.2. Methods

A 2 mL EDTA-anticoagulated blood sample was collected, and the percentage of T-lymphocyte subsets was quantified using a Beckman FC 500 analyzer (Beckman Coulter, Brea, CA, USA) within 2 h after venipuncture. According to the manufacturer's instructions, 10 μL of fluorescently labeled monoclonal antibodies (CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5, Beckman Coulter, Brea, CA, USA) were added to the test tube followed by 100 μL of EDTA dipotassium for anticoagulation, and the mixture incubated for 20 min in the dark. Lysin was used to dissolve red blood cells. Staining with 7-AAD was used as a quality control measure for the percentage of living cells, and all samples were ≥95 % viable. Data were analyzed using CXP software (Beckman Coulter, Brea, CA, USA). Serum CEA levels were assessed by the electrochemiluminescence method using a Cobas e601 analyzer (Roche, Berlin, Germany). The

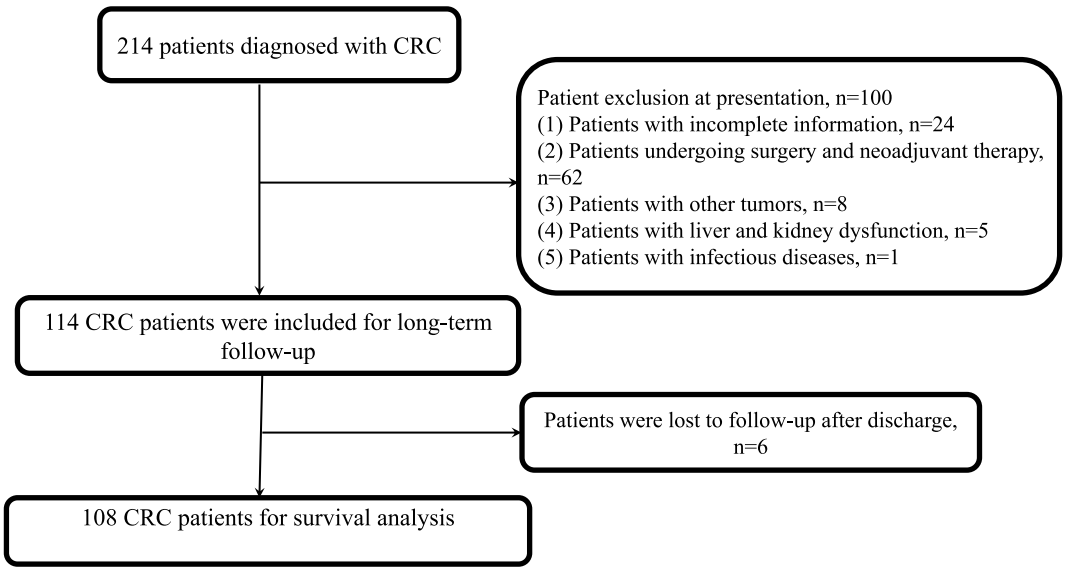

Fig. 1. Patient selection flow chart.

average sampling time of the patients with CRC was 3 days prior to surgery. All fasting venous blood samples were analyzed within 1 h after venipuncture.

2.3. Survival and follow-up

All 114 patients were followed at regular 3–6 month intervals with a program that included outpatient visits, SMS (Short Message Service), telephone calls, and WeChat. Six patients were lost to follow-up after discharge. The OS rate was calculated from the date of diagnosis to the date of death or the end-point of follow-up, through June 30, 2020.

2.4. Statistical analysis

The statistical analyses in this study were performed using SPSS 22.0 (Chicago, IL, USA), GraphPad Prism 7 (San Diego, CA, USA), and MedCalc 9.2 (Mariakerke, Belgium) software. Continuous variables are presented as the mean ± standard deviation or median (quartile). The normality of the calculated variables was assessed using the Kolmogorov–Smirnov test. Student's *t*-test and the Mann–Whitney *U* test were used for normally and non-normally distributed data, respectively. The Chi-squared test was used to compare categorical data. The combined diagnostic ability of $CD4^+/CD8^+$ ratio, DNT percentage, and CEA values was assessed using a binary logistic regression model to calculate overall predictive probability and was further evaluated via a receiver operating characteristic (ROC) curve analysis. A survival analysis was performed using the Kaplan–Meier method and Cox's proportional hazard model. The univariate analysis identified variates with significant differences ($p < 0.05$), which were selected for further multivariate Cox regression survival analysis. A value of $p < 0.05$ was considered statistically significant.

Table 1
Clinicopathological features in patients with colorectal cancer and in healthy controls.

Variables	CRC ($n = 114$)	HC ($n = 107$)
Sex		
Male	66 (57.9 %)	57 (53.3 %)
Female	48 (42.1 %)	50 (46.7 %)
Age (years)	59.82 ± 14.75	63.55 ± 12.09
Smoking	34	38
Drinking	73	59
Location		
Colon	76 (66.7 %)	
Rectum	38 (33.3 %)	
Grade		
High	11 (9.6 %)	
Moderate	79 (69.3 %)	
Low	24 (21.1 %)	
TNM stage		
I + II	69 (60.5 %)	
III + IV	45 (39.5 %)	
Tumor Size (cm)		
<5	86 (75.4 %)	
≥5	28 (24.6 %)	
Lymph Node Metastasis		
Yes	46 (40.4 %)	
No	68 (59.6 %)	
Neoadjuvant Therapy		
Chemotherapy and radiotherapy	41 (36.0 %)	
Chemotherapy	68 (59.6 %)	
Radiotherapy	5 (4.4 %)	
Diabetes	94 (82.5 %)	
No		
Yes	20 (17.5 %)	
Hypertension		
Yes	89 (78.1 %)	
No	25 (21.9 %)	
Genetic history		
No	75 (65.8 %)	
Yes	39 (34.2 %)	

Data are expressed as number (%) and mean ± standard deviation.
Note: CRC: colorectal cancer; HC: healthy controls; TNM: Tumor Node Metastasis.

3. Results

3.1. Baseline characteristics

A total of 114 patients with CRC and 107 HC were included in this study. Among these CRC patients, 76 had colon carcinoma and 38 had rectum carcinoma. In addition, 39.5 % of the patients with CRC were in the TNM III-IV stages, and 40.4 % of the patients had lymph node metastasis (Table 1).

3.2. Comparison of immunobiological indicators between the colorectal cancer group and the healthy control group

To evaluate the proportion of T-lymphocyte subsets in the CRC and HC groups, we used flow cytometry to analyze the T-lymphocyte marker expression in peripheral blood lymphocytes. The percentages of $CD8^+$ T lymphocytes and DNT cells were significantly higher in the CRC group, but the ratio of $CD4^+/CD8^+$ T cells was significantly lower ($p < 0.01$) (Table 2, Fig. 2). The results suggest that the ratios of $CD4^+/CD8^+$, $CD8^+$ percentage, and DNT cell percentage were potential diagnostic tools for CRC.

3.3. Diagnostic values of the ratio of $CD4^+/CD8^+$, percentage of DNT, and CEA levels in colorectal cancer

We used the ROC curve analysis to estimate the diagnostic efficiency of $CD4^+/CD8^+$ ratio, DNT cell percentage, and CEA value, and the results are shown in Fig. 2 and Table 3. The areas under the curve (AUC) of the $CD4^+/CD8^+$ ratio, DNT percentage, and CEA levels were 0.624 (95 % CI: 55.6–68.8), 0.865 (95 % CI: 81.3–90.7), and 0.786 (95 % CI: 72.6–83.8), respectively. DNT cells had the highest diagnostic accuracy, compared with $CD4^+/CD8^+$ ratio and CEA value.

To clarify whether the combination of CEA value, CD4/CD8 ratio, and DNT percentage could improve the diagnostic utility, we used a logistic regression model to combine these values. The combination of DNT cells and CEA improved the sensitivity, specificity, and diagnostic efficiency for CRC, but the additional increase in the $CD4^+/CD8^+$ ratio did not improve diagnostic accuracy (Fig. 3 and Table 4). Thus, the combined detection of DNT cell percentage and CEA value is a potential diagnostic biomarker for CRC.

3.4. The prognostic value of $CD4^+/CD8^+$ ratio and double-negative T cell percentage in patients with colorectal cancer

To further evaluate the prognostic value of $CD4^+/CD8^+$ ratio and DNT cell percentage in CRC, we analyzed the prognoses of 108 patients with CRC. The Kaplan–Meier cumulative survival curves for OS based on the $CD4^+/CD8^+$ ratio and DNT cell percentage are shown in Fig. 4. The low-$CD4^+/CD8^+$ ratio group (<1.2) had a worse prognosis than did the high-$CD4^+/CD8^+$ ratio group (≥1.2) (HR = 0.33, $p = 0.01$). The low-DNT cell percentage group (<8.55 %) had a better prognosis than the high-DNT cell percentage group (≥8.55 %) (HR = 2.06, $p = 0.04$).

The univariate and multivariate analyses are shown in Table 5. In univariate analysis, the Tumor Node Metastasis (TNM) clinical stage, $CD4^+/CD8^+$ ratio, and DNT cell percentage were significantly associated with OS ($p < 0.05$). To identify independent prognostic factors, significant factors according to the univariate analyses were included in a multivariate analysis. By multivariate analyses, the TNM stage (HR = 2.37, 95 % CI: 1.15–4.90), $CD4^+/CD8^+$ ratio (HR = 0.33, 95 % CI: 0.15–0.74), and percentage of DNT cells (HR = 2.29, 95 % CI: 1.11–4.73) were independent prognostic factors of OS ($p < 0.05$).

4. Discussion

Tumor initiation and progression are closely related to systemic immune response. As a vital component of the immune system, the number and proportion of T-lymphocyte subsets are important indicators of the body's immune responsiveness [11]. In our study, we analyzed the T lymphocyte subsets in patients with CRC and found that, relative to healthy people, the percentage of $CD8^+$ T cells was increased, the ratio of $CD4^+/CD8^+$ was decreased, and the percentage of DNT was greatly increased, which is more interesting. We analyzed the percentage of DNT pre-surgery and seven days after surgery in 55 patients with CRC treated between June 2017 and November 2019 in our previous study [12], and we found that the percentage of DNT after surgery reduced significantly compared to the values pre-surgery, suggesting that DNT cells could be used as potential biomarkers for diagnosing CRC. Further analysis showed that the percentage of DNT was not statistically different between different stages of CRC or the location of the tumor in the colon or

Table 2
Comparison of T lymphocyte percentage and carcinoembryonic antigen levels in patients with colorectal cancer and in healthy controls.

Group	*n*	$CD3^+$	$CD4^+$	$CD8^+$	$CD4^+/CD8^+$	DNT	CEA
HC	107	67.23 ± 11.84	34.76 ± 8.70	26.43 ± 8.20	1.59 ± 0.75	4.87 ± 1.90	2.13 ± 2.85
CRC	114	65.30 ± 10.56	32.14 ± 10.26	28.88 ± 10.21	1.31 ± 0.74	9.67 ± 4.66	20.94 ± 70.20
T		1.28	1.77				
Z				2.03	3.18	9.38	7.34
p		>0.05	>0.05	<0.05	<0.01	<0.01	<0.01

Data are expressed as the mean ± standard deviation.
Note: DNT: double-negative T cells; CEA: carcinoembryonic antigen; HC: healthy control; CRC: colorectal cancer.

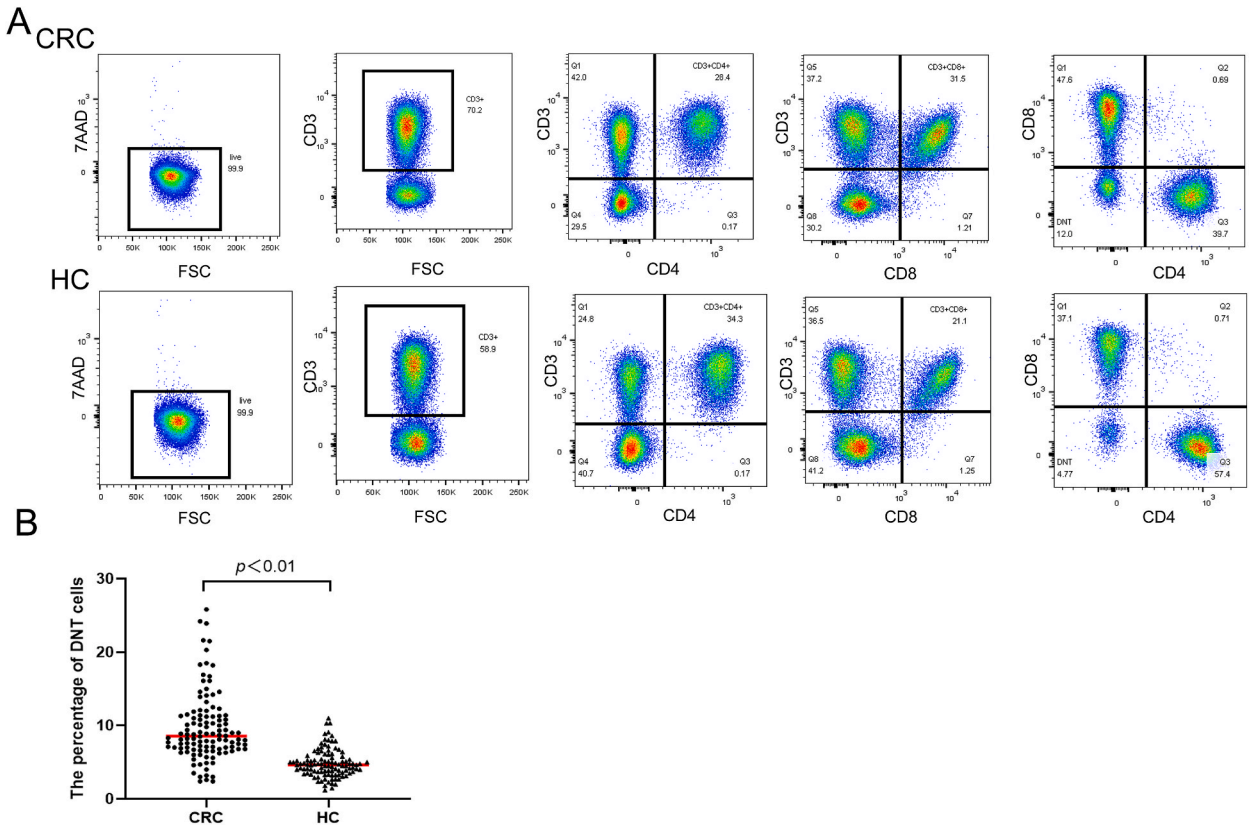

Fig. 2. Characterizations of different subsets of T lymphocytes in patients with colorectal cancer (CRC) and in healthy controls (HCs). A: Representative flow cytometry (FCM) dot plots of a patient with CRC and an HC. B: Comparison of percentage of double-negative T cells in patients with CRC and in HCs.

Table 3
Comparison of variable receiver operating characteristic curves.

Variable	Cut-off	AUC	95 % CI	Sensitivity (%)	Specificity (%)	Youden Index	*Z*	*p*
$CD4^+/CD8^+$	≤1.00	0.624	55.6–68.8	41.23	85.98	0.193	3.35[a]	<0.01
DNT%	≥6.15	0.865	81.3–90.7	85.09	79.44	0.645	5.52[b]	<0.01
CEA	≥2.42	0.786	72.6–83.8	69.30	82.24	0.515	2.04[c]	<0.05

Note: AUC: area under the curve; CI: confidence interval; DNT: double-negative T cells; CEA: carcinoembryonic carcinogen; a: vs. CEA; b: vs. $CD4^+/CD8^+$; c: vs. DNT.

rectum, perhaps because the sample size was not powered to make this assessment. In addition, some studies have found an increased percentage of DNT in malignant tumors, including B-cell chronic lymphoid leukemia, breast cancer, gastric cancer, and liver cancer [13,14], indicating that DNT percentage may reflect a tumor immune response without correlation to tumor type.

Patients with early-stage CRC lack specific symptoms, making prompt diagnosis difficult. Patients in the middle and late stages of the disease are prone to metastasis and have a worse prognosis [15]. Accordingly, early diagnosis and treatment are critical to improving patient prognosis and prolonging patient survival [16]. The serum glycoprotein CEA is a broad-spectrum tumor marker and is the only diagnostic marker recommended for routine screening for CRC [17]. However, the diagnostic efficacy of a single index is limited, but CEA combined with other markers may have better diagnostic utility for CRC. For example, CEA combined with IL-17A and TNF-α had superior diagnostic efficacy [18], while microRNAs combined with the fecal occult blood test (FOBT) and CEA yielded improved accuracy and positivity for CRC diagnosis [19]. CEA combined with neutrophil-to-lymphocyte ratio (NLR), derived neutrophil-to-lymphocyte ratio (d-NLR), and platelet-to-lymphocyte ratio (PLR) had significant diagnostic utility for CRC compared with CEA alone [20]. Our study showed that the combined detection of DNT percentage and CEA level had significantly better diagnostic efficacy for CRC and could be used as an adjunct for early diagnosis of CRC. To our knowledge, this is the first study investigating the diagnostic and prognostic value of DNT percentage in CRC.

DNT cells play different roles depending on the type of tumor and its microenvironment [8], and can exert anti-tumor effects

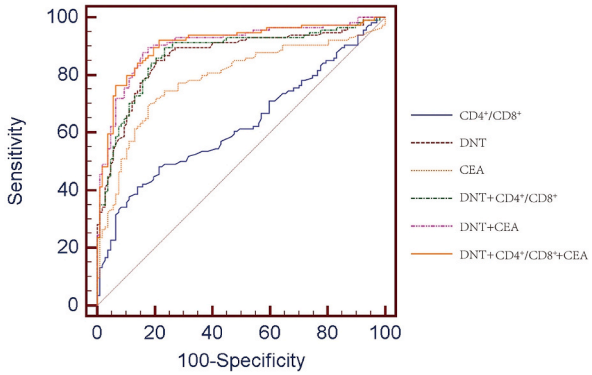

Fig. 3. Receiver operating characteristic curve analysis of the ratio of $CD4^+/CD8^+$ and the percentages of double-negative T cells, value of carcinoembryonic antigen, and the combined detection schemes in patients with colorectal cancer.

Table 4
Comparison of the receiver operating characteristic curves of different combined detection schemes.

Variable	AUC	95 % CI	Sensitivity (%)	Specificity (%)	Youden Index	Z	p
DNT	0.865	81.3–90.7	85.09	79.44	0.645	3.07[a]	<0.01
DNT + $CD4^+/CD8^+$	0.871	82.0–91.2	89.47	79.64	0.691	1.51[b]	>0.05
DNT + CEA	0.905	85.9–94.0	87.72	84.11	0.718	0.77[c]	>0.05
DNT + $CD4^+/CD8^+$ + CEA	0.908	86.1–94.2	92.11	78.50	0.706	3.18[b]	<0.01

Note: AUC: area under the curve; CI: confidence interval; DNT: double-negative T cell percentage; CEA: carcinoembryonic antigen; a: vs. DNT + CEA; b: vs. DNT; c: vs. DNT + CEA + $CD4^+/CD8^+$.

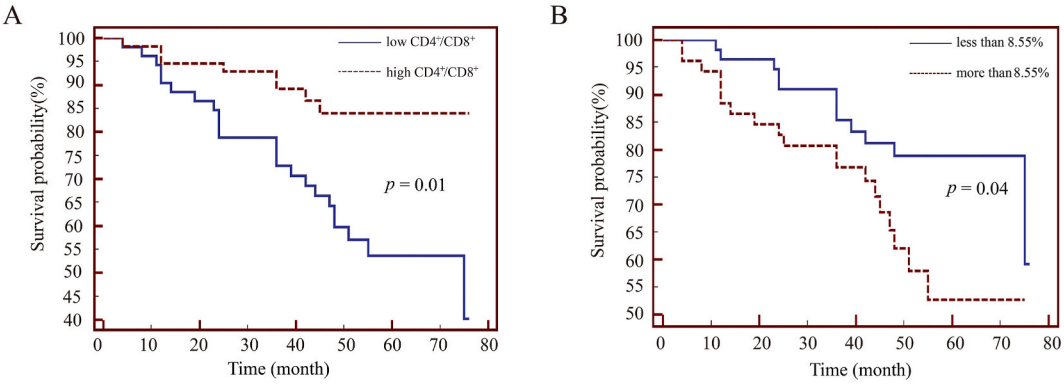

Fig. 4. Survival curves of the ratio of $CD4^+/CD8^+$ and the percentage of double-negative T (DNT) cells. A: The ratio of $CD4^+/CD8^+$; B: The percentage of DNT cells.

through various pathways [21,22]. When DNT cells were isolated from the peripheral blood of healthy individuals and co-cultured with pancreatic cancer or non-small cell lung cancer cell lines in vitro, DNT cells were cytotoxic, inhibiting the proliferation of cancer cells [23,24]. These observations suggest that DNT cells have the potential to be used as a novel adoptive cell therapy. In addition, Tang et al. initiated a Phase I clinical trial testing whether allogeneic DNT therapy is a safe and practical treatment option for patients with Acute Myelocytic Leukemia (AML) who relapse after allogeneic hematopoietic stem cell transplantation (allo-HSCT) [25]. However, some studies found that TCRαβ+DNT cells play an immunosuppressive function in mouse glioma and melanoma models and are increased in the lymph nodes of patients with advanced melanoma, suggesting that DNT may promote progression of tumor metastasis [26,27]. However, the function of DNT in CRC is unclear. In our study, we found that a high percentage of DNT cells in patients with CRC correlated with a poor prognosis, suggesting that DNT cells may promote tumor progression in CRC. However, the DNT cells detected in our experiment were a population of $CD4^-/CD8^-$ T cells, and we did not further characterize these cells.

In fact, the molecular and functional signatures of DNT cells remain poorly understood. Many studies of DNT are descriptive because of the relatively low frequency of these cells and the lack of a standardized definition of this lineage [28–30]. However, a recent study performed single-cell RNA sequencing of cells isolated from mixed splenocytes of male C57BL/6 mice using strict

Table 5
Univariate and multivariate Cox regression analysis for overall survival (OS).

Variables	Univariate analysis for OS			Multivariate analysis for OS		
	HR	95 % *CI*	*p*	HR	95 % *CI*	*p*
Age (≥65 versus <65)	1.33	0.64–2.75	0.45			
Gender (male versus female)	0.81	0.39–1.65	0.56			
TNM	2.38	1.16–4.87	0.01	2.37	1.15–4.90	0.02
Location	0.64	0.31–1.34	0.27			
Smoking	0.64	0.26–1.56	0.39			
Drinking	0.52	0.17–1.57	0.36			
$CD4^+/CD8^+$(<1.20 versus≥1.20)	0.33	0.16–0.66	0.01	0.33	0.15–0.74	0.01
DNT (<8.55%versus≥8.55 %)	2.06	1.01–4.20	0.04	2.29	1.11–4.73	0.03
Diabetes	0.51	0.20–1.28	0.25			
Hypertension	0.39	0.16–0.91	0.10			
Genetic history	1.00	0.47–2.12	0.99			

Note: OS: overall survival; HR: Hazard Ratio; CI: confidence interval; TNM: Tumor Node Metastasis; DNT: double-negative T cells.

fluorescence-activated cell sorting [31]. The researchers compiled a map of the cellular heterogeneity in naive and active DNT cells and found that the DNT subsets could be characterized into helper, cytotoxic, and innate DNT cells. These studies will help to better clarify the intrinsic roles of different functional DNT subsets in the development and progression of tumors.

Increasingly, T-lymphocyte subsets are being recognized for their critical and diverse roles in the immune system. Tumor immune marker analysis and targeted immunotherapy interventions have shown increasing clinical importance [32]. Although endoscopic pathological examination is used extensively, there may be adverse reactions, including perforation and bleeding [33]. Monitoring the expression of T-lymphocyte subsets in patients with CRC, especially the percentage of DNT cells, will enable the anti-tumor immune function of these cells in patients to be further evaluated, potentially facilitating prediction of patient prognosis and design of feasible treatment plans.

Our study has some limitations. First, the sample size was not large, and therefore the study may not have been powered to observe some differences. The DNT level at different sites and stages of CRC was not statistically different, again perhaps because of the sample size. Finally, we also did not analyze the total number of circulating DNTs due to the detection methods used.

5. Conclusion

Our study suggests that the percentage of DNT cells may be used as a potential diagnostic biomarker for CRC. The combination of DNT cells and CEA significantly improved diagnostic accuracy. Moreover, patients with CRC with a high percentage of DNT cells had a poor prognosis. Therefore, the percentage of DNT cells may have important clinical value for diagnosis and prognosis in CRC.

Ethics statement

This study was approved by the institution ethics commission of the 960th Hospital of the PLA Joint Logistics Support Force, China (No. 2022104). Due to the retrospective nature of this study, informed consent (written/verbal) of the patients was waived.

Data availability statement

Data is available on request from the authors.

CRediT authorship contribution statement

Xiao-Cui Liu: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Ke-Na Sun:** Writing – original draft, Investigation, Formal analysis, Data curation. **Hui-Ru Zhu:** Visualization, Data curation. **Yu-Ling Dai:** Data curation. **Xiao-Fei Liu:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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