Identification of key N6-Methyladenosine-Related lncRNAs in Colon Adenocarcinoma using Bioinformatics Analysis

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Research Article

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Abstract

Purpose: The purpose of this study was to investigate the role of m^{6}A-related lncRNAs in colon adenocarcinoma (COAD) and determine their prognostic value.

Material and methods: Gene expression and clinicopathological data were obtained from The Cancer Genome Atlas database. Correlation and univariate Cox regression analysis were conducted to identify m^{6}A-related prognostic lncRNAs. A prognostic signature was established via least absolute shrinkage and selection operator (LASSO) Cox regression analyses. The prognostic value of risk scores was evaluated using the Kaplan-Meier method, receiver operating characteristic curves, and univariate and multivariate regression analyses. Whether the prognostic model could serve as a prognostic indicator for overall survival (OS) in subgroups of patients with different clinical characteristics were explored. Next, We established a competing endogenous RNA network. Gene Set Enrichment Analysis, Kyoto Encyclopedia of Genes and Genomes pathway, and Gene Ontology analysis were performed for biological functional analysis.

Results: 36 lncRNAs that were highly correlated with OS of patients were identified. A prognostic signature comprising 11 m^{6}A-related lncRNAs was constructed, which had significant value in predicting the OS of patients . Univariate and Multivariate Cox regression analyses suggested that the risk score was an independent prognostic factor. This m^{6}A-related lncRNA prognostic model could serve as a prognostic indicator for OS in subgroups of patients with different clinical characteristics. Biological processes and pathways associated with cancer were identified.

Conclusion: We revealed the role and prognostic value of m^{6}A-related lncRNAs in COAD. Our finding refreshed the understanding of m^{6}A-related lncRNAs and provided novel insights to identify predictive biomarkers and develop targeted therapy for COAD.

Introduction

Globally, colorectal cancer (CRC) is the third most common cancer and the second most deadly neoplasm[1]. Colon adenocarcinoma (COAD) is the most common pathological type of CRC, and despite considerable progress in diagnosis and therapeutic strategies for COAD, the prognosis of patients with COAD remains poor due to advanced stage and postsurgical recurrence[2,3]. Therefore, identification of novel biomarkers for early detection and effective therapeutic targets for treating patients with COAD is critical and urgent.

Accumulating evidence has shown that long non-coding RNAs (lncRNAs) had various biological functions and played a crucial role in the oncogenesis and progression of CRC[4]. For example, lncRNA ACTA2-AS1 functions as a competing endogenous RNA (ceRNA) to miR-4428 and promotes the pathogenicity of COAD by regulating BCL2L11[5]. LncRNA MNX1-AS1 acts as an oncogene in CRC by

interacting with RNA binding protein YB1[6]. LINC00337 could facilitate the tumorigenesis and angiogenesis in CRC via recruiting DNMT1 to restrict the expression of CNN1[7].

Increasing evidence suggests that RNA modifications play a critical roles in tumorigenesis and progression of different cancers,including CRC[8,9]. N6-methyladenosine (m^6A), introducing a methyl group in the nitrogen-6 position of adenosine, is found to be the most frequent internal RNA modification in mammals[10]. As a dynamic and reversible process, m^6A RNA modification is primarily regulated by “writers” (adenosine methyltransferases) and “erasers” (demethylases), and performs different functions by interacting with “readers” (m^6A-binding proteins). As identified to distribute extensively in a variety of RNAs, such as messenger RNAs (mRNAs), pri-microRNAs (pri-miRNAs), circular RNAs (circRNAs) and lncRNAs, m^6A is involved in various biological processes related to the occurrence and progression of tumors, including CRC[11]. For instance, the m^6A writer METTL3 stimulates m^6A modification of CCNE1 mRNA and enhances its stability, which CRC proliferation[12]. Overexpression METTL3 facilitates processing of pri-miR-1246 into the miR-1246 through an m^6A DGCR8-dependent method, which activates the MAPK pathway and the progression of CRC[13]. YTHDF3 recognizes and binds to m^6A-modified lncRNA GAS5 and promotes its degradation, which elevates YAP expression and promotes CRC[14].

Here, we analyzed the Cancer Genome Atlas (TCGA) database for m^6A-related lncRNAs involved in COAD and established a m^6A-related lncRNAs prognostic signature. Then, we demonstrated that the m^6A-related lncRNA prognostic model could serve as a prognostic indicator for overall survival (OS) in subgroups of patients with different clinical characteristics. Furthermore, a ceRNA network was constructed based on the m^6A-related prognostic lncRNAs in COAD. The finding in this study revealed the critical role of m^6A-related lncRNAs and shed light on the latent relationship and underlying mechanism between m^6A-related lncRNAs and COAD.

Material And Methods

Acquisition of datasets

The RNA-seq transcriptome data (fragments per kilobase million, FPKM)[15] from 437 samples and clinical information from 385 patients with COAD in TCGA database (http://cancergenome.nih.gov/) were downloaded for our study. Patients with complete clinicopathological and survival information were included for further assessment.

Selection of m^6A-related regulators

Based on published data[8], 24 m^6A-related regulators, including METTL3, METTL14, METTL16, WTAP, VIRMA, KIAA1429, ZC3H13 RBM15, RBM15B, YTHDC1, YTHDC2, YTHDF1, YTHDF2, YTHDF3, HNRNPC, FMR1, LRPPRC, HNRNPA2B1, IGFBP1, IGFBP2, IGFBP3, RBMX, FTO and ALKBH5, were used in our study.

Bioinformatic Analysis

Primarily, correlation analysis was performed between m^{6}A-related regulators and all lncRNAs in COAD. m^{6}A-related lncRNAs were identified based on the following classification parameters: 1) correlation coefficients more than 0.3; 2) *p*-value less than 0.001. Then, to filtrate the m^{6}A-related lncRNAs that were highly correlated with OS, univariate Cox regression analysis was performed. Next, we randomly divided the patients with COAD into two groups: the training group and the testing group. Subsequently, based on m^{6}A-related prognostic lncRNAs identified by univariate Cox regression analysis, the least absolute shrinkage and selection operator (LASSO) Cox regression algorithm was used to identify m^{6}A-related lncRNAs with powerful prognostic significance and construct the prognostic risk model from the training group data. According to the best penalty parameter λ, the m^{6}A-related lncRNAs' coefficients were calculated. The risk score (RS) was estimated using the following formula:

$$RS = \sum_{i=1}^{n} Coef(i)X(i)$$

where Coef(i) is the coefficient and X(i) represents the expression levels of m^{6}A-related lncRNAs. Using the obtained median RS as the demarcation value, patients with COAD were classified in two groups: high-risk group and low-risk group. Kaplan-Meier analysis and the receiver operating characteristic (ROC) curves were used to validate the predictive efficiency[16]. Then, the accuracy of the model was validated from the test group and the combined group by the same method. Furthermore, the differences in clinicopathological features between high-risk group and low-risk group were also explored. Additionally, the prognostic value of the RS was verified using univariate and multivariate Cox regression analyses. The hazard ratio (HR) with 95% confidence intervals and log-rank *p*-value were calculated using the "glmnet" and "survival" R packages[17].

To explore the biological functions associated with m^{6}A-related lncRNAs, Gene Set Enrichment Analysis (GSEA), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) analysis were performed. Genes in different risk groups were functionally annotated using GSEA. Based on the m^{6}A-related prognostic lncRNAs, the target miRNAs were predicted via miRcode database and target mRNAs of these miRNAs were found in different databases, such as TargetScan, miRTarBase, and miRDB. Target mRNAs in the ceRNA network were functionally annotated using GO and KEGG pathway analysis. The flow chart of bioinformatic analysis was shown in Fig. 1.

Statistical analysis

The expression data of m^{6}A-related regulators and all lncRNAs in tumor tissues and adjacent mucosa of COAD obtained from TCGA was compared using one-way analysis of variance (ANOVA); the clinical characteristics of different groups were compared using the chi-square test; the Kaplan-Meier method was used to perform a bilateral logarithmic rank test in OS analysis; *p*-values < 0.05 were regarded as

statistically significant. All statistical analyses were implemented using R v4.0.3 (https://www.r-project.org/).

Results

Identification of m^{6}A-Related prognostic lncRNAs

Firstly, the expression levels of 24 m^{6}A-related genes and all lncRNAs from the TCGA were extracted respectively. Through coexpression analysis, we identified 581 m^{6}A-related lncRNAs (| cor| > 0.3, *p*-value < 0.001). The gene co-expression network of 24 m^{6}A-related genes and 581 m^{6}A-related lncRNAs was shown in Fig. 2A. After conducting univariate Cox analysis, 36 candidate lncRNAs that were highly correlated with OS were identified ($p < 0.05$) (Fig. 2B). The expression of 36 m^{6}A-Related prognostic lncRNAs between tumor tissues and adjacent mucosa was compared (Fig. 2C, D). Among these lncRNAs, two lncRNAs (LINC01555, SNHG16) were prognostic protective factors and others were prognostic risk factors.

Construction and Verification of the m^{6}A-Related lncRNAs Prognostic Signature

Based on 36 candidate lncRNAs that were highly correlated with OS, we used the LASSO method in the training group to construct a m^{6}A-related lncRNAs signature for evaluating the prognosis of patients with COAD. Finally, 11 lncRNAs were chosen to establish a prognostic signature and the risk score was calculated (Fig. 3A, B). Using the median risk score as the demarcation value, patients in the training group (n = 190) were classified into two groups, namely the high-risk group and low-risk group. To test the efficacy of the prognostic model, survival and ROC curves analyses were conducted. Kaplan-Meier analysis showed that the low-risk group had significantly longer survival time than the high-risk group ($p < 0.001$) (Fig. 3C). The area under the curve (AUC) value in the time-dependent ROC curve of 1-, 3- and 5-year was 0.768, 0.814 and 0.873 severally(Fig. 3D), suggesting good prediction performance of the survival model. To further validate this 11 lncRNAs prognostic signature, verification analysis in the test group (n = 189) and the combined group (n = 379) were implemented. As a result, the high-risk group in the test group and the combined group had significantly shorter survival time compared with the low-risk group, which was previously observed in the training group (Fig. 3C). Time-dependent ROC curve of the test group and the combined group also had well-prediction performances, and the AUC value of 1-,3- and 5-year was shown in Fig. 3D. The risk scores, survival time and the expression of 11 m^{6}A-related prognostic lncRNAs of the high-risk group and low-risk group in different subgroups were displayed in Fig. 3E, F, G).

To examine whether the risk score was an independent prognostic factor, univariate and multivariate Cox regression analyses were conducted. This revealed that the risk score ($p < 0.001$) was significantly

associated with OS in patients with COAD, in addition to age at diagnosis ($p < 0.05$) and pathological stage ($p < 0.001$) (Fig. 4A, B).

Subgroup Analysis with Different Clinicopathological Features

The expression of 11 m^6A-related prognostic lncRNAs and the distribution of clinicopathological characteristics in the high-risk group and low-risk group were displayed as a heatmap (Fig. 4C). Evident differences between the two groups according to pathological stage ($p < 0.001$), T stage ($p < 0.001$), M stage ($p < 0.001$) and N stage ($p < 0.001$) were observed. Significant differences of risk score were found between: 1) different pathological stage ($p < 0.001$); 2) different T stage ($p < 0.001$); 3) different M stage ($p < 0.001$); and 3) different N stage ($p < 0.001$) (Fig. 4D).

To evaluate whether m^6A-related lncRNA prognostic model could serve as a prognostic indicator for OS in subgroups of patients with different clinical characteristics, we stratified subgroups by age (age ≤ 65 and age > 65), gender (female and male), clinical stage (stage I-II and stage III-IV), T stage (T1-2 and T3-4), M stage (M0 and M1) and N stage (N0 and N1-3). As the result shown in Fig. 5, the OS of the low-risk patients based on age($p < 0.001$), sex ($p = 0.001$ in female and $p < 0.001$ in male), pathological stage ($p = 0.002$ in stage I-II and $p = 0.004$ in stage III-IV), T stage ($p = 0.014$ in T1-2 stage and $p < 0.001$ in T3-4 stage), M0 stage ($p < 0.001$) and N stage ($p < 0.001$ in N0 and = 0.006 in N1-3) were significantly higher than those of the high-risk patients.

Construction of the ceRNA Network and Functional Enrichment Analysis

To explore the biological function of 36 m^6A-related prognostic lncRNAs, a ceRNA network was constructed based on the mechanism of lncRNAs regulating mRNA expression by sponging miRNAs. 5 lncRNAs were extracted from the miRcode database and 28 pairs of interaction between the 5 lncRNAs and 31 miRNAs were identified. Based on three mRNA predicting database mentioned previously and differentially expressed mRNA between normal group and tumor group of COAD in TCGA, we totally identified 216 target mRNA. Finally, 5 lncRNAs, 31 miRNAs and 216 mRNAs were included to construct ceRNA by Cytoscape software 3.7.1 (Fig. 6A). KEGG pathway and GO analysis were performed to annotate the function of 216 target mRNAs and we found that these target mRNAs were enriched in DNA-binding transcription activator activity, and DNA-binding transcription repressor activity (GO analysis) (Fig. 6B); MicroRNAs in cancer, PI3K-Akt signaling pathway, MAPK signaling pathway, p53 signaling pathway, Cell cycle and Focal adhesion (KEGG pathways) (Fig. 6C).

Furthermore, we used GSEA to predict the functional difference between high-risk group and low-risk group. The results showed that high-risk group was closely enriched in the cancer-related pathways, such as “Focal adhesion,” “ECM-receptor interaction,” and “Wnt signaling pathway.”(Fig. 6D)

Discussion

Recently, an increasing number of studies focusing on the role of lncRNAs in CRC proved that lncRNAs exert a critical oncogenic role based on its dysregulated expression and localization[18,19]. Additionally, as the most abundant posttranscriptional modification in eukaryotic non-coding RNAs (ncRNAs), m^6A has a huge effect on its stability and transport[20–22]. Previous studies have shown that m^6A "writers" and "erasers" could adjust the levels of m^6A modification in mRNAs and ncRNAs to regulate binding sites to m^6A "reader" proteins. Different m^6A "reader" proteins recognize and bind to methylated ncRNAs to realize different functions. For instance, the m^6A mark increases the stability of lncRNA FAM225A, which promotes nasopharyngeal carcinoma progression by acting as ceRNA to sponge miR-590-3p/miR-1275[23]. IGF2BP2 recognizes and binds to m^6A-modified circRNA NSUN2 and increases its export to the cytoplasm[24]. Overexpression METTL3 can significantly increase lncRNA RP11 nuclear localization in CRC cells[25]. Thus, in consideration of the crucial role of lncRNAs and m^6A RNA modification in COAD, these researches call our attention to investigate the gene profile of m^6A-related lncRNAs and molecular mechanisms involved in COAD, explore whether m^6A-related lncRNAs could serve as ideal biomarkers for COAD prognosis and participate in COAD initiation and progression.

In our study, a total of 437 samples, 385 patients with COAD, 24 m^6A-related regulators and 3910 lncRNAs were included to exploit the specific role of m^6A-related lncRNAs in COAD. 36 candidate lncRNAs that were highly correlated with OS of COAD were identified. Then, 11 of 36 m^6A-related prognostic lncRNAs were used to establish a prognostic signature with the LASSO method in the training group. Kaplan-Meier analysis showed that the OS of patients with low risk scores was longer than those of patients with high risk scores. Additionally, the result of ROC curve analysis indicated that the 11-lncRNAs signature could serve as a highly specific and sensitive prognostic survival model in COAD. Moreover, the results were further validated in the test groups and the combined group. This signature can be used as an independent prognostic factor for COAD, suggesting that these 11 lncRNAs may be vital m^6A-related lncRNAs and significant prognostic factors for patients with COAD. Furthermore, this m^6A-related lncRNA prognostic model could serve as a prognostic indicator for OS in subgroups of patients with different clinical characteristics, especially age, gender, pathological stage, T stage, M0 stage and N stage.

To provide a comprehensive analysis of m^6A-related lncRNAs, a ceRNA network consisting of 5 lncRNAs, 31 miRNAs and 216 mRNAs was constructed and differentially expressed genes between the high-risk group and low-risk group were identified for viewing the latent functions of m^6A-related lncRNAs. With the 5 key m^6A-related lncRNAs, LINC00174 and ZEB1-AS1 have been preliminarily studied so far in COAD. LncRNA ZEB1-AS1 acts as a sponge of miR-141-3p/miR-205/miR-455-3p/miR-181a-5p/miR-101 to promote COAD malignant progression[25]. LINC00174 was overexpressed in CRC tissues and cells, and promotes CRC progression via maintaining TAZ overexpression by sponging miR-1910-3p[31]. KEGG pathway and GO analysis showed that 216 target mRNAs were enriched in several biological processes and pathways associated with the occurrence and progression of COAD[32,33], including "PI3K-Akt

signaling pathway," "MAPK signaling pathway," "p53 signaling pathway," "Cell cycle," "Focal adhesion" and so on. Genes in high-risk group and low-risk group were functionally annotated using GSEA. Genes in high-risk group were also enriched in cancer-related pathway,such as "Focal adhesion," "ECM-receptor interaction," and "Wnt signaling pathway."

However, a few limitations and shortcomings in our study should be acknowledged. First, it's beneficial to perform external validation by other genes and clinical datasets. Second, the results of this study is purely computational, and further experimental studies are necessary.

Conclusion

In this study, we constructed a prognostic signature comprising 11 m^6A-related lncRNAs in COAD, which had significant value in predicting the OS of patients with COAD. Additionally, biological processes and pathways associated with m^6A-related lncRNAs were identified, which improved our understanding of the role of m^6A-related lncRNAs in the occurrence and progression of COAD. This work also provides important evidence towards the development of predictive biomarkers and molecular targeted therapy for COAD.

Declarations

Availability of Data and Materials

Publicly available datasets were analyzed in this study, which can be found in the Cancer Genome Atlas (TCGA) database.

Ethics Approval and Consent to Participate

Not applicable

Consent for Publication

All the authors consented to the publication of this research.

Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

YH, PL, FL and YW conceived and designed the study. YH, YY, CH, XJ, and ZY organized the database and performed statistical analyses. YH and YW wrote the first draft of the manuscript. YY, CH and XJ prepared the figures and tables and were involved in manuscript writing. KZ, FL, PL and YW revised and proofread the manuscript. All authors contributed to manuscript revision and approved the submitted version.

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Disclosure of Potential Conflicts of Interest

The author reports no conflicts of interest in this work.

Research involving Human Participants and/or Animals

Not applicable

Informed Consent

Not applicable

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Figures

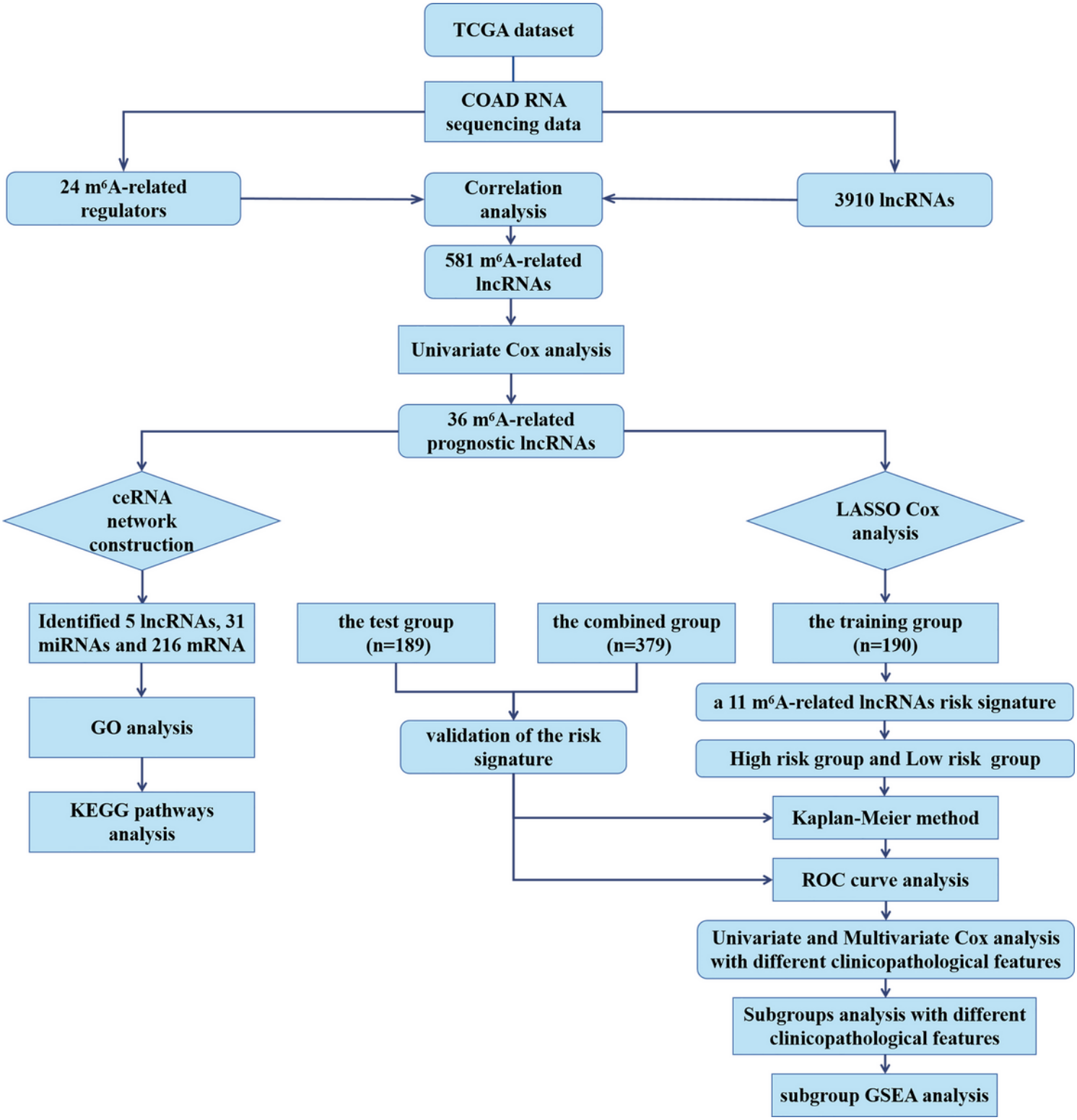

Figure 1

The flow chart of the study design and analysis.

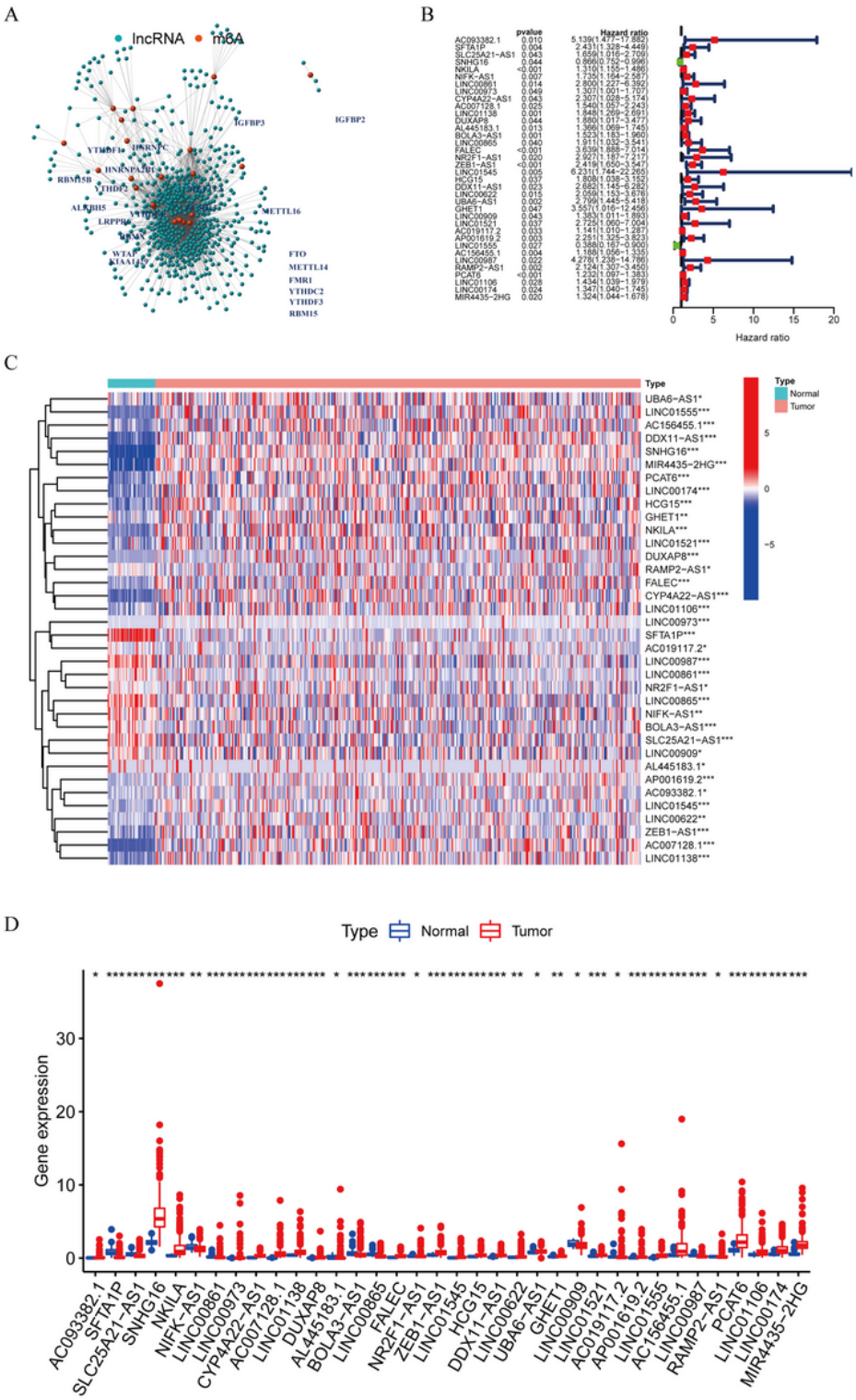

Figure 2

Identification of m6A-related prognostic lncRNAs in COAD patients. (A) The network of the 24 m6A-related regulators and 581 m6A-related lncRNAs. (B) The Hazard ratio (HR) 95% confidence interval (CI) of 36 m6A-related lncRNAs estimated by univariate Cox regression. (C, D) The expression of 36 prognostic m6A-related lncRNAs in TCGA database between the tumor group and the normal group.

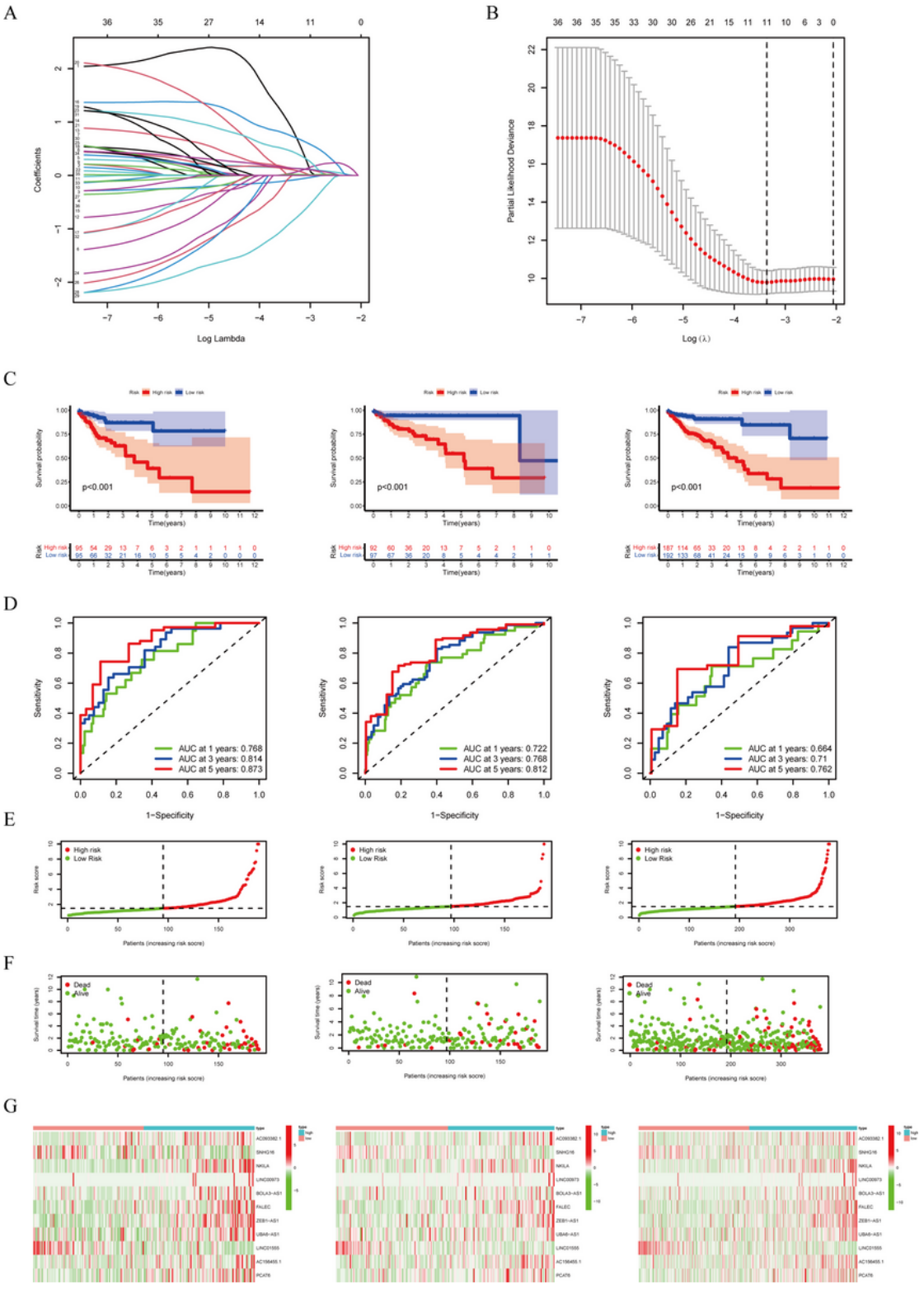

Figure 3

Construction and Verification of the m6A-Related lncRNAs Prognostic Signature. (A) The point with the smallest cross verification error corresponds to the number of factors included in the LASSO regression model. (B) The lines of different colors represent the trajectory of the correlation coefficient of different factors in the model with the increase of Log Lamda. (C) Kaplan-Meier analysis of patients in the high-risk group and low-risk group in the training group, the test group and the combined group. (D) ROC

analysis of 1-,3- and 5-year in the training group, the test group and the combined group. (E) Distribution of patients with different risk scores in the training group, the test group and the combined group. (F) OS status of patients with different risk scores in the training group, the test group and the combined group. (G) Heatmap of the prognostic signature scores in the training group, the test group and the combined group.

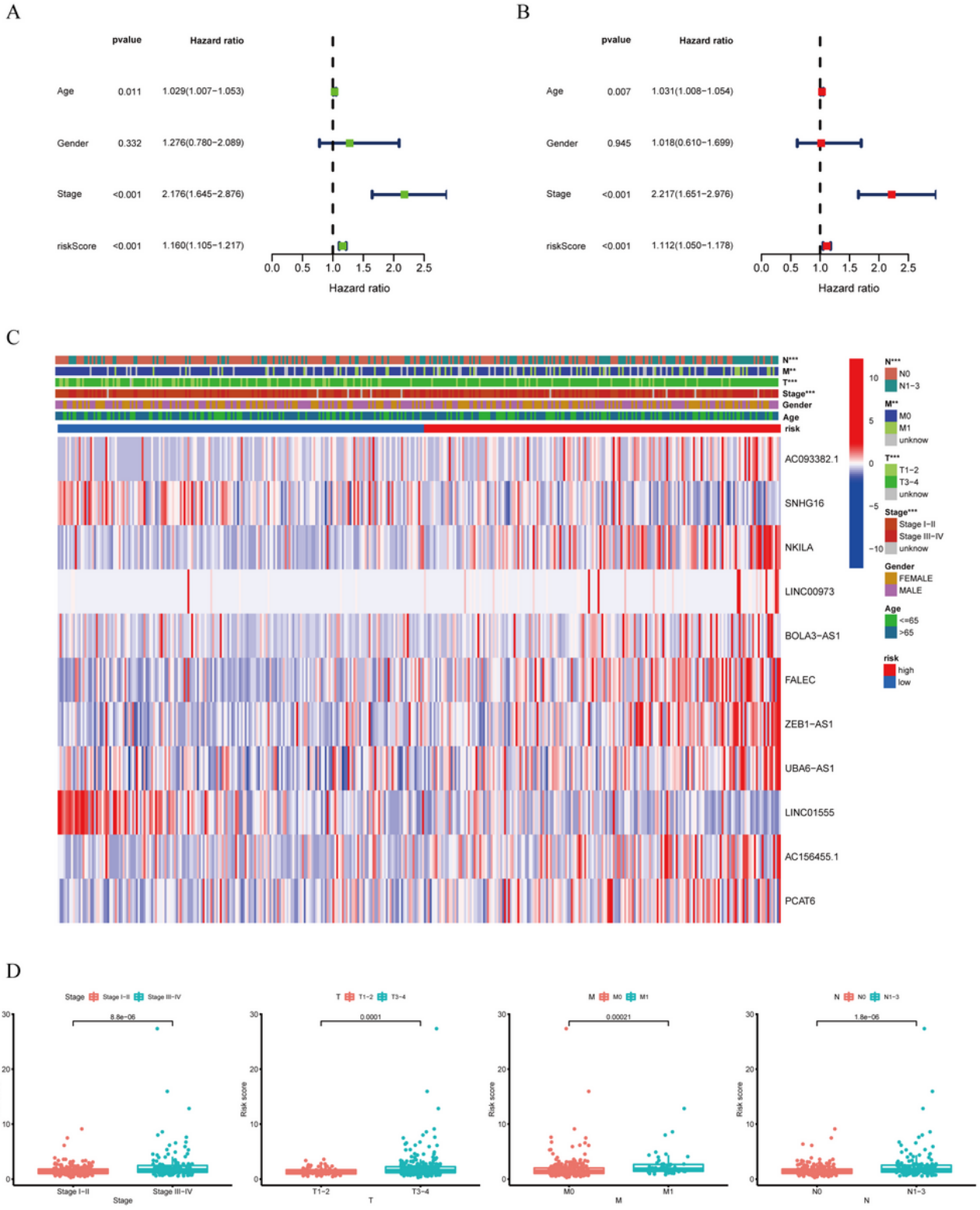

Figure 4

Relationship between the risk score and clinicopathological characteristics. (A) Univariate Cox regression analysis of the association between clinicopathological factors (including risk score) and overall survival of patients in the training group, the test group and the combined group. (B) Multivariate Cox regression analysis of the association between clinicopathological factors (including risk score) and overall survival of patients in the training group, the test group and the combined group. (C) The heatmap showed the clinicopathological characteristics in high-risk group and low-risk group. The distribution of clinicopathological characteristics was compared between the high-risk group and low-risk groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (D) Pathological stage, T stage, M stage and N stage were significantly different in high-risk and low-risk group.

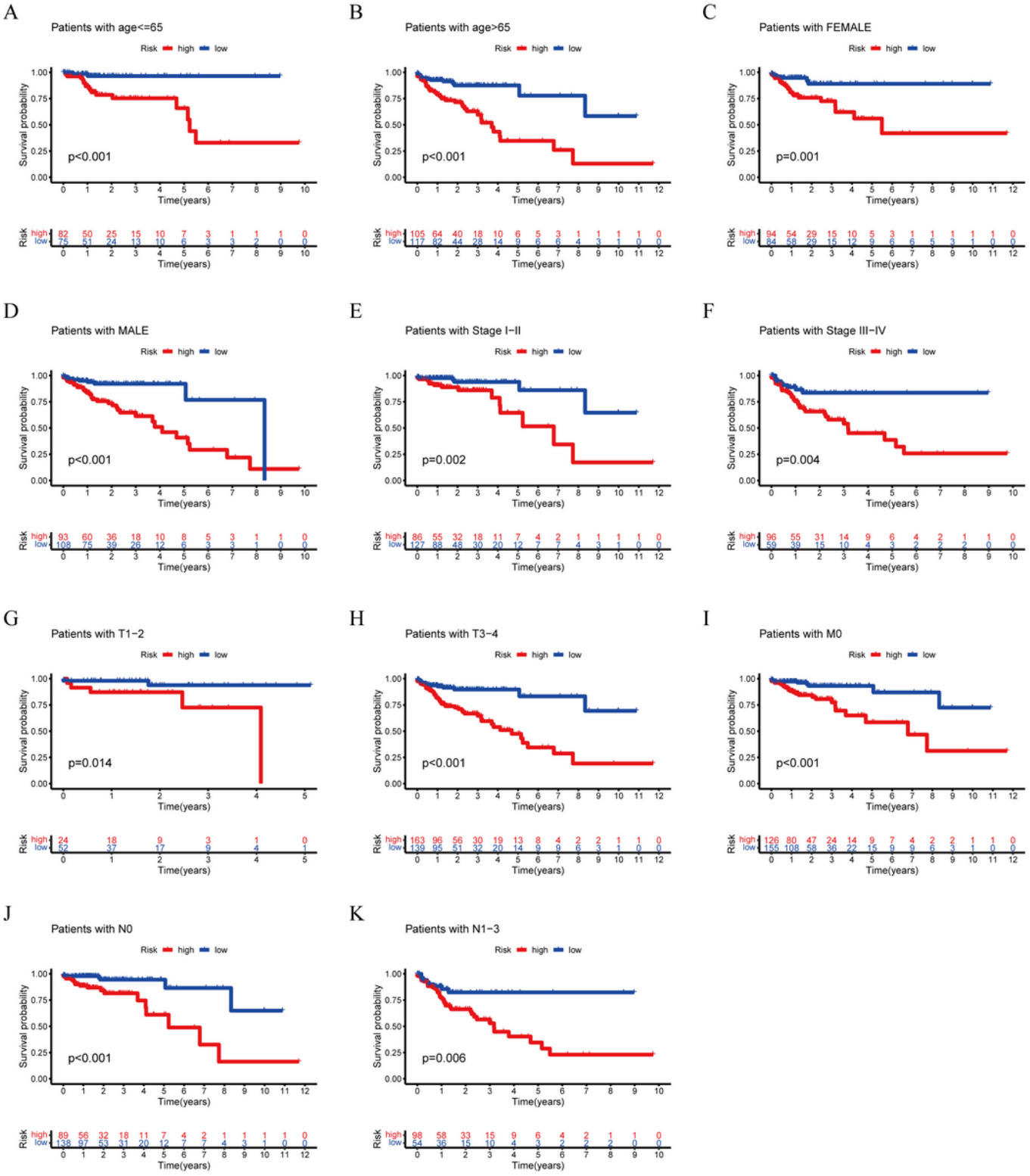

Figure 5

Subgroup Analysis with Different Clinicopathological Features in COAD. (A)Age ≤ 65. (B)Age > 65. (C)Female. (D)Male. (E)stage I-II. (F)stage III-IV. (G)T1-2 stage. (H)T3-4 stage. (I)M0 stage. (J)N0 stage. (K)N1-3 stage.

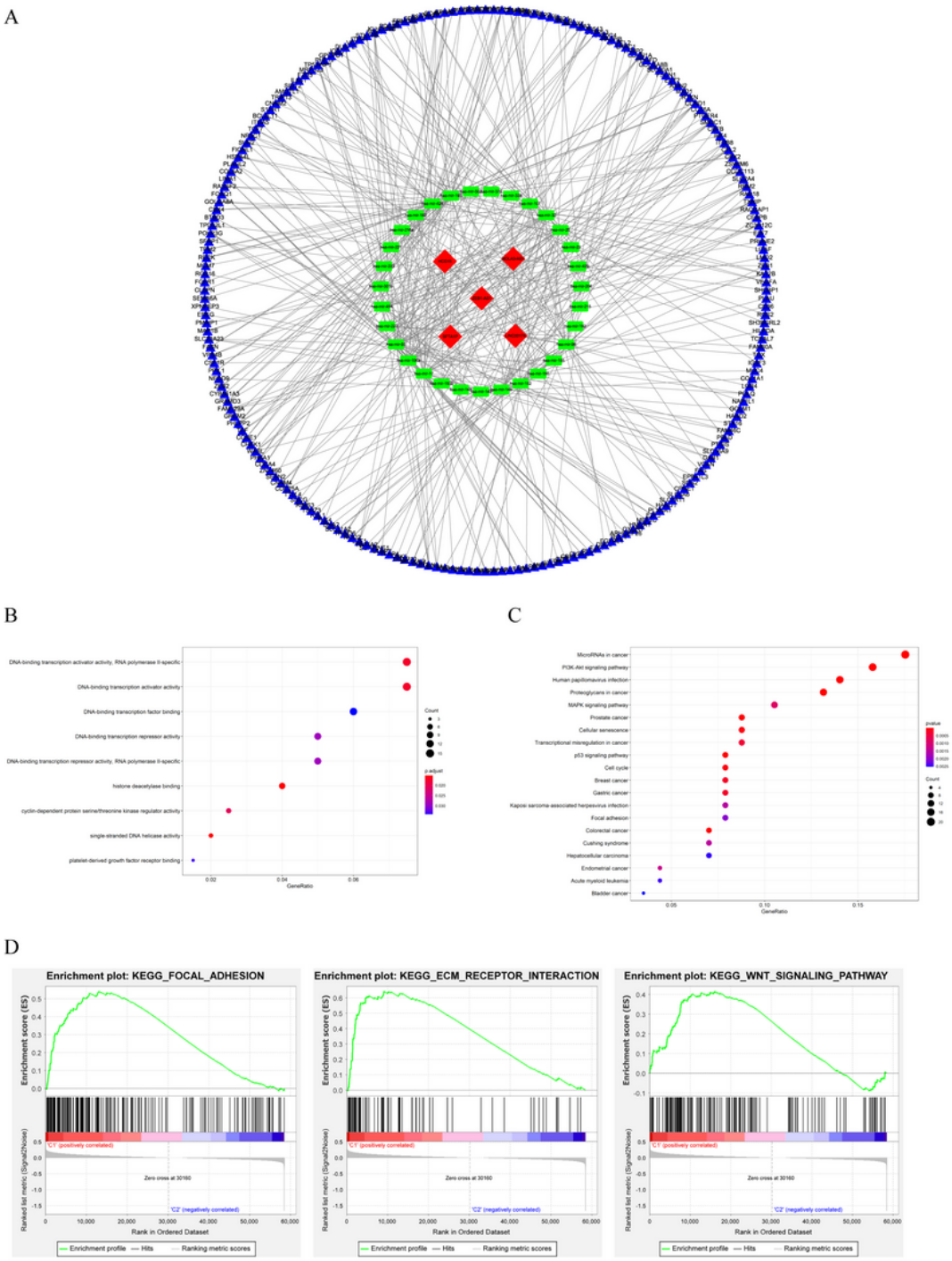

Figure 6

The ceRNA network construction and biological function analysis. (A)The ceRNA network of 5 lncRNAs (red) and their target miRNAs (green) and mRNAs (blue).(B) GO analysis in 216 target mRNAs. (C) KEGG pathways analysis in 216 target mRNAs. (D) The high-risk groupwas associated with “Focal adhesion,” “ECM-receptor interaction,” and “Wnt signaling pathway.”