

Evaluated the Role of N6-Methyladenosine-associated lncRNAs in Prognosis and Immune Microenvironment of Colorectal Cancer

Congfei Yuan

Lianshui County People's Hospital

Caidong Liu

Nanjing Medical University

Shuli Zhao

Nanjing Medical University

Xishan Zhang

Lianshui County People's Hospital

Haifeng Jia

Lianshui County People's Hospital

Baiyu Chen

Lianshui County People's Hospital

Maojin Zhang

Lianshui County People's Hospital

Yuan Zheng

Lianshui County People's Hospital

Xiaowei Wei (✉ gswxw@njmu.edu.cn)

Nanjing Medical University

Jin Zhou

Nanjing Medical University

Yanzhi Bo

Lianshui County People's Hospital

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Abstract

Background: The role of N6-methyladenosine mediated long non-coding RNAs (lncRNAs) is elusive in colorectal cancer (CRC).

Materials and Methods: We identified m6A-associated lncRNAs by using the data gathered from The Cancer Genome Atlas (TCGA) and stratified CRC patients into different subgroups. Cox-regression analysis were performed to construct a m6A-associated lncRNA signature. And the role of this signature in prognosis and immune microenvironment was dissected subsequently. Finally, a gene set enrichment analysis (GSEA) were executed to predict the possible bio-mechanisms on the basis of the signature.

Results: Three m6A-associated clusters were constructed from 866 differentially expressed lncRNAs. Cluster 2 had poor prognosis and low immune cell infiltration. A m6A-associated lncRNA signature consisting of 14 lncRNAs was constructed, and recognized as an independent prognostic indicator in CRC by using survival analysis and receiver operating characteristic (ROC) curves. The clinical features and immune cell infiltration status were significantly different in the patients stratified by risk score. Furthermore, GSEA showed that P53 pathway and Natural killer cell mediated cytotoxicity were more enriched in the low risk group.

Conclusion: Our data revealed that m6A-associated lncRNAs could be potential prognostic and immunogenicity indicators in CRC.

Introduction

Colorectal cancer is the third prevalent gastrointestinal malignancies worldwide¹. A significant number of CRC patients will ultimately relapse after curative treatments²⁻³. Hence, there is an urgent requirement to investigate alternative prognostic markers in CRC patients.

N6-methyladenosine (M6A) is the most common post-transcriptional abundant methods in RNAs⁴. Recent studies have highlighted that m6A RNA modification plays important roles in many biological processes including cancer pathogenesis⁵. Aberrant expressions of m6A regulators (e.g. METTL14, METTL3, KIA, ALKBH5, FTO and YTHDF1/2/3) have been identified in numerous tumors⁶⁻¹⁰. Varieties of biological functions, ranging from tumor initiation, invasion, metastasis to tumor stem cell pluripotency, could be mediated by m6A methylation¹¹⁻¹². Long non-coding RNAs (lncRNAs) are important epigenetic regulators, which play critical roles in diverse physiological and pathological processes¹³⁻¹⁴. Studies have reported that many lncRNAs participate in tumor initiation and progression¹⁵⁻¹⁷. Despite extensive efforts to define the pathogenesis of lncRNAs, the roles of lncRNAs in the m6A modification remains largely elusive in CRC.

Immune microenvironment has been found to be closely implicated in the clinical outcome of immunotherapy and tumor development. In the present study, the co-expression network of the m6A-

associated lncRNAs to obtain 68 m6A-associated prognostic lncRNAs was generated. Then we established three m6A-associated clusters in CRC, analyzed the characteristics of immune cell infiltration among tumor cells and investigated whether m6A-associated lncRNAs clusters have prognosis values in CRC patients. Furthermore, we constructed 14 m6A-associated lncRNAs signature which could predict the prognosis of CRC patients.

Results

The differential expressions of m6A-associated lncRNAs.

A total of 19604 mRNAs and 14086 lncRNAs were screened from TCGA database. 1590 m6A-associated lncRNAs were obtained ($R > 0.4$ and $p < 0.05$) according to 23 reported m6A-associated genes, of which, 866 differentially expressed m6A-associated lncRNAs in CRC were detected with a log/fold change (FC) > 0.5 and a $p < 0.001$ (Supplementary data 1).

Identification of m6A-associated lncRNAs with prognostic value

As shown in Figure 1A, we annotated m6A-associated lncRNAs and clinical characteristics, then investigated the role of each lncRNA on the prognostic outcome of the patients with CRC. And there are 68 m6A-associated lncRNAs with obvious prognostic value were detected for further study.

Establishment of m6A-associated lncRNA clusters

To classify the different tumor clusters with m6A phenotypes on the basis of lncRNAs, we mapped 68 m6A-associated lncRNAs to expression profile of CRC samples to perform consistent clustering with Consensus Cluster Plus (CCP) tool. As shown in Figure 1B, the number of clusters was sequential set from 1 to 9 and CCP analysis indicated that the results were most stable when these m6A-related lncRNAs were separate into three tumor clusters (Figure 1C, D). The OS data of each cluster was calculated using Kaplan-Meier method, and the results displayed that there were significant difference among the survival of the three clusters (Figure 1E).

Clinical characteristics and Immune Score of each cluster in CRC

As compared with cluster 1 and cluster 3, cluster 2 had the highest N stage, M stage and TNM stage (Figure 2A). ESTIMATE-algorithm was employed to accurate estimate score (tumor purity), immune score and stromal score in accordance with the gene expression profiles of CRC patients. Our findings showed that compared with cluster 1 and 3, the cluster 2 had the lowest estimate score, immune score and stromal score (Figure 2B).

m6A-associated lncRNAs signature construction

As shown in Figure 3A, a total of 14 m6A-associated lncRNAs that had a co-expression relationship with 8 m6A-associated genes were recognized as effective independent prognostic factors. Among them,

AC137932.3, AL391422.4, AC092123.1, AC156455.1, AC132192.2, AC008760.1, RPARP-AS1, LINC02657, AP001619.1, AC003101.2, AL161729.4, TNFRSF10A-AS1, AL121906.2 and AC074117.1 were found to be favorable prognostic factors (Supplementary data 2). The risk score of each CRC patient = $AC137932.3 * (-1.4041) + AL391422.4 * 0.9484 + AC092123.1 * (-1.3865) + AC156455.1 * 0.1977 + AC132192.2 * (-0.4822) + AC008760.1 * 0.5973 + RPARP-AS1 * 0.3572 + LINC02657 * 0.7205 + AP001619.1 * 0.8025 + AC003101.2 * 1.0959 + AL161729.4 * 0.3047 + TNFRSF10A-AS1 * (-0.2329) + AL121906.2 * 1.02629 + AC074117.1 * 0.25582$. Based on the median risk score, 426 CRC patients were classified into two groups (low risk vs high risk). The Kaplan-Meier curves and the distributions of survival status confirmed the poor outcome in the high risk group (Figure 3 B-D). Our findings showed that the mortality occurrence was closely associated with the risk score. Moreover, the area under the curve (AUC) is measured and the value for prognostic risk score was 0.764 which is higher than AUCs of the other clinicopathological factors (Figure 4E). And the AUC values corresponding to 1, 3, and 5 years of OS were 0.764, 0.743 and 0.753, respectively (Figure 3F). These data accomplished good prediction accuracy of this model.

The validation of the signature in CRC

The prognostic value of the m6A-associated lncRNA signature was investigated in CRC patients classified by various clinical parameters, consisting of gender, age, T, N, M and TNM stage. In almost all subgroups, the patients with low risk score tended to have higher OS rate than high-risk group (Figure 4).

Next we evaluated the independence and effectiveness of this model in predicting prognosis of CRC patients. Our findings showed that this m6A-associated lncRNA signature could be an effective and independent factor for predicting the outcome of the patients with CRC (Figure 5 A-B). Then, a nomogram was conducted to predict 1, 3 and 5-years OS of the patients with CRC based on the results of univariate and multivariate Cox-regression analyses, including age, TNM stage and risk score (Figure 5C). The calibration curves proved well prediction accuracy of this nomogram in CRC patients (Figure 5 D-F).

Gene set enrichment analysis

Finally, we tried to detect the potential biological mechanisms associated with the risk model by GSEA. As shown in Figure 6, the P53 signaling pathway (NOM p-val=0.0019, FDR q-val=0.155) and Nature Killer cells mediated cytotoxicity (NOM p-val=0.0172, FDR q-val=0.195) were more enriched in low risk group. Our study suggested that the risk-related model might contribute to organize the personalized treatment for CRC patients.

Discussion

Previous advances have demonstrated the pivotal roles of m6A modification in various cancers including CRC¹⁸⁻²⁰. Investigating the potential prognostic role of m6A-associated lncRNAs will facilitate understanding the molecular mechanisms of CRC. In our work, 68 prognostic m6A-associated lncRNAs were obtained, then three m6A-associated lncRNAs cluster groups were constructed using 426 CRC

samples from TCGA database. Compared with cluster1 and cluster3, cluster2 had worst OS time and later pathological stage. In addition, ESTIMATE analyses further revealed that the immune score was remarkably reduced in Cluster 2. Our data suggested that m6A-associated lncRNAs might serve as a predictive biomarker.

It is generally known that there are currently some CRC prognostic indicators, including TNM stage and tumor grade. However, More accurate prognostic factors are required to predict and analyze the OS rate in CRC patients. Current studies have indicated that lncRNAs may play important roles in predicting the outcome and prognosis of various cancers. For instance, Dan Yin, et al²¹ reported that overexpression of LINC01133 was related to the poor prognosis in the patients with hepatocellular carcinoma. Shujun Feng, et al²² reported that lncRNA-CTS was aberrantly expressed in gastric cancer tissues, and the upregulation of CTS was closely associated with tumor volume, tumor histology, lymph node metastasis and the poorer prognosis. Recently, numerous m6A-associated lncRNAs are reported to be potential markers for the prediction of various cancers, Haixu Wang, et al²³ established a 11 m6A-associated lncRNA signature, and confirmed that it had a good prognostic value and could act as a valid marker for gastric cancer. Feng Xu, et al²⁴ established a risk model consisting of 12 m6A-associated lncRNAs, and demonstrated that this model might be a promising prediction of prognosis in lung adenocarcinoma patients. In the present study, a m6A-associated lncRNA signature consists of 14 lncRNAs could screen the patients with poor prognosis by the degree of risk. Moreover, we assessed the clinical value of the signature in gender, age, T, N, M and TNM stage, and identified that the signature was closely associated with the progression of CRC. Meanwhile, the GSEA analysis preliminary displayed that these lncRNAs were closely involved in P53 pathway and NK cell mediated cytotoxicity. Further studies are needed to demonstrate the bio-mechanisms involved in this lncRNA signature.

Conclusion

In summary, our work defined an innovative m6A-associated lncRNA signature which could provide a new perspective on predicting the prognosis of CRC patients. Furthermore, our m6A-associated lncRNA signature provides an important clue for the development of individual treatment.

Methods

Data processing of the CRC dataset

The public RNA sequencing (RNA-seq) were downloaded from TCGA. Patients without survival information were removed.

Identification of m6A-associated lncRNAs in CRC

The m6A-associated genes were gathered from TCGA database and selected based on previously published articles. And the m6A-associated lncRNAs were screened by spearman correlation coefficient

formula with $R_value > 0.6$ and $p_value < 0.001$.

Consensus clustering of m6A-associated lncRNAs

On the basis of m6A-associated lncRNAs expression levels, the CRC patients were separately divided into three groups according to the optimal k-means clustering. Cluster analysis was performed with Consensus Cluster Plus R package. The OS data of each cluster was calculated using Kaplan-Meier method. The correlation between m6A-associated lncRNAs and clinical characteristics was analyzed according to the TCGA database. And ESTIMATE-algorithm was employed to estimate the tumor immune microenvironment.

m6A-associated lncRNA signature construction

The prognostic m6A-associated lncRNAs were indentied via univariate cox regression analysis. And the prognostic signature was established via mutivariate cox regression analysis. The risk scores of CRC patients were calculated by following formula, Risk score = $\sum \text{Exp}_i * \beta_i$, here Exp_i represents the expression and β_i represents the coefficient of m6A-associated lncRNAs. And the accuracy of the m6A-associated lncRNAs was assessed via the ROC curve analysis.

Statistical analysis

All data were analyzed via by using R statistical soft-ware version 4.0.3. A p_value less than 0.05 was statistically significant.

Abbreviations

lncRNAs: long non-coding RNAs

CRC: colorectal cancer

TCGA: The Cancer Genome Atlas

GSEA: gene set enrichment analysis

ROC: receiver operating characteristic

M6A: N6-methyladenosine

FC: fold change

CCP: Consensus Cluster Plus

AUC: area under the curve

RNA-seq: RNA sequencing

Declarations

Acknowledgments

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Ethical Approval and Consent to participate

TCGA belong to public databases. The patients involved in the database have obtained ethical approval. Our study is based on open source data, so there are no ethical issues.

Consent for publication

Not applicable.

Availability of data and materials

The data that support our findings are openly available in TCGA (<https://portal.gdc.cancer.gov/>) repository.

Competing interests

No author has conflict of interest.

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Authors' contributions

Conception and design: Xiaowei Wei, Jin Zhou and Yanzhi Bo

Development of methodology: Congfei Yuan and Caidong Liu

Acquisition of data: Congfei Yuan and Caidong Liu, Shuli Zhao

Analysis and interpretation of data: Congfei Yuan, Xishan Zhang, Haifeng Jia, Baiyu Chen, Maojin Zhang and Yuan Zheng

Writing, review, and/or revision of the manuscript: Congfei Yuan, Caidong Liu, Xiaowei Wei, Jin Zhou and Yanzhi Bo

Study supervision: Xiaowei Wei

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Figures

Figure 1

Unsupervised clustering of CRC using m6A-associated lncRNA expression data. (A) . The forest plot of 68 prognostic m6A-associated lncRNAs. (B). Consensus Cumulative Distribution Function(CDF) curve of unsupervised clusters analysis. (C). Delta area under CDF curve of cluster analysis. (D). Cumulative distribution function graph of the consistency matrix at K=3. The white and blue heatmap exhibits sample consensus. (E). Survival curve analysis of three clusters.

The signature for CRC patients on the basis of m6A-associated lncRNAs. (A). The network of 14 m6A-associated lncRNAs. (B). Kaplan-Meier analysis between low/high group was employed. (C-D). The distribution of risk scores and the survival state of selected m6A-associated lncRNAs. (E). The AUC of risk score and other clinicopathological factors were presented. (F). The AUC for 1, 3 and 5-years survival were 0.764, 0.743, 0.753, respectively.

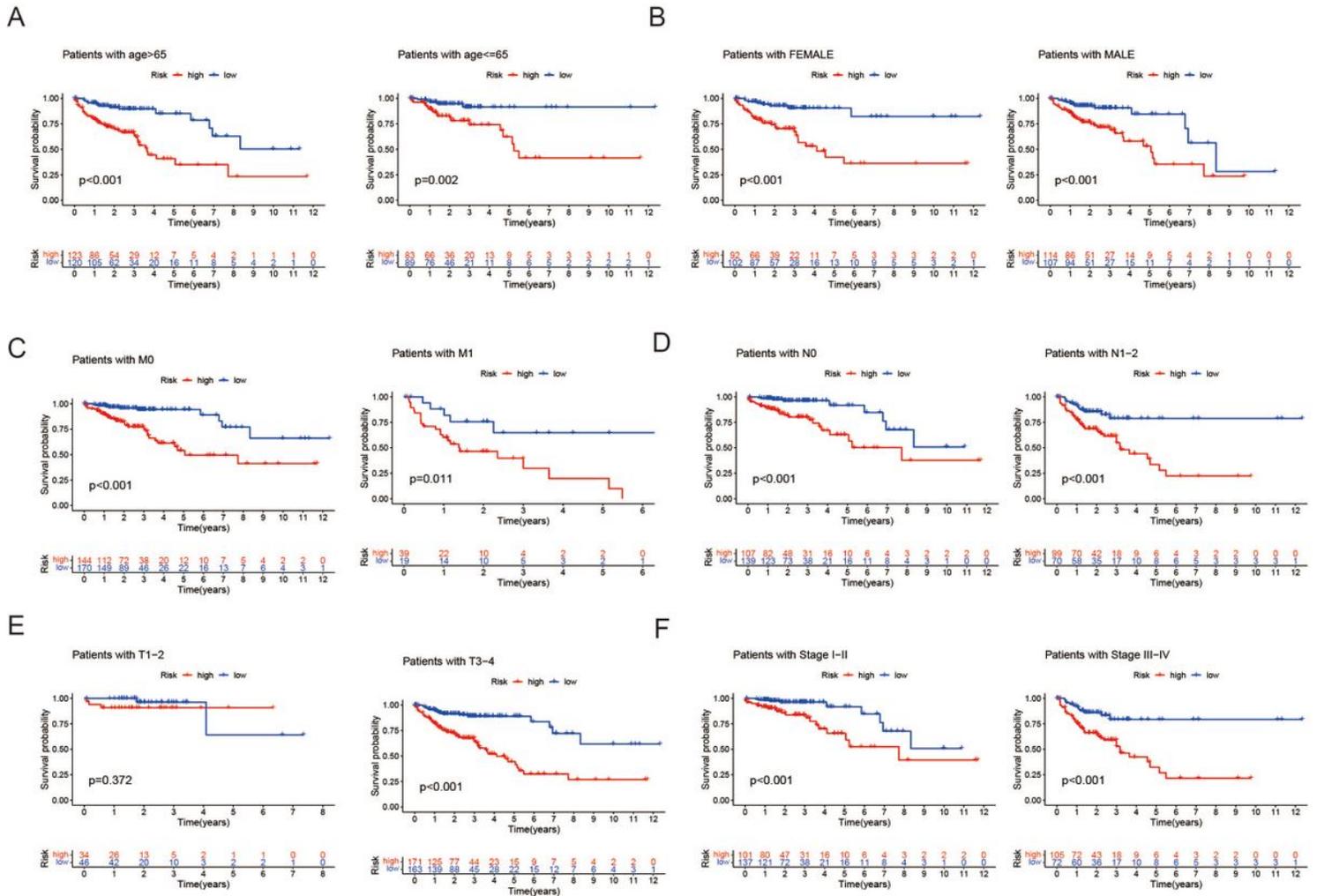


Figure 4

The prognostic value of the m6A-associated lncRNA signature in CRC patients. Kaplan-Meier analysis for the different risk groups classified using clinical factors including age (A), gender (B), M stage (C), N stage (D), T stage (E) and TNM stage (F).

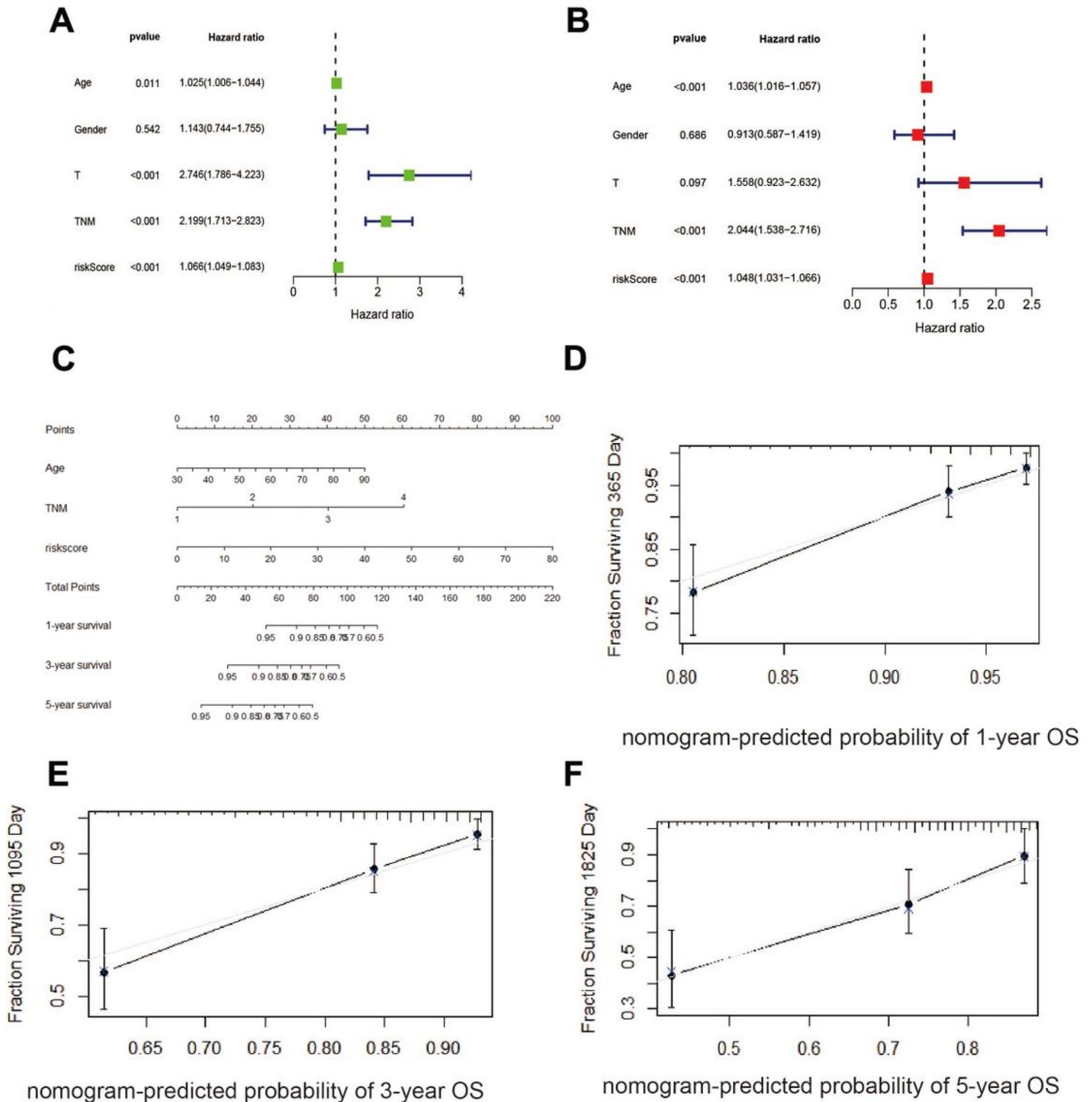


Figure 5

The independence and effectiveness of this model in predicting prognosis of CRC patients. Forest plots of univariate (A) and multivariate (B) Cox regression analysis in CRC. Nomogram model (C) to predict 1, 3 and 5-years survival rates of CRC patients. Calibration graph showed that predicted 1 (D), 3(E) and 5-years (F) survival rates were close to actual survival rates.

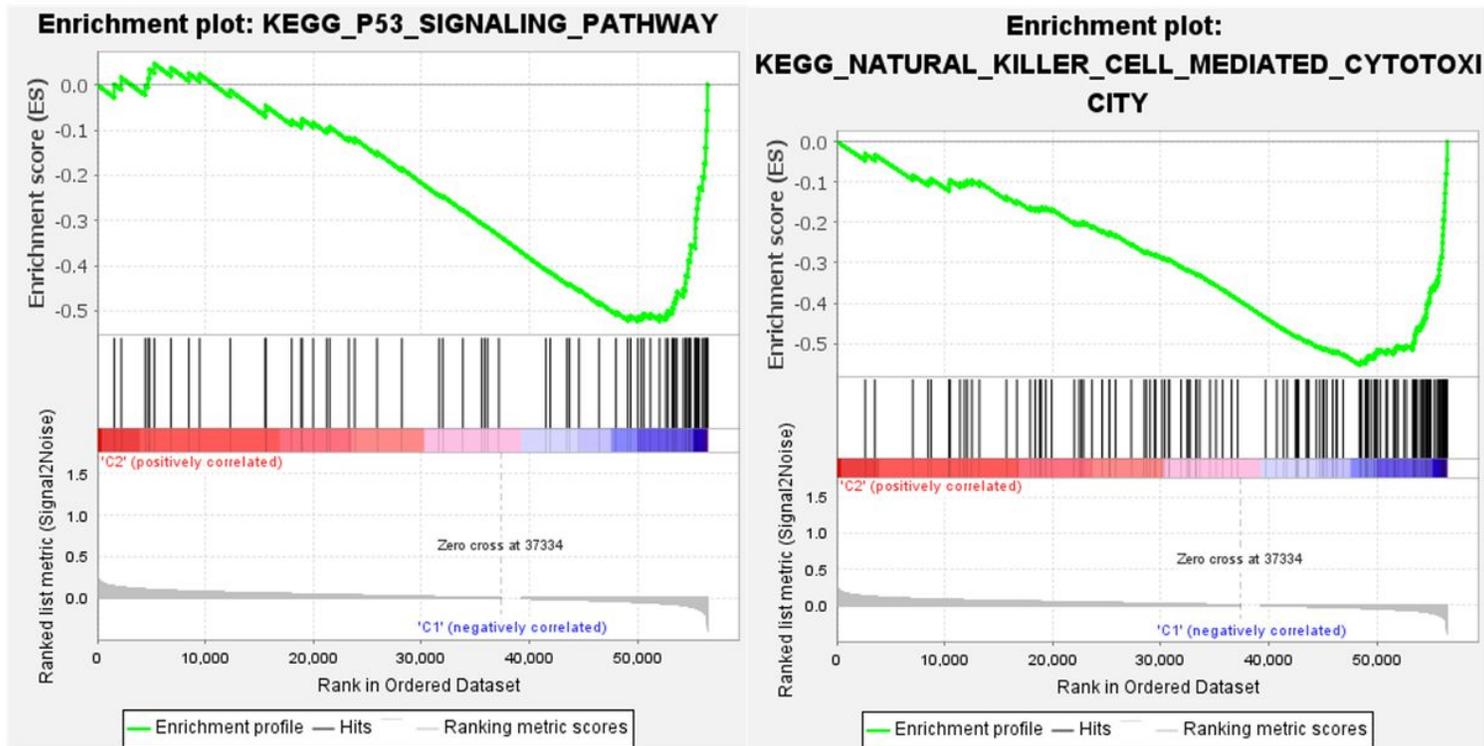


Figure 6

The enriched signaling pathways in the low risk group. The GSEA results of the P53 signaling pathway and nature killer cells mediated cytotoxicity.

Supplementary Files

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