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Research Article

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M7G-related miRNA biomarkers for predicting overall survival outcomes for colon carcinoma

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Abstract

Background

Colon cancer (COAD) is a highly malignant disease with poor prognosis, more effective treatment strategies need to be explored urgently. M7G methylation can molecularly affect transcriptional processes leading to cancer development. However, the prognostic value of m7G-related gene miRNAs in colon cancer remains to be further investigated.

Methods

In this study, miRNA, mRNA expression profiles and corresponding clinical data of COAD patients were downloaded from the public database TCGA. A total of 109 m7G-related gene miRNAs were differentially expressed between COAD and adjacent normal tissues in TCGA ($p<0.05$ and $|logFC|>=1$), and 13 differentially expressed genes (DEGs) were correlated with overall survival in univariate Cox regression analysis. Based on Least Absolute Shrinkage and Selection Operator (LASSO) Cox regression, a polygenic prognostic model was constructed, and the risk score was validated.

Result

Five gene biomarkers (hsa−miR−21−3p, hsa−miR−887−5p, hsa−miR−9−5p, hsa−miR−378d, hsa−miR−31−5p) were associated with colon cancer prognosis. The patients were divided into high and low risk groups according to the risk score. The OS of patients in the high-risk group was significantly higher than that of the patients in the lower risk group ($P < 0.001$). The time-dependent ROC curve analysis confirmed the predictive ability of tumor biomarkers. Risk score was an independent predictor of OS ($HR>1$, $P<0.001$). Functional enrichment analysis showed that immune-related pathways were involved in the regulatory process.

Conclusion

five novel m7G-related gene miRNAs biomarkers can be used for prognosis prediction of colon cancer. Targeting m7g-related miRNAs may be an alternative treatment for COAD.

Key Words : Colon cancer, m7G methylation, microRNA, biomarker, prognosis

Introduction

Colon cancer is the fourth deadliest cancer in the world, accounting for about 10% of all cancer cases in both men and women [1, 2]. The incidence of colon cancer has increased significantly in recent years, global mortality rate of colon cancer is about 50%, which is closely related to dietary habits, genetic factors, age, and smoking [3-6]. The current main treatment modalities include surgery, chemotherapy and radiotherapy, and targeted therapy, more effective treatment strategies are imminent. The search for novel diagnostic biomarkers and effective treatment strategies, and the discovery of COAD-related pathogenesis can provide new insights into the prediction, development, and progression of COAD, and improve optimal adjuvant therapy for patients in routine clinical practice.

M7G RNA methylation (N7 methyladenosine, m7G) is a modification in which methyl is added to the seventh position N of RNA

guanine (g) under the action of methyltransferase [7]. M7G modification is one of the most common forms of base modification in post transcriptional regulation. It is widely distributed in tRNA, rRNA and 5 hats of eukaryotic mRNA, and plays an important role in maintaining RNA's processing, metabolism, stability, nucleation and protein translation [8]. Recent studies claim that METTL1 modifies miRNAs through m7G methylation to regulate cell migration [9] , and can also promote post-ischemic angiogenesis in peripheral vascular lesions [10]. On the other hand, WDR4 has been confirmed to promote the proliferation, metastasis and drug resistance of liver cancer, and can be used as an effective therapeutic target for liver cancer [11]. In addition, the study claims that WDR4 is indispensable for embryonic stem cell self-renewal and differentiation [12]. Critical role of M7G in cancer development is considered as a new cancer treatment.

MiRNA (microRNA) is a small molecule encoded by higher eukaryotic genomes, similar to siRNA [13]. The microRNA-guided silencing complex (RISC) degrades mRNA or hinders its translation by base-pairing with the mRNA of a target gene [14]. MicroRNAs are negative regulators of gene expression. It regulates more than 30% of mRNA and plays a role in fundamental processes such as development, apoptosis, differentiation, cell proliferation, and stress responses [15]. Gain and loss of miRNA function contribute to cancer development through various mechanisms. Changes in miRNA expression directly regulated by hepatocyte growth factor (HGF) enhance cancer development and invasive characteristics by regulating HGF-induced translation of functional molecules [16, 17], also COAD, lung cancer. Although many studies have reported the effect of miRNAs on the occurrence and development of colon cancer [18, 19], no study has reported the use of m7g-related miRNAs to predict the prognosis of colon cancer patients. Therefore, whether m7G-related miRNAs are related to the prognosis of COAD patients is still unclear, and it is very necessary to explore the molecular markers that use m7G-related miRNAs to predict the prognosis of colon cancer patients.

This study firstly downloaded the colon cancer miRNA expression data and related clinical data from public databases, Then, five miRNA molecular biomarkers associated with colon cancer prognosis were obtained through differential analysis, univariate Cox, and multivariate Cox regression model construction. Finally, the prognosis-related miRNAs were predicted to target genes, and the functional enrichment analysis of the target genes was performed to explore the potential mechanisms of these miRNAs.

Materials and Methods

COAD data acquisition and processing

The miRNA and mRNA expression data and related clinical data of colon cancer patients from the TCGA database were downloaded with counts format through the R software (version4.1.1)" TCGAbiolinks" "Summarized Experiment" package. Gene expression profile normalization was achieved by the "limma" R package. And perform data matching, data filtering, data correction, and also filter and match relevant clinical data for subsequent analysis.

Identification of Differentially Expressed M7g-Related Mirnas

To obtain m7G-related miRNA differential expression matrix, we extracted two m7G genes from previous reviews: METTL1, WDR4, the miRNA expression genes of COAD include 8 normal tissues and 457 tumor tissues, then integrated two m7G genes and miRNA expression genes of COAD using "limma" packaged in R, according to the screening criteria $|logFC|\geq 1$ and FDR <0.05, the up-regulated and down-regulated miRNAs were obtained. The top 20 most meaningful genes were selected to draw heatmaps and volcano plots by R [7].

Construction of M7g-Related Mirnas Prognostic Model

To evaluate m7G-related differentially expressed miRNAs prognostic value, we further employed Cox regression analysis to evaluate the association between each gene and survival in the TCGA clinical data. Batch univariate Cox regression analysis was performed on the obtained differentially expressed miRNAs. A LASSO Cox regression model (R package "glmnet") was used to screen the range of prognostic genes and build a prognostic model. According to the results of LASSO Cox regression analysis, the miRNAs with significant correlation were selected to construct a multivariate Cox regression model, and the forest plot was drawn through the R package "forestplot". The normalized expression level of each gene and its corresponding regression coefficient were used to calculate the patient's Risk Score. Colon cancer patients were classified as high- and low-risk group based on the median risk score. We performed PCA and t-SNE cluster analysis to further evaluate the accuracy of risk scores by R package "Rtsne". Survival analysis is performed on the genes model according to the risk score, and the prognosis of the high and low risk groups is judged by R software. Time-dependent ROC curve analysis to assess the predictive power of gene signatures was performed by the R package " timeROC ". At the same time, we also draw the risk score distribution map including riskpoint and riskline to further observe the characteristics of the model intuitively.

Assessment of Risk Model Accuracy and Predictive Powers

After removing duplicate and incomplete clinical data, we combined the risk score obtained by the genes model with clinical factors (age, gender, stage, T, N) to perform univariate Cox and multivariate Cox regression analysis. According to the results of Cox regression analysis, to judge whether the risk score can become an independent prognostic factor, the nomogram and calibration curve are drawn using the R package "regplot" "rms".

mRNA Differential Genes Acquisition and Functional Enrichment Analysis

The risk scores were divided into high-risk and low-risk groups according to the median risk score. Then, differential analysis was performed on the mRNA expression data of colon cancer to obtain mRNA differential genes according to specific criteria ($|log2FC| \geq 1$ and FDR< 0.05). Additionally, colon cancer immune score data were downloaded from the online database ESTIMATE (https://bioinformatics.mdanderson.org/) and differences were grouped according to immune score (high vs low). Finally, the intersec-

tion genes of the two groups were obtained through the R package "VennDiagram" for further analysis, and a Venn diagram was drawn. Based on these DEGs, we performed functional enrichment analysis including gene ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG) and DO to understand the relationship between genes and function, which was achieved by applying the R package "clusterProfiler"[20], "org. Hs.eg.db" , "enrichplot", "DOSE", "ggplot2". Perform ssGSEA analysis through the "gsva" package, calculate immune scores for immune infiltrating cells and immune function, to understand immune-related pathways, and further explore the link between m7G differentially expressed miRNAs and the immune microenvironment.

Statistical Analysis

One-way ANOVA was used to compare gene expression levels between colon normal and tumor groups, while Pearson's chi-square test was used to compare categorical variables. To compare patient OS between subgroups, we used the Kaplan-Meier method and the two-sided log-rank test. In order to assess risk-independent prognostic value models, we used univariate and multivariate Cox regression models. The Mann-Whitney test was used to compare immune cell infiltration and immune pathway activation between the two groups. All statistics were analyzed using R software (v4.1.1).

Results

Identification of m7g-related differential genes

In this study, we obtained 465 miRNA expression data in colon cancer, including 8 normal and 457 tumor samples. Two m7G genes METTL1, WDR4 related differential genes were obtained, including a total of 109 differential expressed genes (|logFC| ≥ 1, FDR <0.05), of which 64 genes were up-regulated and 45 genes were down-regulated (figure 1A), These miRNAs were aberrantly expressed in colon cancer tissues compared to normal tissues. The top 20 DGEs were selected to make a heatmap in figure 1B (green: low expression level; red: high expression level), among which 8 genes were highly expressed (hsa−miR−379−5p, hsa−miR−16−5p, hsa−miR−186−5p, hsa−miR−429, hsa−miR−423−3p, hsa−miR−378a−5p, hsa−miR−194−3p, hsa−miR−21−3p), low expression of 12 genes (hsa−miR−139−3p, hsa−miR−495−3p, hsa−miR−766−3p, hsa−miR−1976, hsa−miR−139−5p, hsa−miR−96−5p, hsa−miR−455−5p, hsa−miR−582−5p, hsa−miR−486−5p, hsa−miR−185−5p, hsa−miR−32−5p, hsa−miR−2355−5p).

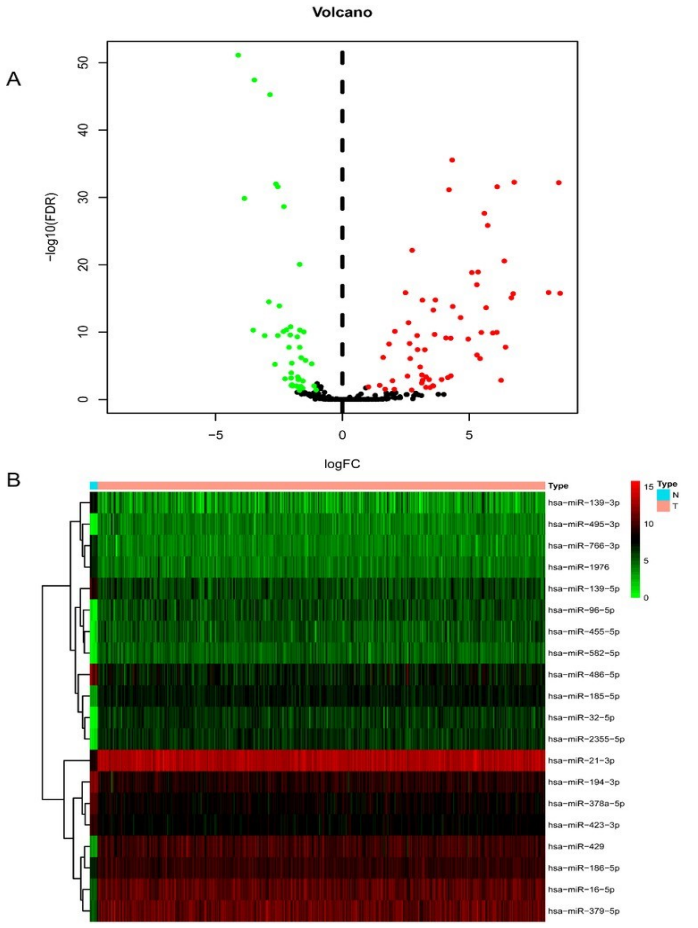

Figure 1: Identification of 109 m7G-related differentially expressed miRNAs. (A) volcano plot for 64 up-regulated and 45 down-regulated differentially expressed miRNAs genes; (B) heatmap of the top 20 differentially expressed miRNAs.

Building a Prognostic Gene Model in the TCGA Cohort

A total of 109 COAD m7G differentially expressed miRNAs were associated with corresponding patients with 438 complete survival information. The detailed clinical characteristics of colon cancer patients are summarized in Table 1. The initial screening of survival-related genes was performed using univariate Cox regression analysis, a total of 13 differential genes (p<0.05) were obtained for further study. Among these genes, 3 genes (hsa-miR-194-3p, hsa-miR-21-3p and hsa-miR-200c-5p) were protective genes with HRs < 1, while the other 10 genes (hsa-miR-200c-5p, hsa-miR-221-3p, hsa-miR-150-3p, hsa-miR-361-3p, hsa-miR-9-5p, hsa-miR-149-3p, hsa-miR-216a-5p, hsa-miR-378d, hsa-miR-628-3p, hsa-miR-31-5p) were associated with an increased risk of HR > 1(figure 2A). 11-gene signatures were constructed based on the optimal λ value by performing least absolute shrinkage and selection operator (LASSO) Cox regression analysis (figure 2C,2D). The above 11 miRNAs were selected to construct a multivariate Cox regression model. The results showed that there were 6 miRNAs (hsa-miR-194-3p, hsa-miR-221-3p, hsa-miR-150-3p, hsa-miR-149-3p, hsa-miR-216a-5p, hsa-miR- −628−3p) were

not statistically significant, while the other five miRNAs (hsa−miR−21−3p, hsa−miR−887−5p, hsa−miR−9−5p, hsa−miR−378d, hsa−miR− 31−5p) were statistically significant at $p<0.05$(figure 2B). These five m7G-related differential miRNAs deserve further study. Risk scores were calculated based on the normalized expression level of each gene and its corresponding regression coefficient. The risk score was calculated as follows: risk score = (0.3596*hsa−miR−194−3p exp.) + (0.0113*hsa−miR−21−3p exp.) + (0.0200*hsa−miR−887−5p exp.) + (0.2321*hsa−miR−221−3p exp.) + (0.7881*hsa−miR−150−3p exp.) + (0.0199*hsa−miR−9−5p exp.) + (0.3526*hsa−miR−149−3p exp.) + (0.0650*hsa−miR−216a−5p exp.) + (0.0026*hsa−miR−378d exp.) + (0.2787*hsa−miR−628−3p exp.) + (0.0022*hsa−miR−31−5p exp.). Patients were divided into high-risk groups (n = 205) or low-risk groups (n = 204) according to the median risk score cutoff. The higher risk group was found to be significantly associated with T stage and N stage in the TCGA cohort (Table 2). PCA and t-SNE cluster analysis showed that patients in different risk groups were significantly separated and distributed in different directions (figure 3A, 3B). The riskline and the riskpoint also show the same characteristics (figure 3C, 3D). As shown in figure 4A, patients in the high-risk group had a lower probability of survival and shorter survival times in the lower-risk group($p<0.001$). Moreover, evaluating the predictive performance of the OS risk score model using a time-dependent ROC curve, we found that the area under the curve (AUC) reached 0.737 at 1 year, 0.738 at 3 years, and 0.703 at 5 years respectively (figure 4B). The results of the ROC curve show that the predictive ability of the model has a certain predictive ability

Table 1: Clinical characteristics of COAD patients used in this study

characteristics	TCGA cohort	percentage
Total number of patients	459	
Age (median, range)	60.50(31–90)	
Sex		
Female	216	47.1%
Male	243	52.90%
Status		
Die	93	20.3%
Live	360	79.7%
Stage		
I	76	16.6%
II	178	38.8%
III	129	28.1%
IV	65	14.2%
unknown	11	2.3%
T stage		
T1	11	2.4%
T2	78	17.0%
T3	313	68.2%
T4	57	12.4%
N stage		
N0	270	44.70%
N1	106	3.20%
N2	83	52.10%
M stage		
M0	337	79.30%
M1	65	14.70%
unknown	57	6%

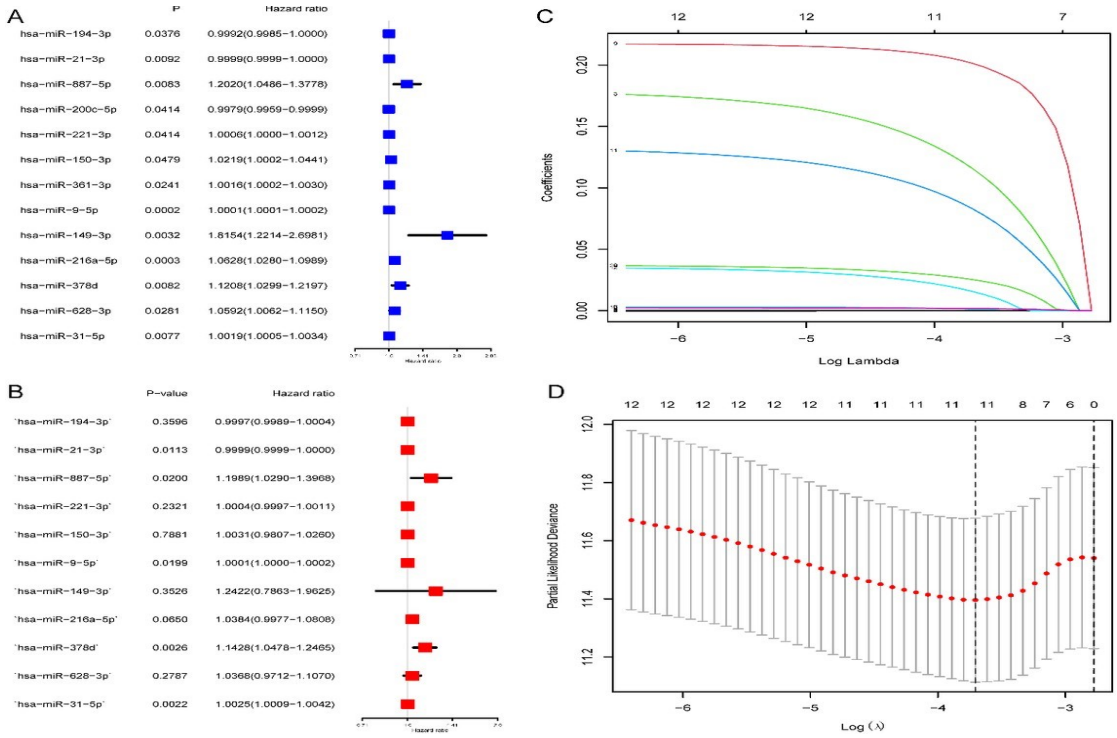

Figure 2: Building a prognostic genetic model in the TCGA cohort. (A) A total of 13 differential miRNAs were obtained by univariate Cox regression analysis; (B) Five statistically significant m7G-related differential miRNAs were obtained by multivariate Cox regression analysis; (C, D) LASSO regression analysis to construct 11 prognostic miRNAs genes

Table 2: Baseline characteristics of patients in different risk groups

Clinicopathological	features content	low risk	high risk	P
Age(year)	< 60	61	44	0.051
	>=60	143	161	
Sex	Female	97	98	0.921
	Male	108	107	
Stage	I + II	113	121	0.458
	III + IV	91	84	
T	T1 + T2	44	35	< 0.001
	T3 + T4	60	170	
N	N0	121	37	0.023
	N1	50	95	
	N2	33	73	

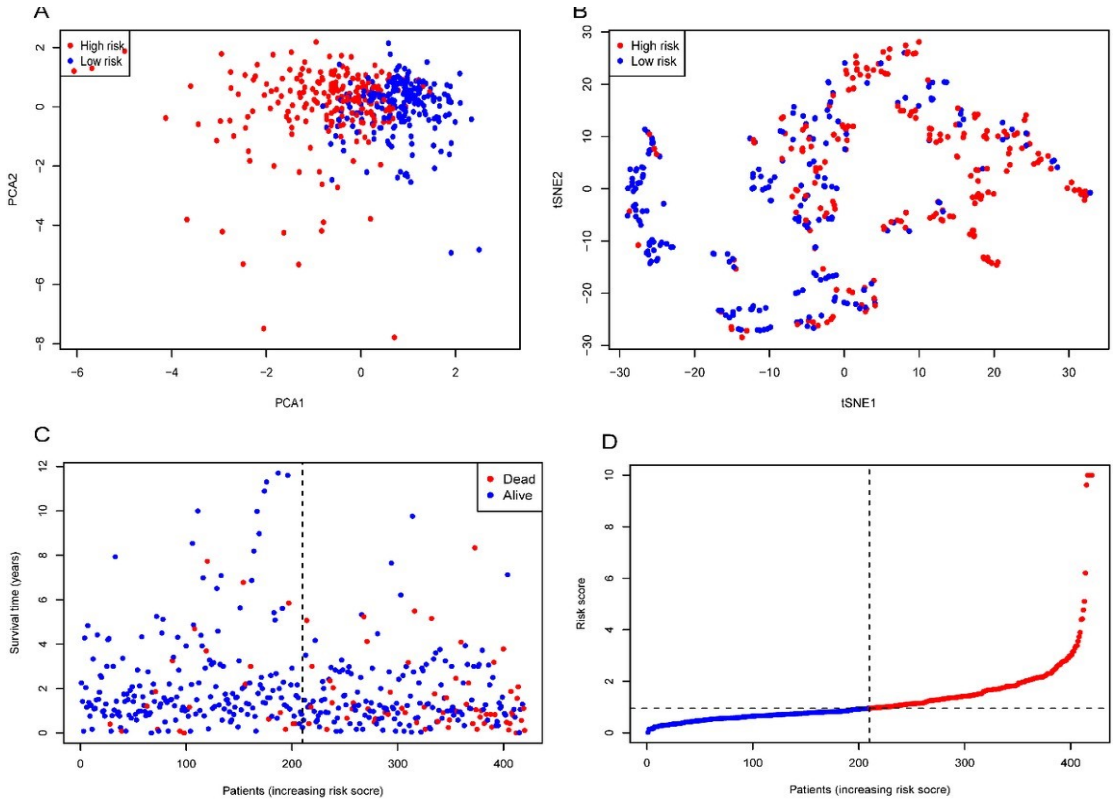

Figure 3: Analysis of the accuracy of the prognostic risk model. (A, B) PCA and t-SNE cluster analysis showed that patients in different risk groups were significantly separated in different directions; (C, D) Drawing of risk score distribution map: riskline and the riskpoint.

Validation of the predictive performance of risk scoring model

To test whether the risk score of this model could serve as an independent prognostic factor, we performed univariate Cox and multivariate Cox regression analyses combining the risk score with clinical factors (age, sex, stage, T, N). The results showed that the risk score was statistically significant($p<0.001$) in uni/multivariate Cox regression model while other factors make no sense (figure 4C, 4D). The nomogram showed that the final total score was 374 points according to different factors. The risk score had a significant effect on survival time, the higher the risk score, the worse the prognosis of colon cancer. The calibration curve also shows that the risk score results are reliable (figure 4E, 4F).

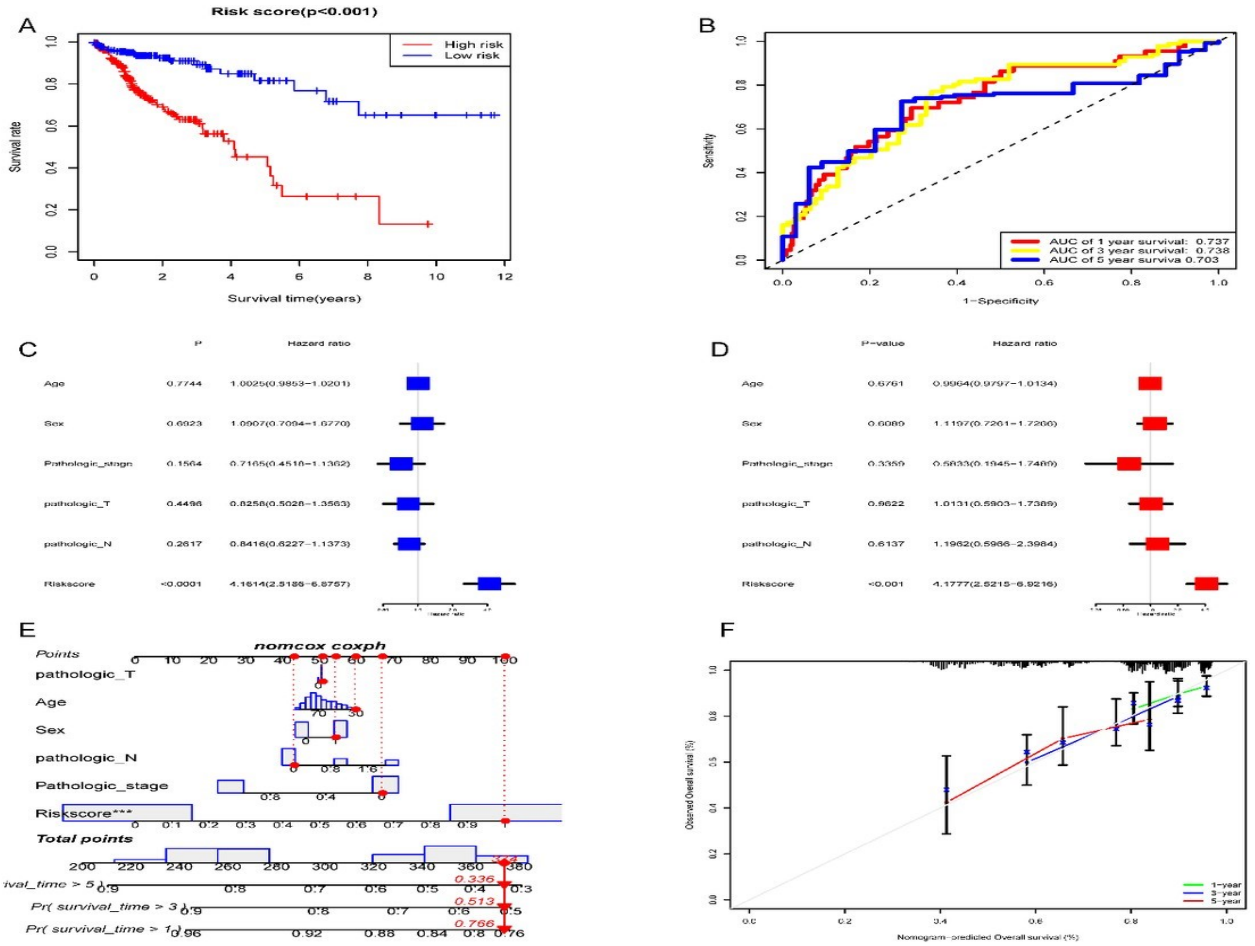

Figure 4: (A) Overall survival analysis of risk score; (B) Time-dependent ROC curve results, area under the curve (AUC) reached 0.737 at 1 year, 0.738 at 3 years, and 0.703 at 5 years respectively; (C) Univariate COX analysis to identify the risk factors; (D) Multivariate COX analysis to identify the risk factors; (E) Drawing a nomogram to evaluate risk scoring models; (F) Evaluation of Risk Scoring Models by Drawing Calibration Curves.

Functional analysis based on risk models and immune scores in COAD cohort

mRNAs associated with risk scores and colon cancer immune scores were selected to predict target genes. We obtained 403 differential genes in COAD mRNA expression data according to risk score, 1584 genes related to colon cancer immune score were downloaded from the online database ESTIMATE, and applied R software "venn" package to obtain a total of 163 intersecting genes (Figure 5A). Then Gene Ontology (GO) enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, DO disease enrichment analysis were performed based on these intersection genes for functional analysis. GO enrichment which comprised biological process (BP), molecular function (MF) and cellular component (CC), the results of enrichment analysis showed that these target genes were significantly enriched as follows: female pregnancy antimicrobial humoral immune, keratin filament, G protein−coupled receptor binding (figure 5C). KEGG enrichment analysis showed that these target genes were associated with the Neuroactive ligand-receptor interaction pathway (figure 5B). The 5 most correlated genes in disease enrichment analysis were obesity, overnutrition, pre−eclampsia, absolute aortic aneurysm, nutrition disease (figure 5D). To further explore the correlation between risk scores and immune status, we used single-sample gene set analysis (ssGSEA) to enrich scores for different immune cell subsets and associated functions. Interestingly, in the analysis of immune cells and immune function, Macrophages and T-helper cells scored the highest (score=0.82), showing a positive correlation. Conversely, Treg and p-DCs were negatively correlated (score=-0.02) (figure 6A, 6B). On the other hand, when the association between the selected prognostic genes and 29 immune cells was studied, we found that PRSS56 was negatively correlated with 16 types of immune cells, CALML5 was also negatively correlated with Treg, but not others (figure 6C).

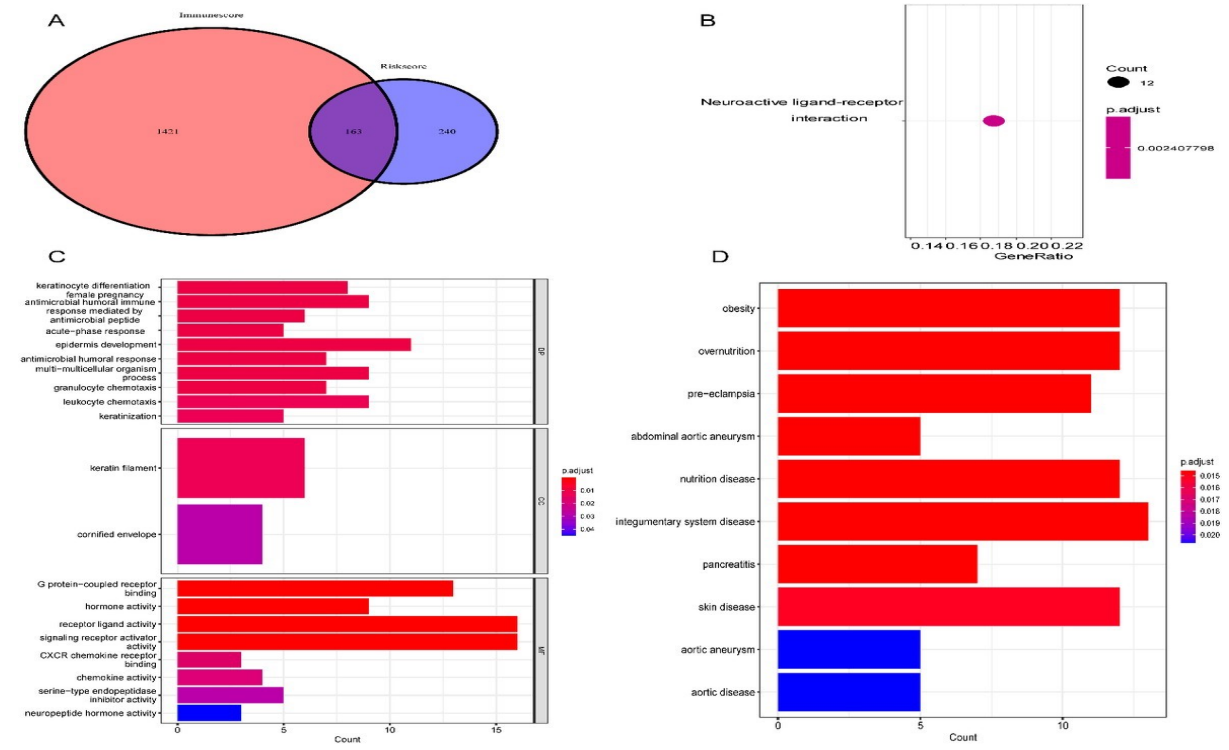

Figure 5: (A) Intersection genes Venn diagram;(B) KEGG enrichment pathway results; (C) The results of GO analysis of intersection genes; (D) Intersection gene DO analysis results.

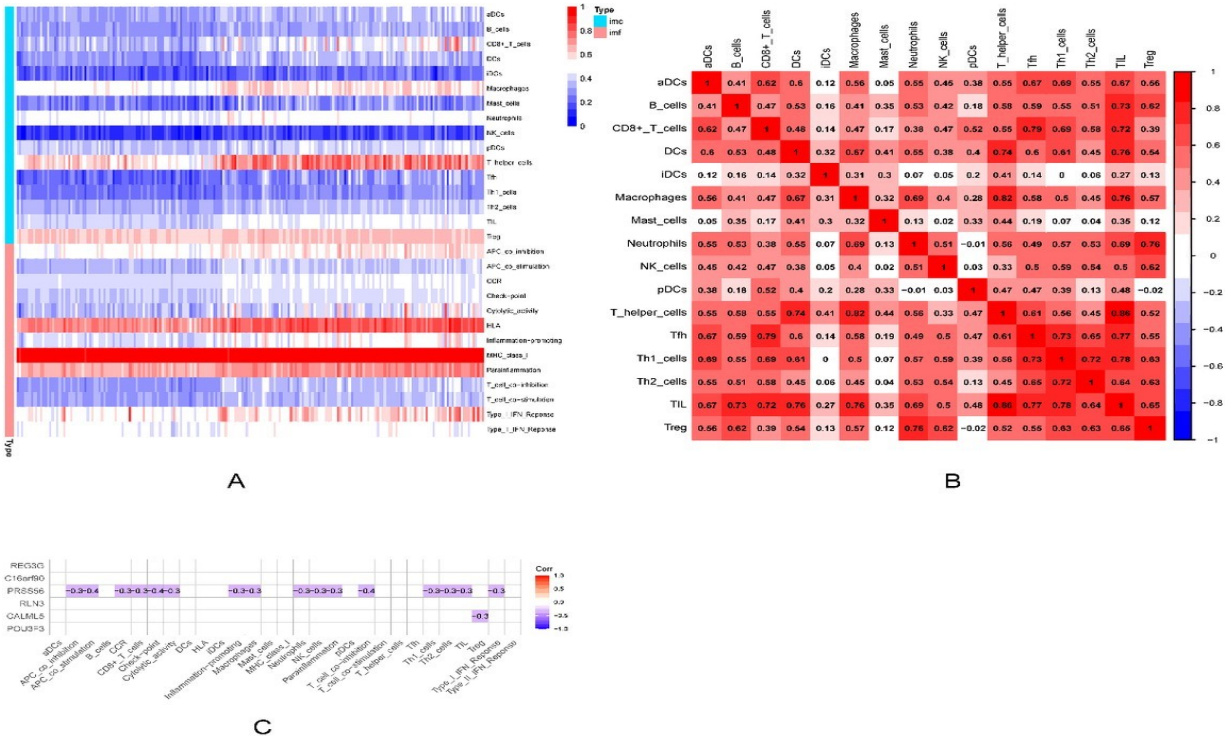

Figure 6: Correlation analysis results of immune cells and immune function. (A) Expression of immune cells in colon cancer cohorts; (B) The correlation between immune cells and immune cells; (C) Correlation between target genes and 29 types of immune cells.

Discussion

Although there are many studies on the use of miRNAs to predict the prognosis of colon cancer patients, no study has systematically used m7G-related miRNAs to predict the prognosis of colon cancer patients. To our knowledge, this study is the first to explore the use of m7G-related miRNAs as molecular markers for predicting prognosis in colon cancer patients.

M7G methylation is a novel transcriptional regulatory mechanism with dual roles in tumor development, has become a research hotspot in recent years. On the one hand, normal cells are stimulated by transcription factors to change the structure of m7G[21, 22], which leads to activation of downstream promoters and enhanced expression. On the other hand, regulating m7G methylation stabilization may be a new therapeutic target[23, 24]. In COAD, how m7G-related regulatory gene miRNAs function and whether it is related to survival remains unknown.

Some previous studies have shown that m7G-related genes may regulate tumor progression and tumor resistance [25, 26], but there are few studies on the specific regulatory mechanism and prognosis. In this study, we systematically investigated the differential expression of two m7G-related miRNA genes in colon cancer tissues and their association with clinical data. To our surprise, a total of 109 m7G-related differentially expressed gene miRNAs were statistically significant in COAD, and 13 of them were associated with OS in univariate Cox regression analysis. These results imply the possibility of m7G-related regulatory gene miRNAs as effective biomarkers and prognostic models in COAD. To further evaluate the value of these m7G-related regulators, we constructed an 11-gene risk model by Cox multivariate analysis and LASSO Cox regression analysis, and then validated that risk score can be an independent prognostic factor in colon cancer. Functional analysis showed that DEGs between low-risk and high-risk groups were associated with immune-related pathways. This immune cell infiltration and activation pathway was compared between the low-risk group and the high-risk group, and we found that the high-risk group generally had decreased levels of infiltrating immune cells and decreased their activity compared with the low-risk group, and immune-related pathways were activated.

Our study yielded five statistically significant m7G-related miRNA genes (hsa−miR−21−3p, hsa−miR−887−5p, hsa−miR−9−5p, hsa−miR−378d, hsa− miR-31-5p), and found that they could predict OS in COAD patients. Dysregulation of hsa-miR-21-3p can lead to autism and may be a biomarker for esophageal squamous cell carcinoma[27, 28], Overexpression of hsa-miR-21-3p results in downregulation of the tumor suppressor gene STARD13, which leads to gene activation in breast cancer pathways leading to carcinogenesis[29]. Also, a circRNA-hsa-miR-21-3p-mRNA network identifies an interacting gene circ-YOD1 via an online database that could be a biomarker in coronary artery disease[30]. Hsa−miR−9−5p is recognized as a regulatory molecule associated with the blood system, a posttranscriptional regulator of acquired etoposide resistance in leukemia therapy[31, 32]. Yang et.al [33] demonstrated that Hsa-miR-9-5p can be a useful biomarker through bioinformatic analysis of CRC miRNA data. Hsa−miR−9−5p overexpression induces NF-κB channel activation and inhibits B cell transcription factors to promote Hodgkin lymphoma progression [34]. Jiang et.al claims [35] that miRNA-378d regulates polyadenylation factor dysregulation and causes structural changes in APA that lead to the occurrence of CRC. has-miR-378d can participates in NC-DE-miRNAs-mRNAs regulation axis in HCC and inhibits cancer progression [36]. Hsa−miR-31-5p has been extensively studied, K et.al claimed that glioma cell invasion was inhibited by co-overexpression of has-miR-145-5p and has-miR-31-5p[37]. Interestingly, has-miR-31-5p plays an important role in the inflammatory response. Low expression can inhibit helper T cells to stabilize the human immune response[38]. It can also inhibit the effect of RA-FLS on cell growth and inflammatory response through the activation of the circ0003353/miR-31-5p/CDK1 to treat rheumatoid arthritis [39]. High expression of hsa−miR-31-5p in bladder cancer attenuates the tumor-promoting effect of circ-BPTF and binds to RAB27A target genes to regulate carcinogenesis [40]. Population-based data from 141 CRCs suggest that hsa-miR-31-5p may also be associated with CRC pathological stage and death [41]. However, there is no related research report on hsa-miR-887-5p. The mechanism about these m7G related miRNAs play a role in affecting the prognosis of COD patients remains to be elucidated.

To further explore the function of the risk scoring model in colon cancer, we integrated the colon cancer immune score data to perform GO and KEGG functional enrichment analysis based on the obtained intersection genes, and unexpectedly found immune-related enrichment. The biological process is consistent with the research results of the five genes mentioned above, indicating that m7G-related regulation may be closely related to the immune response. Interestingly, in a gene-to-disease enrichment analysis (DO), we found that obesity is significantly associated with disease, as we already know, colon carcinogenesis is inextricably linked to diet. Both hsa-miR-21-3p and hsa-miR-9-5p are associated with vascular diseases, similar to our results by DO enrichment analysis, it is very likely that these biomarkers are associated with the occurrence of vascular diseases. In the analysis of immune cells and immune function, macrophages and Treg cells were associated with the highest proportions. Previous studies have shown that increased macrophages [42] or Treg cells have marked immune invasion in HCC patients [43]. In addition, higher risk scores imply stronger immune compromise, suggesting a poor prognosis. In the correlation analysis of 29 inflammatory factors and genes, PRSS56 was negatively correlated with 16 kinds of cells. Some studies have shown that it plays an important role in the occurrence of inflammation and further leads to the occurrence of cutaneous neurofibromas [44]. There is still a lack of sufficient research in colon cancer. The above results strongly demonstrate that m7G-related regulated miRNAs play an important role in colon cancer.

There are inevitably several flaws in this study. First, our prog-

nostic model was constructed and validated with data from public databases. More prospective data are needed to validate its clinical utility. Secondly, the role of the obtained five prognostic miRNAs in colon cancer needs further experimental verification, which is also the focus of our next study. Furthermore, it should be emphasized that the relationship between risk score and immune activity has not been experimentally validated.

Finally, our study defines a novel prognostic model of 13 m7G-related gene miRNAs. The model was shown to be independently correlated with the analysis score, and the five miRNA biomarkers can potentially predict colon cancer prognosis. The miRNAs associated with m7G methylation and tumor immunity are still poorly understood, and further proof investigations are required in the future.

Data Availability Statement

All available data were analyzed in this study. These can be found here: TCGA (https://portal.gdc.cancer.gov/), R(https://www.r-project.org/), ESTIMATE (https://bioinformatics.mdanderson.org/)

Ethics Statement

Ethical review and approval were not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

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Author Contributions

LB wrote the paper. CF, ZW edited the paper. All authors contributed to the article and approved the submitted version. All authors have no related financial or non-financial conflicts of interest.

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