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Original Article

Low mutation burden and differential tumor-infiltrating immune cells correlate with lymph node metastasis in colorectal cancer

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Abstract: Background: Tumor immunotherapy has become an important means of cancer treatment. A response depends on the interaction of tumor cells with immune regulators in the tumor microenvironment, which plays an important role in inhibiting or enhancing the immune response. However, lymph node (LN) metastasis leads to major changes in the tumor microenvironment of patients with colorectal cancer, directly affecting prognosis. Methods: Using data downloaded from the Cancer Genome Atlas (TCGA) database, we studied the microenvironmental differences between LN-negative and positive populations by bioinformatic methods. Results: Patients in the LN-positive group had significantly lower immune scores, cytolytic activity scores, and overall survival than the LN-negative group. In addition, a high mutation burden and a new antigen burden could inhibit lymph node metastasis of CRC. In particular, in the LN positive group, the ratio of monocytes to M1 macrophages was significantly downregulated. After the differentially expressed mRNAs between the LN positive and negative groups were determined, a new CRC model was constructed based on multivariate Cox proportional hazard regression analysis to examine the prognosis of patients. The analyses showed that the model was stable and robust. Conclusions: We used multiple scores and details of immune cell infiltration as indicators to assess changes in the tumor microenvironment of CRC patients before and after lymph node metastasis, and quantify and model the immune cells in the microenvironment to predict the overall survival of CRC patients.

Keywords: CRC, microenvironment, lymph node metastasis, tumor mutation load, prognosis

Introduction

Colorectal cancer (CRC) has the second highest mortality rate among all cancers, with mortality ranking fourth (9.0%) and third (9.5%) among male and female cancer patients, respectively [1]. Surgical resection is a common treatment in clinical practice [2]. However, due to the absence of a national colorectal cancer screening program, most CRC patients in China already have metastasized tumors at the time of treatment, and chemotherapy sensitivity has also led to poor therapeutic efficacy.

In recent years, tumor immunotherapy has become an important means for cancer treatment [3], and it is an important topic in cancer research. For example, blocking the binding of PD-L1 to PD-1 can enhance the cellular immune response by increasing T cell apoptosis, indicating that this is a highly promising therapy [4]. However, a study showed that the expression of PD-L1 was independent of lymph node (LN) metastasis, leading to limited or no response to this treatment in some patients [5]. This is because the occurrence of metastasis involves the interaction of many factors, including molecular factors triggered by changes in tumor microenvironment [6]. Tumor immunotherapy positive reactions depend on the interaction of tumor cells with immune regulators in the tumor microenvironment. Tumor-infiltrating immune cells (TIICs) are a major non-tumor component of the tumor microenvironment and are of great

value in the diagnosis and prognosis assessment of glioblastoma [7], breast cancer [8], and CRC [9]. Pan-immunochemical analysis shows that many adaptive immune-related TIICs, such as activated CD8+ T cells, resting memory CD4+ T cells, and effector memory CD4+ T cells, are associated with improved patient prognosis [10]. In addition, changes in characteristics such as immunosuppression of the tumor microenvironment can cause lymph node metastasis in patients with colorectal cancer, which directly affects prognosis. The metastasis incidence and tumor invasion depth in lymph nodes are prognostic factors of CRC metastasis [11, 12]. To achieve a precise immunotherapy, studies have defined tumor mutation burden (TMB) by the number of mutations per megabase of DNA, which is used as a predictive biomarker for the evaluation [13]. The higher the TMB is, the more likely the tumor is to be effective for immunotherapy [14]. Therefore, studying TMB and TIIC in patients with metastatic CRC can help slow tumor progression and improve patient prognosis.

Taking advantage of CRC cohorts from the TCGA database, we assessed some characteristics of the tumor microenvironment, which include immune scores, TMB, neoantigen burdens, TIICs, and gene expression. We hypothesized that significant changes in these characteristics were associated with lymph node metastasis in CRC patients.

Materials and methods

Data sources and preprocessing

All the genomic, clinical, and mutation annotation format (MAF) data were obtained from the TCGA CRC cohort in February 2019. We first discarded the clinical information with a survival time less than 30 days or with unknown lymph node metastasis information; then, we removed the samples without complete clinical information or MAF data, and all normal samples. In total, we obtained 426 tumor samples. Gene expression data in the FPKM format were also acquired from the TCGA and converted to transcripts per million (TPM). Immune scores were calculated by using the ESTIMATE algorithm based on the expression values in TPM [15].

Functional analysis in silico

The immune cytolysis activity score of each sample was obtained by the geometric mean of the TPM type expression values of *GZMA* and *PRF1* [16]. After filtering out variants occurring in intergenic or noncoding regions and in synonymous variants, the maftools package [17] was used for mutation burden analysis and mutational spectral visualization. We then analyzed the new neoantigens and their derived proteins between the LN-negative and positive groups based on data downloaded from the TCIA database (https://tcia.at/home). To evaluate the composition of TIICs in CRC, a CIBERSORT deconvolution algorithm [18] was used to estimate the fraction of 22 immune cell types in the LN-negative and positive groups based on the gene profile.

Differentially expressed mRNAs were screened using edgeR [19] with the criterion of $|\log_2 \text{fold change}| \geq 1.5$ and a false discovery rate (FDR) < 0.01. The TIMER online database [20] was used to analyze and visualize the abundance of TIICs of differentially expressed genes. Two plugins for Cytoscape software, ClueGO and CluePedia [21], were used for the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.

To obtain and analyze mRNAs that are significantly related to the overall survival (OS) of CRC patients, we first performed univariate Cox proportional hazards regression on differentially expressed mRNAs, removed factors that were not statistically significant, and then performed a multiple Cox regression model analysis.

Statistical analyses

All statistical analyses were conducted using R software (version 3.5.0) and Bioconductor (https://www.bioconductor.org/). A chi-squared test was used to compare the differences in various values (such as cytolytic activity scores) and the sample mutation rates. Kaplan-Meier (KM) analysis using the survival package was performed to analyze the correlation between the mRNA and OS. *P*-values less than 0.05 were considered significant. In the comparison study, receiver operating curve (ROC) was executed using SPSS 24.0.

Table 1. Clinicopathologic features of 426 patients with colorectal cancer

	LN-negative	LN-positive	p
N	253	173	
Age (years) (%)			0.021
> 60	186 (73.5)	109 (63.0)	
≤ 60	67 (26.5)	64 (37.0)	
Gender (%)			0.285
Male	142 (56.1)	88 (50.9)	
Female	111 (43.9)	85 (49.1)	
Race (%)			0.057
WHITE	113 (44.7)	89 (51.4)	
ASIAN	10 (4.0)	2 (1.2)	
BLACK OR AFRICAN AMERICAN	28 (11.0)	27 (15.6)	
Unknown	102 (40.3)	55 (31.8)	
Pathologic stage (%)			< 0.001
Stage I	75 (29.6)	0 (0.0)	
Stage II	163 (64.4)	0 (0.0)	
Stage III	0 (0.0)	118 (68.2)	
Stage IV	8 (3.2)	51 (29.4)	
Unknown	7 (27.8)	4 (2.3)	
Pathologic T (%)			< 0.001
T_1	10 (4.0)	0 (0.0)	
T_2	67 (26.5)	9 (5.2)	
T_3	162 (64.0)	127 (73.4)	
T_4	14 (5.5)	37 (21.4)	
Pathologic M (%)			< 0.001
M_0	220 (87.0)	98 (56.6)	
M_1	8 (3.2)	51 (29.5)	
M_X	25 (9.8)	24 (13.9)	
Vital status (%)			< 0.001
Alive	225 (88.9)	120 (69.3)	
Dead	28 (11.1)	53 (30.7)	

Results

Sample demographic statistics

Our analysis is mainly derived from the sequencing data and clinical information of 426 CRC patients published in the TCGA database. After pretreatment as described in the materials and methods section, we obtained data including 253 LN negative samples and 173 LN positive samples. The detailed clinical and pathologic characteristics in these samples, including age, gender, race, pathologic stage, tumor (T), and metastasis (M), are summarized in **Table 1**.

Characteristic analyses between the LN-negative and positive groups

Immune scores represent the infiltration of immune cells in tumor tissue. We found that the LN-negative group had a higher immune score than the LN-positive group ($P < 0.018$) (**Figure 1A**). We further examined the association between grouping and cytolytic activity scores in CRC. Interestingly, the distribution of cytolytic activity scores was the same as that of immune scores in the LN-negative and positive groups (**Figure 1B**).

To explore the association of TMB with lymph node metastasis in CRC, we compared TMB between the LN-negative and positive groups. We found that the performance of TMB in both groups was consistent with the performance of immune and cytolytic activity scores ($P = 0.0002$) (**Figure 1C**). To determine the correlation of overall survival with lymph node metastasis, we performed KM survival curve analysis and found that the overall survival of samples in the LN-negative group was longer than that of the LN-positive group ($P < 0.0001$ in log-rank test) (**Figure 1D**).

The landscape of immune infiltration in the LN-negative and positive groups

During tumor progression, TIICs respond to anticancer therapies and change dynamically, and their presence is often associated with improved clinical outcomes. To further analyze the differences in immune cell invasion between the LN-negative and positive groups, we used the CIBERSORT algorithm to study the difference in the proportion of 22 immune cell subsets (**Figure 2A**, **2B**). We found that gamma delta T cells did not exist in any sample. We also assessed the difference in the proportion of TIICs between these two groups. The number of monocytes and M1 macrophages in the

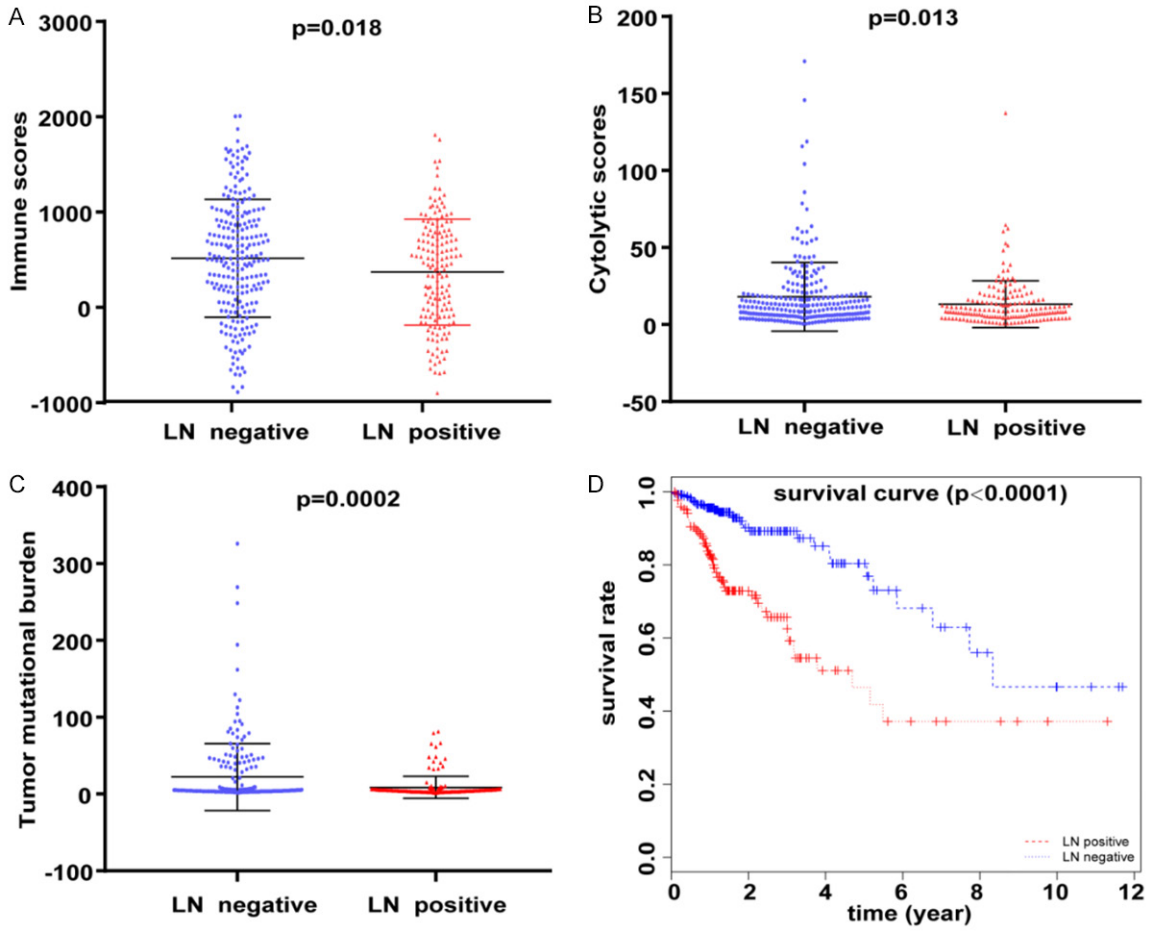

Figure 1. Characteristic analysis between lymph node metastasis negative and positive groups. A. Distribution of immune scores. B. Distribution of cytolytic scores in the lymph node metastasis negative and positive groups. C. Distribution of tumor mutation burden. D. Survival analysis of CRC patients in the LN-negative and positive groups.

LN-negative group was significantly greater than that of the other group (**Figure 2C**).

Mutation burden and neoantigen burden related to lymph node metastasis

A high mutation burden is a part of the characteristics of malignant tumors. To obtain the burden of nonsynonymous mutations in the LN-negative and positive groups, we analyzed somatic mutations in these two groups based on the results of Mutct2 software. The median nonsynonymous mutation burden is 111.5 in the LN-negative group and 101.5 in the other group. Our study validates that CRC is accompanied by a high mutation load when lymph node metastasis occurs, and there are significant differences in the mutation patterns of highly mutated genes before and after metastasis. As shown in **Figure 3A**, **3B**, *APC*, *TTN*, *TP53*, *KRAS*, *MUC16*, and *PIK3CA* are in the top 10 mutated genes in these two groups. We also found that most of the mutations were single base substitutions.

We also investigated whether the mutational and neoantigen pattern were modified with respect to immune scores. The neoantigen counts and neoantigen origin protein counts for each CRC sample were available from the TCIA database. The LN-positive groups had significantly lower neoantigen burden (**Figure 3C**) and neoantigen origin protein burden (**Figure 3D**) than those in the other group, which suggested that the LN-negative group has a higher number of mutations accumulated in the tumor cell genome, resulting in a corresponding increase in neoantigen burden, and thereby activating more T cells and producing a stronger immune response.

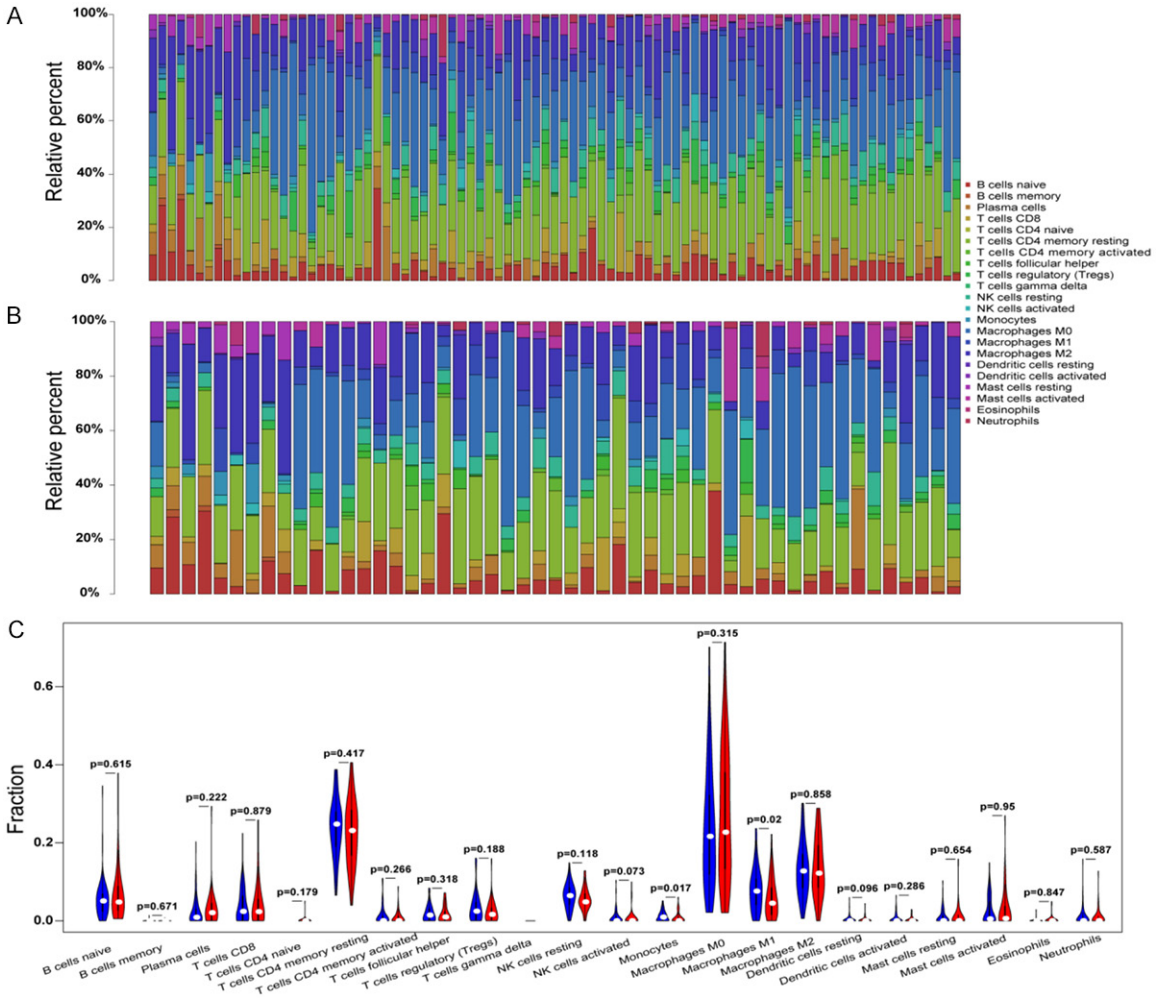

Figure 2. Landscape and prognostic analysis of immune infiltration. A. Bar chart summarizing the immune cell subset proportions in the lymph node metastasis negative group. B. Bar chart summarizing the immune cell subset proportions in the lymph node metastasis positive group. C. Difference in tumor infiltrating immune cell (TIIC) proportion between the lymph node metastasis negative and positive groups.

Comparison of gene expression profiles before and after metastasis

To reveal the correlation between gene expression profiles and lymph node metastasis, a differential analysis of count data of genes in the samples of the LN-negative and positive groups was performed. Volcano plots in **Figure 4A** show that in high score group, 68 mRNAs are upregulated and 261 mRNAs are downregulated compared with those in the low score group. To outline the function of differentially expressed genes, functional enrichment analysis of the 329 mRNAs (**Figure 4B**) was performed.

We first performed univariate Cox proportional hazards regression analysis and found that 27 differentially expressed mRNAs were significantly correlated with OS ($P < 0.01$). Then stepwise multivariate Cox proportional hazard regression analysis was used to find that 9 of these candidate mRNAs were significantly and independently related to prognosis. Finally, we constructed a prognostic signature based on the expression levels and regression coefficients of these best prognostic mRNAs. The formula is as follows: risk score = (0.24783 × the expression level of *NLGN1*) + (0.12994 × the expression level of *CDK5R2*) + (0.13208 × the expression level of *NTF4*) + (0.14851 × the expression level of *TMEM213*) + (-0.13580 × the expression level of *SLC6A15*) + (0.34611 × the expression level of *IRX4*) + (-0.34510 × the expression level of *COLEC10*) + (0.25542 × the expression level of *CT45A1*) + (0.10484 × the

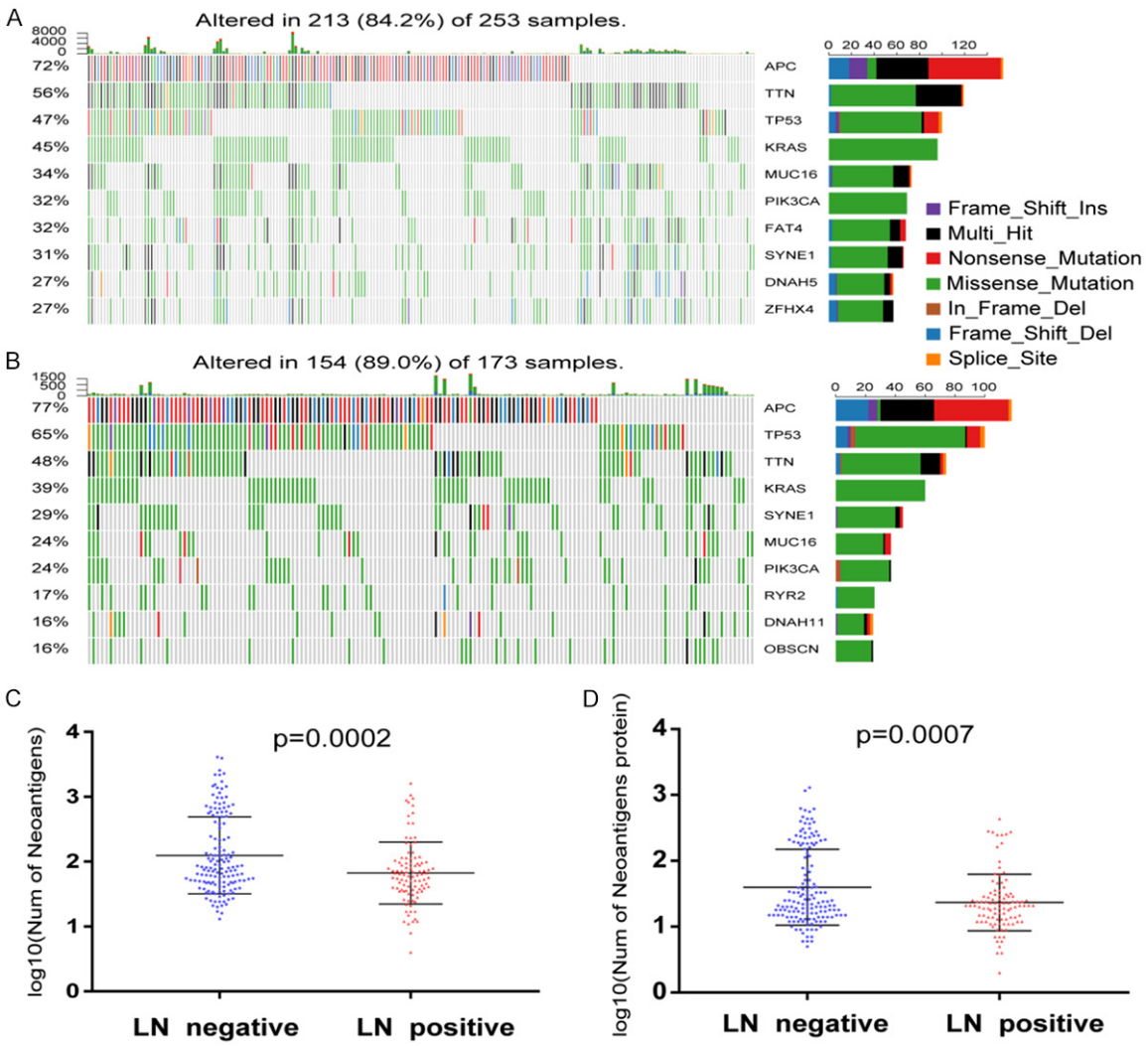

Figure 3. Landscape of mutations in CRC and neoantigen comparison between the lymph node metastasis negative and positive groups. Individual mutations for the top 10 most mutated genes for the lymph node metastasis negative group (A) and lymph node metastasis positive group (B). (C) The graph shows that neoantigen burdens are significantly different between the lymph node metastasis negative and positive groups (P<0.05). (D) Graph shows the number of neoantigen-related proteins.

expression level of *XAGE2*). Among these 9 mRNAs, *SLC6A15* and *COLEC10* showed negative coefficients, suggesting they are protective factors, as their high expressions predicted low risk. The other 7 mRNAs with positive coefficients, seemed to be risk factors, as their high expressions predicted high risk. Studies have shown that *NTF4* is involved in the metastasis of colon cancer [22]. Interestingly, factors such as *NLGN1*, *CDK5R2*, *TMEM213*, *IRX4*, and *CT45A1* have been shown to trigger the tumorigenesis and metastasis of tumors, including leiomyosarcoma, lung cancer, and others [23-25].

KM analysis showed that the prognosis of the low-risk group was significantly improved compared with the high-risk group ($P < 0.001$, **Figure 4C**). Subsequent time-dependent ROC analysis showed that the area under the ROC curve (AUC) of the 9 mRNAs was 0.763, indicating that the signature performed well in predicting the prognosis of CRC patients (**Figure 4D**).

Discussion

To investigate changes in tumor microenvironment before and after lymph node metastasis in colorectal cancer, we analyzed RNA sequencing data of 426 samples published in the TCGA

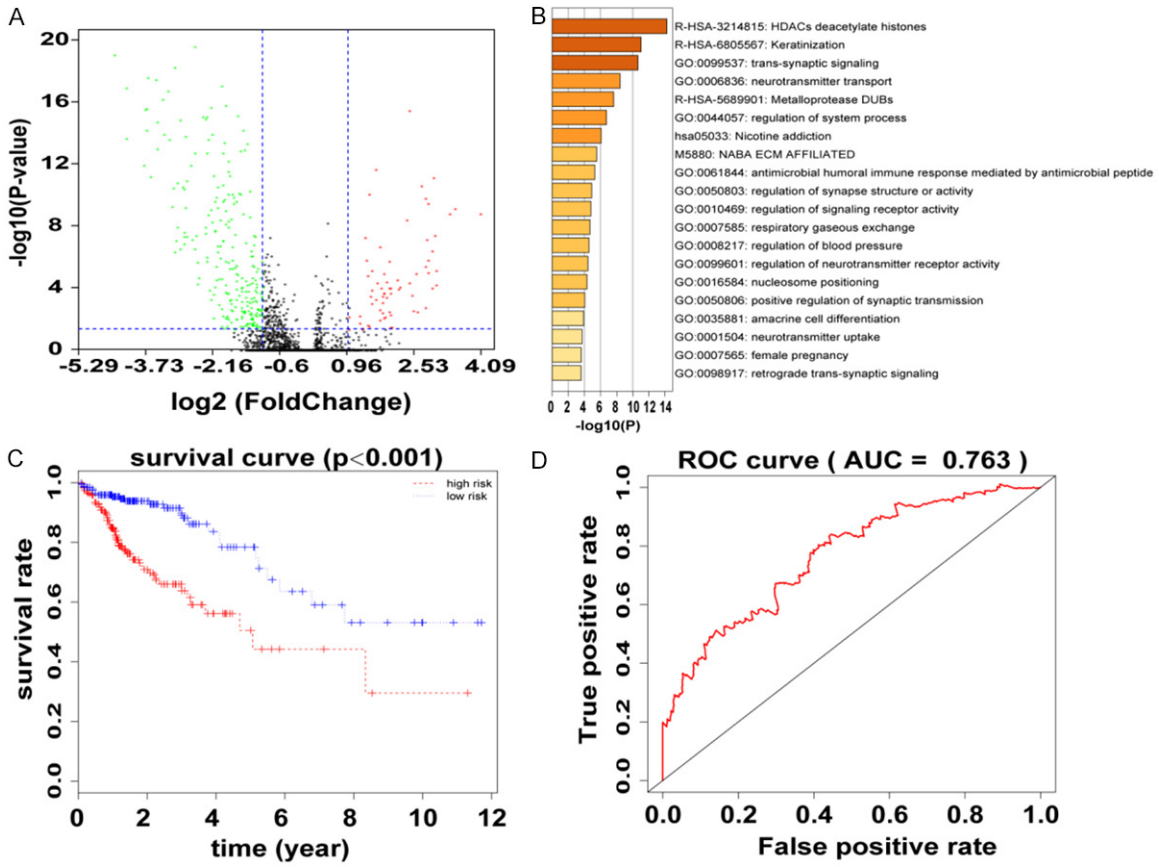

Figure 4. Gene expression spectrum. A. Volcano plots of differentially expressed genes between the lymph node metastasis negative and positive groups. The three colored dots represent different types of mRNAs, among which green represents a significant down-regulation, red represents a significant up-regulation, and black represents no significant differential expression. B. GO analysis of 329 differentially expressed genes. C. Kaplan-Meier analysis of patients' overall survival in the high-risk (n = 210) and low-risk (n = 211) subgroups. D. The receiver operating characteristic (ROC) analysis of the risk scores for the prediction of the overall survival. The area under the curve was calculated for the ROC curves.

database. Our results reveal many associations between the occurrence of metastases and changes in tumor microenvironment-related factors.

In this study, we found that the LN-negative group had higher immune scores, cytolytic activity scores, tumor m burden (TMB), neoantigen burden, and neoantigen origin proteins compared to those in the LN-positive group. Previous studies indicated that immunization scores can determine the high risk of recurrence and metastasis in cancer patients [15], and the cytolytic activity was involved in immune response and improved prognosis [26]. Therefore, we believe that the LN-negative group has a good prognosis and a low risk of recurrence. Furthermore, lymphocyte infiltration will be prominent due to increased neoantigen burden, and more stromal cells and cytolysis activity can be found in tumor tissue [27]. This means that the samples in the LN-negative group have a good cytolytic immune response and relatively abundant stromal cells because of the higher levels of the tumor-infiltrating immune cells (TIICs). In metastatic melanoma, features of the immune microenvironment such as tumor mutation burden (TMB), neoantigen negativity, and cytolytic activity have been demonstrated to be related to CTLA4 blockade [28]. In addition, in the LN-negative group, CRC patients survived longer than those in the LN-positive group. These results were in agreement with the corresponding findings of Wang et al., which state that patients with higher TMB are more likely to have improved survival [29].

However, whether patients with high TMB and increased effector immune infiltration are more susceptible to the blocking of immune checkpoints requires further research.

Recently, immunotherapy has aimed to activate the immune system and has shown high efficacy in the treatment of some cancers [30]. Using CIBERSORT, we analyzed the proportion changes in 22 immune cell subsets in the LN-negative and positive groups. Tumor-associated microenvironments, including immune cells, can inhibit malignant cells. Numerous studies have shown that factors such as the degree of infiltration of immune cells and tissue localization are significantly related to the progression and survival rate of CRC in certain stages. For example, among patients with stage III CRC, the ones with lower TIIC values had significantly lower 5-year OS than those in the high TIIC group [31]. Our study also showed that in the LN-negative group TIIC was significant, and the OS of CRC patients also improved significantly. In addition, our data reveal the details of the infiltration of 22 TIIC subsets in CRC. The proportion of macrophages and resting memory CD4+ T cells was the highest, and gamma delta T cells did not even exist in all samples. Resting memory CD4+ T cells can help CD8+ T cells inhibit tumors, and block CD8+ T cell activation, and NK cell killing [32]. We found that the number of monocytes and M1 macrophage changed significantly after lymph node metastasis. Our results show that tumor metastasis comes from these different internal mechanisms such as changes in the content of TIIC cells in the tumor microenvironment.

Neoantigen burden is an effective biomarker in cancer immunotherapy, which stimulates the development of new therapeutic approaches to T cell reactivity against this class of antigens [33]. Tumors with a high mutation burden are often accompanied by a high number of CD8+ T cells [34]. T cell reactivity against neoantigens is widespread in melanoma [35]. In CRC, we found that the neoantigen peptide burden in the samples before lymph node metastasis was significantly higher than in the samples after metastasis. In the LN-negative group, the proportion of T cells such as CD4 memory activation was significantly increased, possibly due to the higher neoantigen burden on HIM samples. Considering their higher nonsynonymous mutation burden, we believe that the nonsynonymous mutation burden in tumors will form more neoantigens, resulting in higher immunogenicity.

We also found that some genes are highly mutated before and after lymph node metastasis. The tumor suppressor gene *TP53* showed a discontinuous mutation distribution in both groups. *TP53* plays a regulatory role in cell proliferation and apoptosis [36]. The loss of *TP53* may lead to the development and progression of CRC through a multistep process [37]. Some authors showed an association between *TP53* mutations and the worst outcomes in stage III CRC [38]. We found that TP53 mutations occurred in approximately 47% (213) of the samples in the LN negative group, rising to 65% (154) in the LN positive group. We also noticed that *APC* has a mutation frequency ranking first in these two groups (72% and 77% respectively).

Deregulation of expression of genes is vital for extensive investigation into the initiation and progression of colorectal carcinogenesis. We identified 329 lymph node metastasis-related genes. Functional enrichment analysis showed that these genes are mainly involved in immune response and cell adhesion. TNM staging, which can reflect the invasion and metastasis capacity and degree of tumor, has been used to determine the progression and prognosis of colorectal cancer [39]. However, CRC is a highly heterogeneous malignant tumor with a unique genetic and epigenetic background that determines the complex clinical biologic behavior and prognosis of CRC [40]. We thus constructed a new CRC prognostic analysis model consisting of 9 mRNAs based on Cox regression analyses. Several factors, such as *NTF4*, *NLGN1*, *CDK5R2*, *TMEM213*, *IRX4*, and *CT45A1* are associated with metastasis in multiple cancers. The consequences of Kaplan-Meier survival analysis showed that low-risk groups showed significant advantages in OS. Subsequently, the value of AUC demonstrated that the risk models were robust and reliable for predicting the survival of CRC patients. In short, the model we built is a good complement to TNM staging. However, this study has some limitations because all the conclusions were obtained in silico. Further in vivo studies will

contribute to fully understanding the molecular characteristics of CRC before and after lymph node metastasis.

In conclusion, we demonstrate that some immune-related features change significantly after lymph node metastasis in CRC patients. When metastasis occurs, the immune score, TMB, neoantigen burden, OS of CRC patients, and the proportions of monocytes and M1 macrophage are all significantly reduced. Given the low TMB and the significant changes in effector immune infiltrates in the LN-positive group, whether or not it would be more difficult to block the immune checkpoint needs further study.

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Disclosure of conflict of interest

None.

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