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Investigating the prognostic value of m6A methylation-related genes in renal cell carcinoma patients

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Abstract. – OBJECTIVE: The objective of this research was to explore the importance of N6-methyladenosine (m6A) methylation-associated genes concerning the clinical outcome of patients with renal cell carcinoma (RCC) by employing the Cancer Genome Atlas (TCGA) database along with various bioinformatics methodologies.

MATERIALS AND METHODS: The transcriptome and clinical data of RCC patients were obtained from the TCGA database. We identified the differential expression of 13 genes and selected potential predictive genes for further analysis of their prognostic values.

RESULTS: Ten genes (*YTHDC2, FTO, YTHDF2, METTL3, KIAA1429, ZC3H13, METTL14, ALKBH5, WTAP,* and *RBM15*) exhibited altered expression levels in RCC. Subgroup analysis based on m6A methylation-related gene expression levels revealed no significant differences in survival rates, but significant differences were observed in grade, T stage, and gender. Five potential predictors (*FTO, RBM15, YTHDC2, ZC3H13b,* and *ALKBH5*) demonstrated independent predictive value. Multivariate analysis selected two regulators (*METTL14* and *METTL3*), and based on these, prognostic signals for RCC were constructed, independent of potential confounding factors. The model clearly distinguished between samples with good and poor prognoses.

CONCLUSIONS: The expression levels of m6A methylation-related genes in RCC patients were found to differ and were associated with survival rates and prognosis. These findings suggest that m6A methylation-related genes could serve as prognostic indicators and promising therapeutic targets for RCC patients.

Key Words:
m6A, Renal cell carcinoma, Regulators, Prognosis, TCGA.

Introduction

Renal cell carcinoma (RCC) is a primary adenocarcinoma that originates from the renal tubular epithelial cells[1], accounting for approximately 3% of all cancer types[2]. This kidney malignancy is considered among the most immunogenic cancers[3-5] and displays heterogeneity in renal tubular epithelial cells[6]. In fact, over the last 20 years, RCC incidence has risen by roughly 2% annually. With respect to gender, RCC is the tenth most frequently occurring cancer in women and the sixth in men[7], exhibiting a female-to-male ratio of 1:1.5. The highest RCC incidence is observed in individuals aged 60 to 70 years[8], making it a principal contributor to cancer-related fatalities worldwide[6].

Over the past 50 years, the diagnosis of RCC has risen due to the emergence of "modern" risk factors and advancements in imaging techniques[9]. While surgical interventions such as partial nephrectomy or radical nephrectomy can effectively treat early-stage, non-metastatic RCC, there remains a lack of reliable biomarkers strongly associated with RCC[10]. Consequently, treating advanced RCC poses significant challenges. Presently, there are no specific tumor markers for RCC diagnosis other than imaging examinations, and the clinical use of renal tumor-puncture biopsy is limited due to the risk of needle-path metastasis[11]. Therefore, identifying and developing valuable biomarkers to enhance the prognosis of RCC patients is of paramount importance, shifting the focus of clinical research on RCC from diagnosis and biomarker discovery to exploring novel therapeutic targets.

Epigenetics plays a crucial role in life sciences, with RNA modification being the most abundant type of epigenetic alteration[12]. Natural RNA molecules contain a variety of chemically modified nucleosides, and N6 methyladenosine (m6A) is one of the most prevalent post-transcriptional modifications in RNA, accounting for over 50% of total RNA methylation[13,14]. Consequently, m6A has garnered increasing attention[15]. Disruptions in m6A can lead to abnormal RNA expression, which may contribute to disease development[16].

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m6A is implicated in the pathogenesis and progression of numerous diseases[17-19]. For instance, the m6A methyltransferase *METTL3* has been identified as a poor prognostic biomarker in hepatocellular carcinoma[20], and the m6A-binding protein *YTHDF2* is closely associated with gastric cancer[21]. *FTO* is related to poor bladder cancer prognosis, and its elevated expression significantly increases bladder cancer risk. *FTO* knockout suppresses bladder cancer cell proliferation, while *FTO* overexpression promotes it through the *FTO/Mir-576/CDK6* pathway[22]. Miao et al[23] highlighted the functional importance of *KIAA1429* as a potential treatment target and prognostic indicator for gastric cancer. *YTHDC2* has been found[24] to inhibit tumorigenesis in non-small cell lung cancer (NSCLC), indicating its potential as a promising therapeutic target for NSCLC. Furthermore, knocking down the *METTL14* gene significantly increases tumorigenicity and metastasis of colorectal cancer cells *in vivo* while promoting their proliferation and invasion *in vitro*[25].

In our investigation, we gathered epidemiological information from 530 RCC cases by utilizing the Cancer Genome Atlas (TCGA) tumor genome database. We examined the connection between m6A methylation-associated genes and RCC development, identifying critical regulatory elements linked to overall survival (OS) in RCC patients. Based on these regulatory components, we established prognostic markers for RCC and evaluated the impact of m6A regulatory elements on RCC incidence, progression, and prognosis.

Materials and Methods

Study Population

We acquired transcriptome data along with relevant clinical information for RCC patients from the TCGA database (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga), which contained mRNA expression data of 530 tumor samples and 69 normal tissues. Additionally, the database provided clinical details such as gender, age, clinical stage, grade, and TNM stage of the 530 RCC patients (Table I). The accession number for these datasets is 20200711_180040.

Bioinformatic Analysis

We analyzed the differential expression of 13 m6A methylation-associated genes (*METTL3, METTL14, WTAP, KIAA1429, RBM15, ZC3H13, YTHDC1, YTHDC2, YTHDF1, YTHDF2, HNRNPC, FTO,* and *ALKBH5*) in RCC and normal samples using R software (Version 3.6.3, Auckland, New Zealand). Subsequently, we generated a heatmap for these 13 genes and conducted Pearson's correlation analyses to determine the inter-gene correlations.

Construction of Protein-Protein Interaction (PPI) Network

We collected numerous PPIs from the STRING database (https://cn.string-db.org/), which comprises experimental and bioinformatics prediction data. Differentially expressed genes were imported into STRING, with "Homo sapiens" as species. High-confidence PPI data (confidence score > 0.7) were saved in TSV format, and Cytoscape software was employed to construct and analyze the protein-protein interaction network. Utilizing Cytoscape's default layout algorithm, we generated a graphical network with nodes representing proteins and edges representing interactions. Network parameters such as degree, clustering coefficient, and shortest path length were computed using Cytoscape's built-in tools, while ClusterONE and cytoHubba plugins were employed for protein complex prediction and hub proteins identification, respectively.

Table I. Clinicopathological characteristics of RCC patients from TCGA database.

	RCC patients (N = 530)	
Characteristics	**NO**	**%**
Age		
≤ 60 (Median)	264	49.81
≥ 60 (Median)	266	50.19
Gender		
Female	189	35.66
Male	341	64.34
Pathologic stage		
I	267	50.38
II	55	10.38
III	122	23.02
IV	83	15.66
Unknow	3	0.57
Grade		
G1	14	2.64
G2	225	42.45
G3	205	38.68
G4	78	14.72
GX	5	0.94
Unknow	3	0.57
Survival time		
Long (> 5 years)	117	22.08
Short (< 5 years)	413	77.92
OS status		
Dead	169	31.89
Alive	361	68.11

This streamlined methodology effectively revealed complex relationships among proteins and their biological significance, providing a valuable tool for studying protein functions and discovering novel biological pathways, and potential drug targets[26].

Prediction of Potential Mark Gene

We employed the 'pROC' package in R software to generate receiver operating characteristic (ROC) curves based on differentially expressed genes in RCC and normal samples. Genes with an area under the ROC curve (AUC) greater than 0.7 were screened as potential marker genes for RCC clinical prediction and diagnosis, indicating their ability to distinguish between tumor and normal tissue samples with relatively high accuracy. This approach provides an efficient methodology for identifying promising biomarker candidates in RCC and other cancer types.

Principal Component Analysis (PCA) and Subgroup Survival Analysis

Using R's ConsensusClusterPlus package, we identified different subgroups of 530 tumor samples by synthetically expressing the 13 genes. We validated the grouping results with principal component analysis (PCA) and employed the Kaplan-Meier (KM) method to create survival curves for the two subgroups.

Prognostic Value of m6A Methylation-Related Genes

The prognostic value of 13 m6A methylation-related genes for RCC patients was assessed. We carried out univariate Cox regression analysis on these genes and chose candidates with $p < 0.05$. Following this, the "glmnet" package in R software was used for LASSO regression to analyze high-dimensional data and identify the most valuable prognostic factors. Associated risks for five selected genes were calculated and based on the median expression of m6A methylation-related genes, patients were categorized into high- and low-risk groups. The KM survival method was employed to analyze the relationship between m6A-related genes and survival rate, while the log-rank test was utilized to calculate the *p*-value of the KM survival curve. To ensure the accuracy of the model, a ROC curve was constructed. Determination of RCC prognostic factors was achieved through univariate and multivariate Cox regression analyses, and a heatmap displaying the association between clinical risk factors and m6A methylation-related genes was created. This all-encompassing approach allows for the evaluation of m6A methylation-related genes' prognostic value in RCC patients, offering valuable insights for future research and clinical applications.

Prognosis Prediction Model Construction

We used multivariate Cox regression analysis to identify regulatory factors of mRNA expression significantly associated with RCC OS. These important regulators were analyzed by LASSO Cox regression to develop potential prognostic features of RCC.

Protein-Protein Docking

The methylation-related genes of m6A with high prognostic value were selected, and the protein sequences were searched using UniProt database. The protein structure was modeled through swiss-smodel (https://swissmodel.expasy.org/interactive). Then, the protein structure was optimized with Schrodinger software (removal of solvents and ligands, side-chain completion, and energy minimization), and protein docking was performed.

Nomogram Analysis Based on RCC Patients

The rmsr software package was used for nomogram analysis, and the factors significantly correlated with the OS of RCC patients were fitted into multivariate analysis. This ability to predict nomograms was evaluated using a calibration plot of AUC[27].

Results

Expression of m6A Methylation-Related Genes in RCC

We built a heatmap of 13 m6A methylation-related genes to gain insights into their expression in RCC. Ten genes (*METTL3, YTHDF2, YTHDC2, FTO, KIAA1429, ZC3H13, METTL14, ALKBH5, WTAP,* and *RBM15*) exhibited significant alterations in tumor samples compared to adjacent normal tissue according to TCGA (Figure 1A). These genes had varying expression levels between normal and tumor tissues (Figure 1B). Pearson's correlation analysis revealed that some genes had positive correlations, while others showed no correlation (Figure 1C). The highest correlation coefficient was 0.66 between *YTHDC1* and *METTL14.*

ROC Analysis

The specificity and sensitivity of potential genes were evaluated through ROC analysis, which established their diagnostic value (Table II). Genes

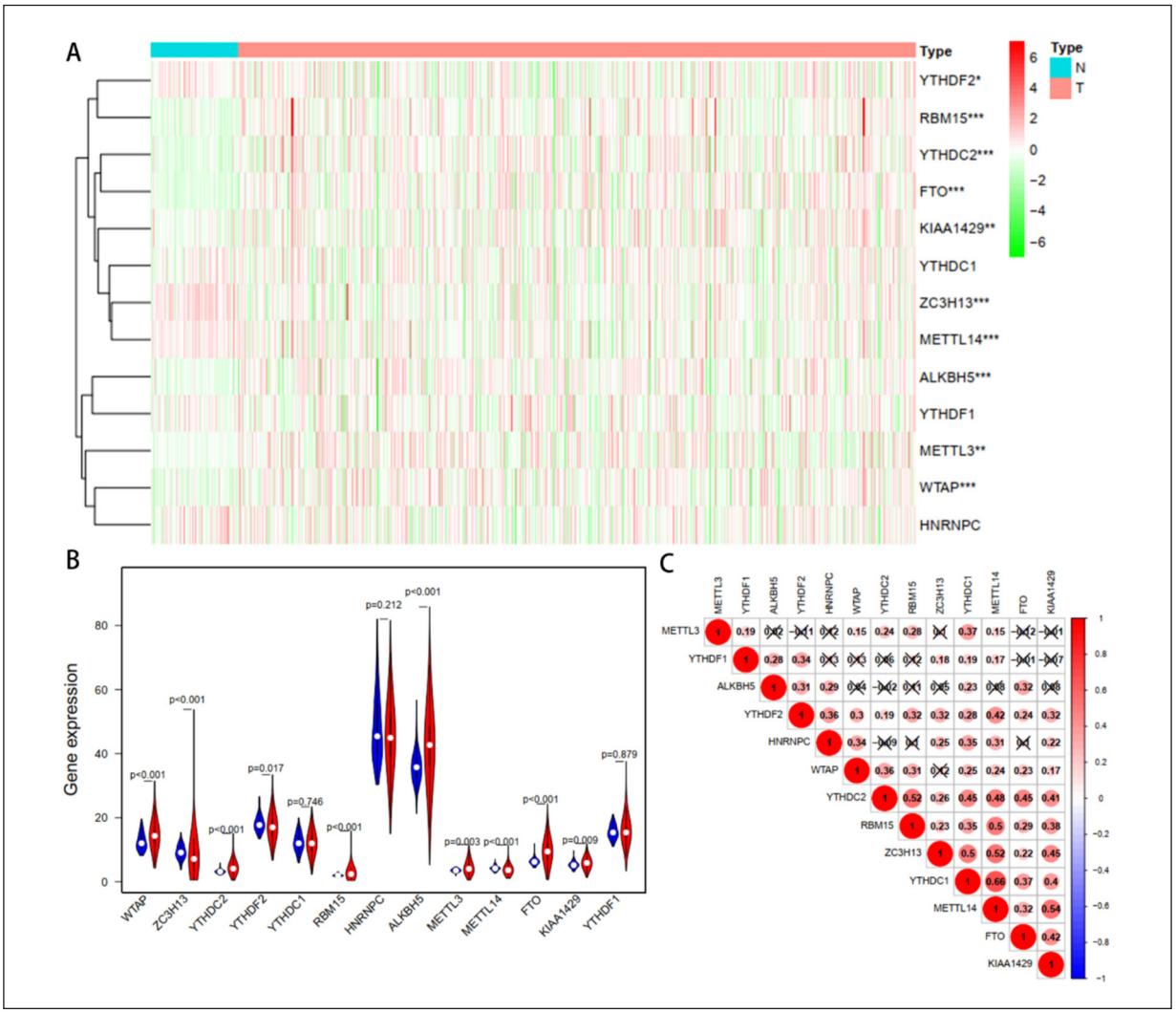

Figure 1. Expression, correlation, and prognostic information of m6A methylation-related genes. **A**, Heatmaps of m6A methylation-related genes expressed in tumors and adjacent normal tissue. (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$). **B**, DEGs in tumors and adjacent normal tissue. **C**, Correlation matrix of selected genes. Negative correlation: blue. Positive correlation: red.

Table II. ROC curve analysis of m6A methylation-related genes.

Gene	Sensitivity	Specificity	AUC
FTO	0.942	0.699	0.790
HNRNPC	0.29	0.829	0.546
KIAA1429	0.783	0.498	0.591
METTL3	0.986	0.427	0.612
METTL14	0.652	0.643	0.650
YTHDF1	0.971	0.13	0.506
YTHDF2	0.957	0.293	0.588
ZC3H13	0.855	0.513	0.740
RBM15	0.841	0.528	0.706
WTAP	0.638	0.664	0.671
YTHDC1	0.667	0.412	0.512
YTHDC2	0.870	0.598	0.741
ALKBH5	0.870	0.620	0.735

exhibiting an AUC exceeding 0.7 were regarded as possessing high sensitivity and specificity, suggesting strong predictive capabilities. This criterion was fulfilled by five genes (*FTO, ZC3H13, RBM15, YTHDC2,* and *ALKBH5*), which were identified as the most pertinent potential genes for RCC clinical prediction and diagnosis (Figure 2).

Consistent Clustering of m6A RNA Methylation Regulators Identified two RCC Clusters

Two RCC clusters were identified through unbiased grouping of 530 RCC patients based on the expression profile of m6A RNA methylation regulators using consistent clustering. PCA was employed to compare transcriptional profiles between the two clusters, but minimal OS differences between cluster 1 and cluster 2 were observed (Figure 3B). Despite significant differences in grade, T stage, and gender between the clusters, OS differences remained minimal (Figure 3C).

PPI Network LASSO Model Construction

The PPI network consisted of 10 nodes and 39 edges. The average node degree was 7.8. The local clustering coefficient was 0.91 (Figures 3C, 4, Table III). Univariate Cox regression analysis and LASSO Cox regression modeling identified seven prognostic factors for RCC (*ZC3H13, YTHDF2, YTHDC1, METTL3, METTL14, FTO,* and *KIAA1429*) (Figure 5A-C). Based on the median expression of these seven genes, patients were classified into high-risk and low-risk groups, with the low-risk group exhibiting a better prognosis (Figure 5D). ROC curves compared the prognostic efficiency of risk factors, resulting in an AUC of 0.705 (Figure 5E), indicating that m6A methylation-related genes could serve as RCC prognostic biomarkers.

Prognostic Significance of m6A Methylation-Associated Genes

Univariate analysis revealed that factors including T stage, N stage, M stage, clinical stage, grade, age, and m6A methylation-related gene risk score significantly influenced patients' prognosis ($p < 0.05$). Gender, however, did not correlate with RCC prognosis ($p > 0.05$) (Figure 6A). Multiple regression analysis identified age and m6A methylation-related gene risk score as independent prognostic factors for RCC ($p < 0.05$) (Figure 6B). Figure 6C demonstrates that the protective gene *METTL14* tended to be upregulated in low-risk patients, while *METTL3* was more likely to be overexpressed in high-risk patients. T stage, M stage, clinical stage, grade, and gender correlated with risk level, whereas N stage, age, and risk level showed no significant differences ($p > 0.05$).

Interaction between METTL3 and METTL14

The primary forces acting between *METTL3* and *METTL14* were hydrogen bonds and salt bridges. In-depth information was provided in Table IV and Figure 7. The binding score of -993.486 suggested that *METTL3* and *METTL14* formed a strong bond.

Nomogram Development and Validation

A nomogram was established to predict 1-, 3-, and 5-year OS probabilities for RCC patients, utilizing significant factors such as age and risk score from multivariate Cox regression analysis (Figure 8A). The AUC values for 1-, 3-, and 5-year OS predictions were 0.706, 0.688, and 0.757, respectively (Figure 8B-D), demonstrating the nomogram's effectiveness in predicting RCC OS. These findings suggest that the risk score could augment epidemiological traits to improve RCC prognosis evaluation.

Table III. Information of different expressed m6A methylation-related genes.

Rank	Name	Degree
1	*ZC3H13*	5
2	*YTHDC2*	6
3	*YTHDF2*	7
4	*FTO*	7
5	*ALKBH5*	8
6	*KIAA1429*	9
7	*METTL3*	9
8	*WTAP*	9
9	*RBM15*	9
10	*METTL14*	9

Table IV. Bonding and binding sites between *METTL3* and *METTL14*.

Bonding	*METTL3*	*METTL14*
hydrogen bond	ASP 242	CYS500
	ARG298	GLU438
	ASN477	LYS420
	ASP312	LYS420
	ARG255	GLU454
	ASP312	GLY479
	ARG255	ASP501
	ARG245	ASP501
salt bridge	ASP 242	CYS500

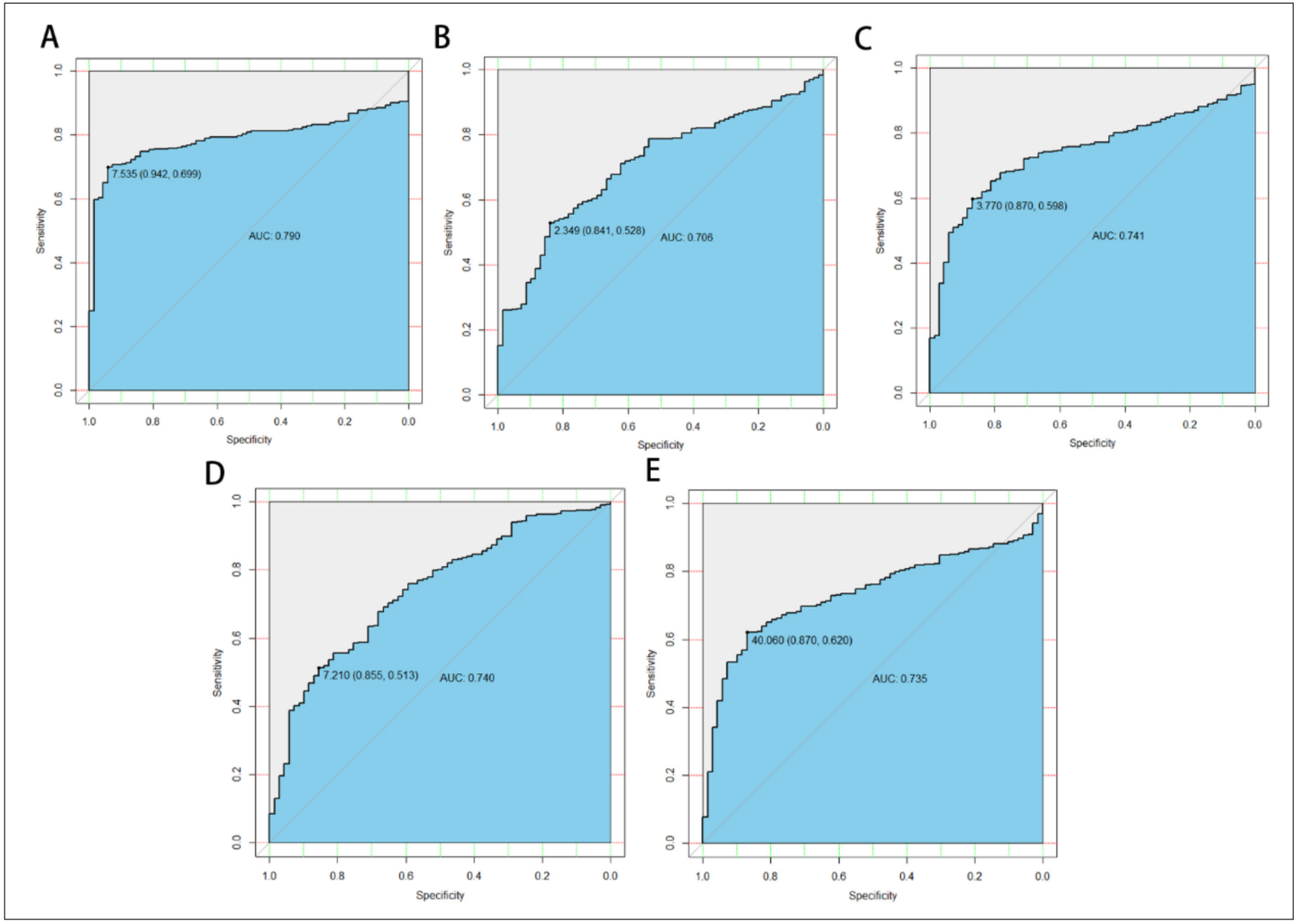

Figure 2. ROC curve of 5 marker genes (AUC > 0.7) screened.

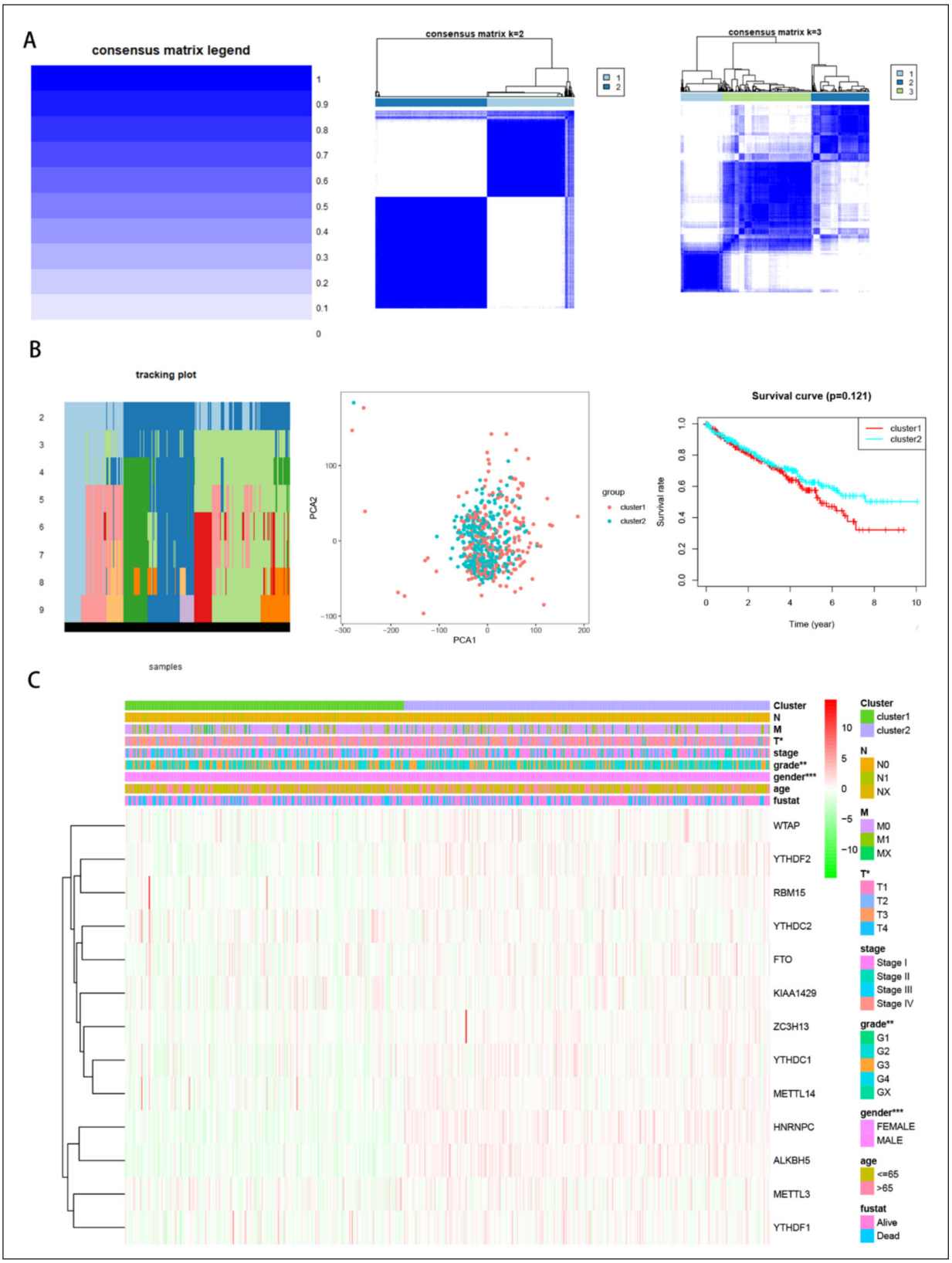

Figure 3. Clinic pathological features and OS of OC in the cluster 1-9 subgroups. **A**, Consensus clustering matrix for k = 2. **B**, Kaplan-Meier OS of 530 cases. **C**, Heatmap and clinic pathologic features of the three clusters defined by the m6A RNA methylation regulators' consensus expression.

Discussion

m6A methylation is a prevalent RNA modification occurring at the nitrogen atom of the adenine's 6th position[28]. Research[29-31] has indicated that m6A methylation is closely tied to tumor pathogenesis, with the expression level of m6A regulatory genes potentially directly impacting tumor pathological processes. This modification plays an important role in regulating cell behaviors[32]. In recent years, following the discovery of the first m6A demethylase (*FTO*), m6A modification has garnered increased attention and in-depth study[33].

Analogous to histone modification and DNA methylation, writer enzymes can add m6A RNA modification, while eraser enzymes can remove it. Nonetheless, in various tumors, distinct functions may be exhibited by the same methylation regulator[34]. The connection between m6A and renal cell carcinoma (RCC) patients is not yet well understood. Recent studies[35] suggest that bioinformatics analysis can offer numerous potential functions of novel genes in cancer research. Consequently, the potential of commonly employed m6A regulatory factors for evaluating the prognosis of RCC patients was investigated through bioinformatics analysis.

Transcriptome expression data and corresponding clinical information for RCC patients were acquired from the TCGA database, from which expression data for 13 m6A methylation regulators were extracted for further analysis. The relationship between RCC clinical prognosis and m6A regulatory genes was scrutinized using Cox regression analysis, which uncovered an association between *METTL14* and *METTL3* with RCC prognosis. Patients with overexpression of *METTL14* and low expression of *METTL3* tended to have better outcomes, implying a strong connection between *METTL14*, *METTL3*, and RCC development.

A prognostic risk model containing seven m6A methylation regulatory factors was constructed based on multivariate analysis. The model's effectiveness and accuracy were validated using Kaplan-Meier, LASSO regression, and ROC curve analysis. In conjunction with RCC patient clinical data, factors impacting prognosis were analyzed and identified. The results demonstrated that high-risk model scores and patient age were independent risk factors affecting RCC prognosis.

Differential expression analysis revealed 10 m6A regulatory factors with significant differences in expression between adjacent normal samples and tumor samples. Six m6A methylation-related genes were upregulated, and four were downregulated in RCC patients. Five genes (*FTO*, *ZC3H13, RBM15, YTHDC2,* and *ALKBH5*) were identified as the most relevant potential genes in RCC patients, suggesting these m6A regulators may influence RCC initiation and warrant further analysis[36].

Our research indicated that m6A methylation-associated genes were expressed in tumor tissues, playing a crucial role in predicting RCC prognosis. Additionally, the PPI network demonstrated strong associations between m6A methylation-associated genes, highlighting their cooperative role in cancer development. PCA

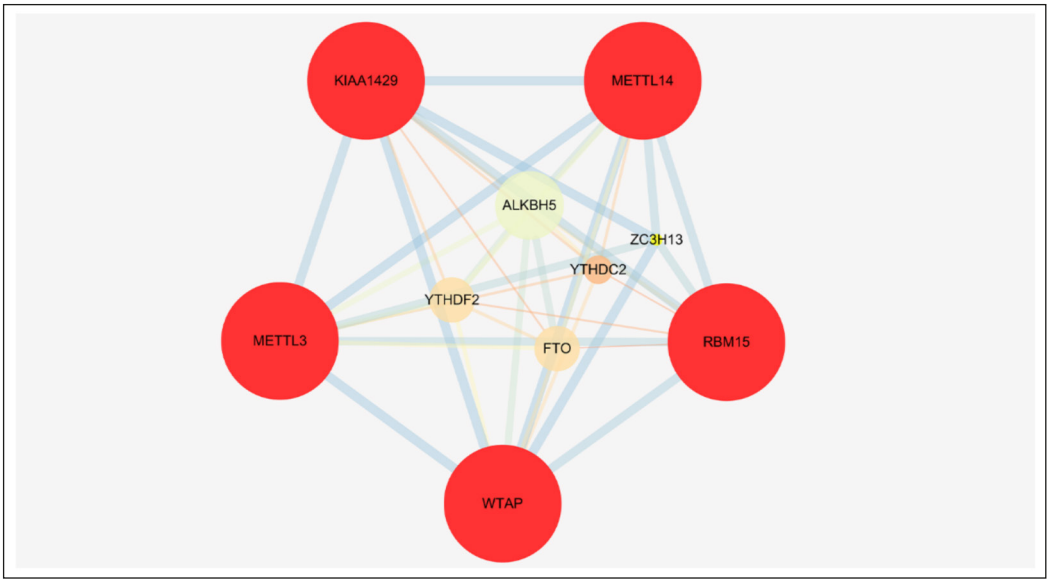

Figure 4. PPI network of m6A methylation-related genes.

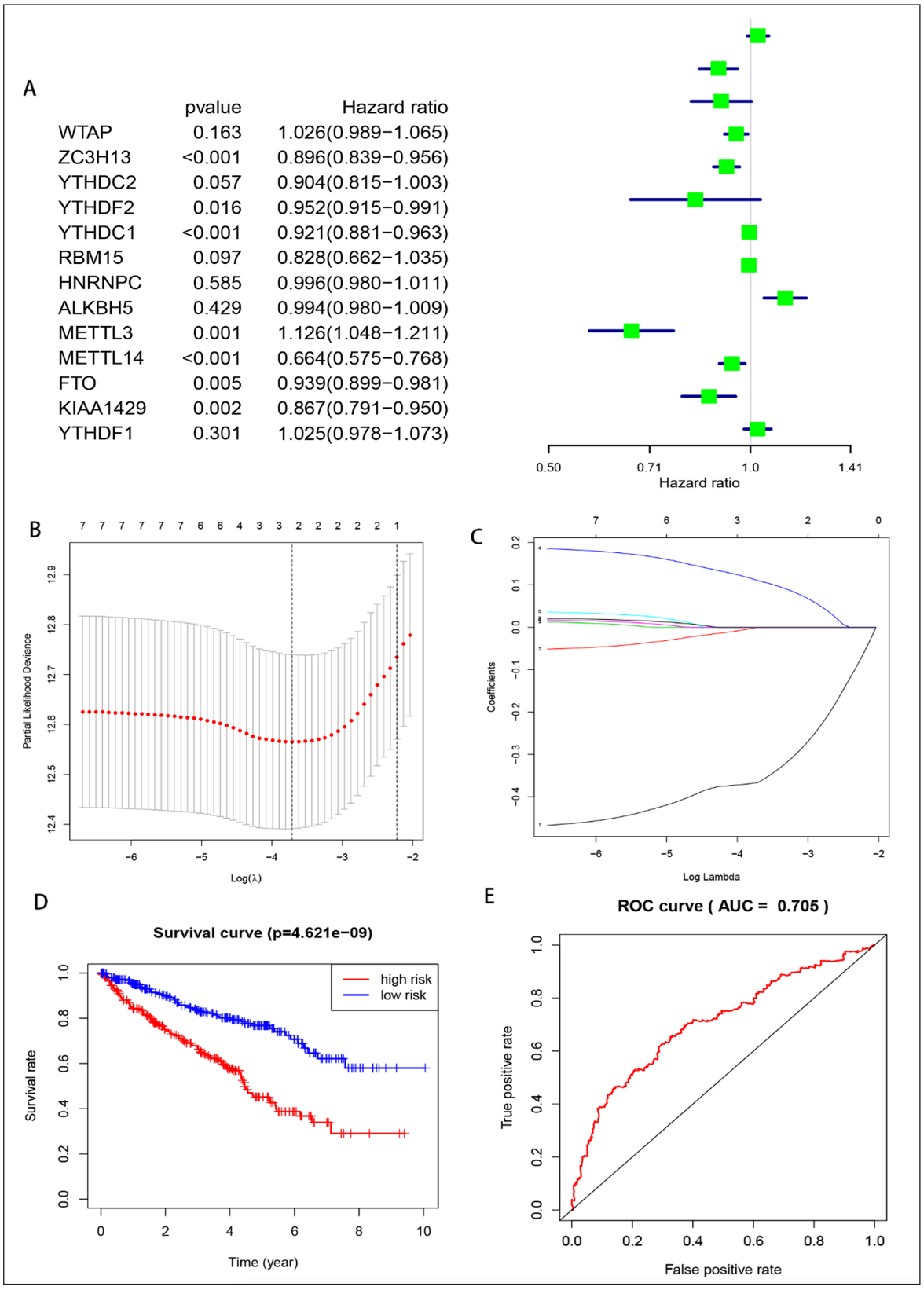

Figure 5. Gene selection and survival analysis. **A**, Forest plots of survival-associated m6A methylation-related genes. **B**, Partial likelihood deviance *vs.* log (λ) was drawn using LASSO Cox regression model. **C**, Coefficients of selected features were shown by lambda parameter. **D**, Kaplan-Meier survival plots of the two groups. **E**, ROC curves of the survival model in RCC (AUC = 0.705).

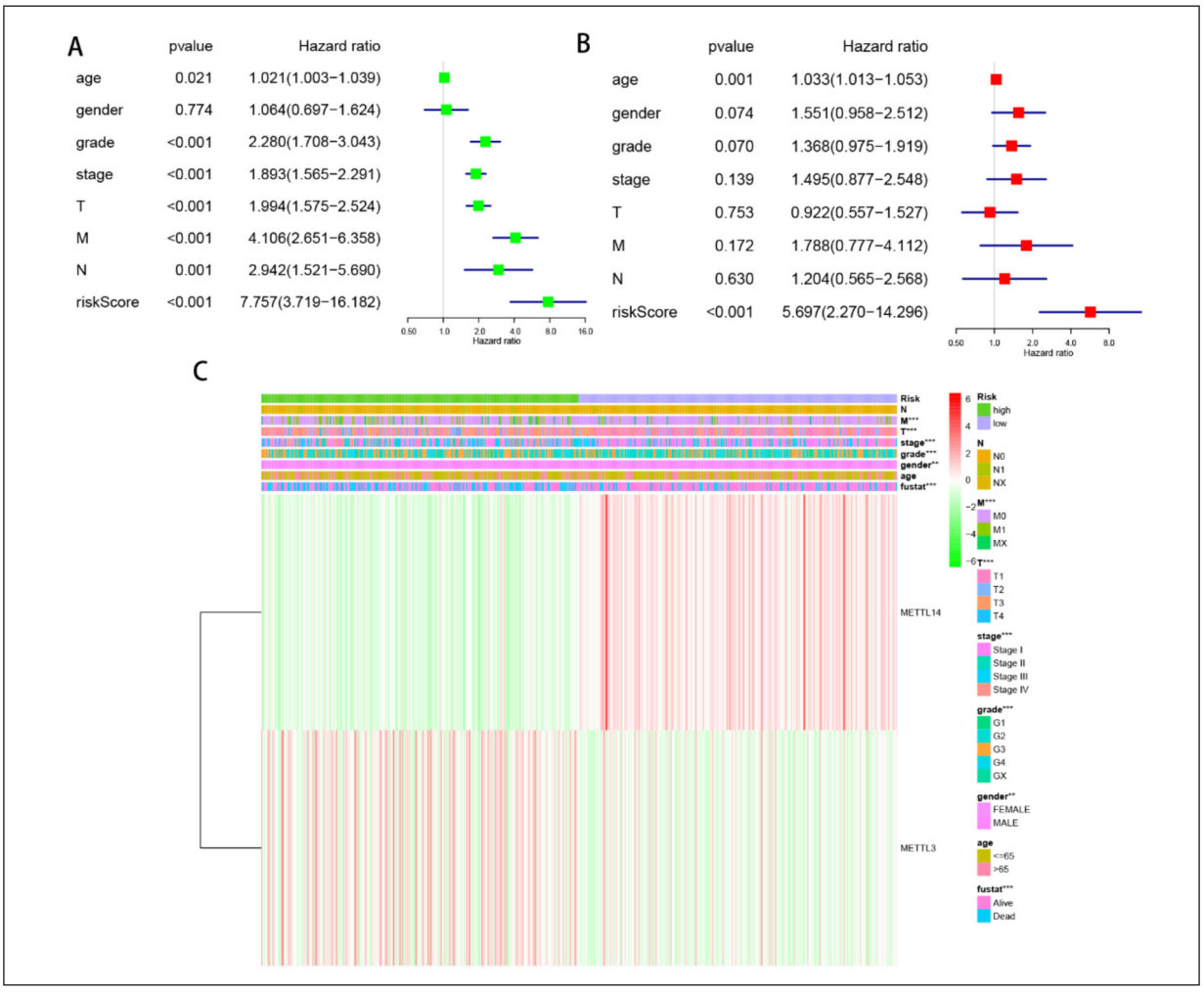

Figure 6. Forest plot and heatmap of m 6 A methylation-related genes and clinical risk factors. **A**, Forest plot of univariate Cox regression analysis in RCC. **B**, Forest plot of multivariate Cox regression analysis in RCC. **C**, Heatmap of m6A methylation-related genes and clinical risk factors. (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$).

exhibited partial separation between subgroup 1 and subgroup 2, with clinical characteristics correlating to the comprehensive expression level of m6A methylation-related genes.

Utilizing the LASSO algorithm, all independent variables were simultaneously analyzed to identify the most influential ones[37], proving to be more accurate than traditional regression methods. LASSO analysis identified 7 of the 13 genes (*ZC3H13, YTHDF2, YTHDC1, METTL3, METTL14, FTO,* and *KIAA1429*) as prognostic factors for RCC. The predictive ability of m6A methylation-related genes for RCC prognosis was evaluated through ROC curve analysis, which demonstrated the involvement of m6A methylation-related genes in the survival of RCC patients. Risk scores associated with methylation-related genes have the potential to act as powerful biomarkers for the survival of RCC patients, with *METTL14* serving as a protective gene and high *METTL3* expression indicating a poor prognosis.

A multicomponent methyltransferase complex, composed of *METTL3*, *METTL14*, and WTAP, adds a methyl group to substrate RNA[38]. As a vital component of the m6A methyltransferase complex, *METTL3* plays crucial roles in numerous biological processes, including spermatogenesis, embryogenesis, and neurodevelopment[39]. Serving as a catalyst in the methyltransferase complex, *METTL3* primarily transfers a methyl group to its substrate using S-adenosylmethionine (SAM) as a methyl donor. This dynamic mRNA modification is regulated by methyltransferases and demethylases[40]. *METTL3* can regulate the translation of oncogenes, thus playing a significant role in various tumors[41].

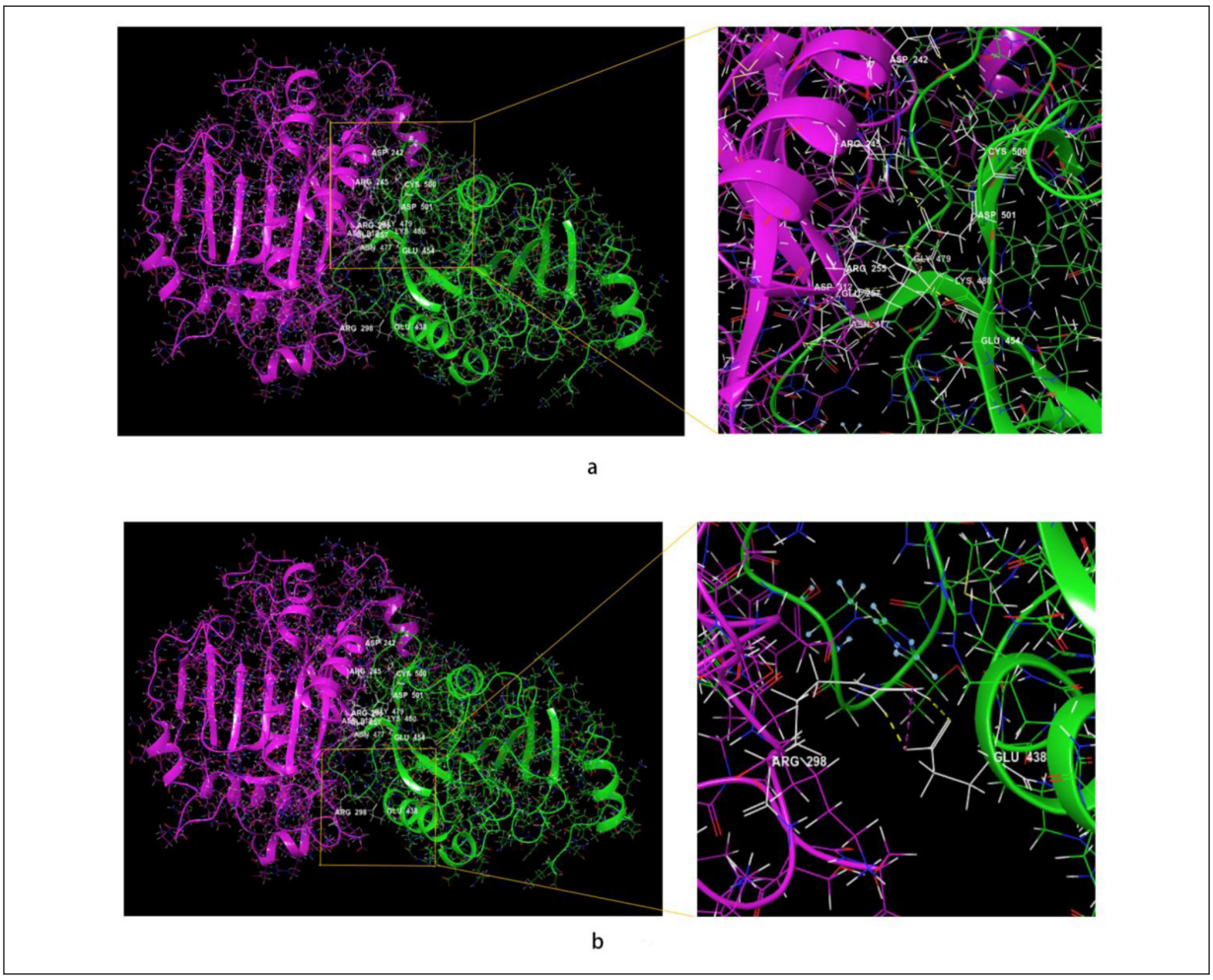

Figure 7. The docking diagram of *METTL3* and *METTL14*.The overall picture of docking and a partially enlarged view. The purple protein is *METTL3* and the green protein is *METTL14*.

Chen et al[42]. reported that *METTL3* is increased in human hepatocellular carcinoma and predicts poor prognosis. Furthermore, it has been observed that *METTL3* is notably upregulated in both bladder cancer tissues and associated cell lines. In their research, Cheng et al[43] uncovered that *METTL3* plays a role in fostering the proliferation of bladder cancer cell lines, specifically 5637 and SV-HUC-1. An investigation by Han et al[44] implies that *METTL3* might have a part in bladder cancer development by interacting with the microprocessor protein *DGCR8*, as well as actively regulating the PRI-mir221/222 process, which is reliant on the m6A mechanism. This groundbreaking study offers the first in-depth analysis of the effects of *METTL3* on tumor growth by altering m6A modifications within noncoding RNA molecules, which could potentially shed new light on the therapeutic approaches for bladder cancer. Bi et al[45]'s study emphasized that *METTL3* fosters ovarian cancer development and offers new treatment targets.

As an m6A-related gene, *METTL14* has been found[46-48] to be associated with oncogenesis promotion in various human cancers. However, unlike *METTL3*, *METTL14* inhibits tumor growth in the majority of cancer types. Acting as a tumor-suppressor gene, *METTL14* generally prevents oncogenesis and progression in several types of cancer, including hepatocellular and colorectal cancer. In hepatocellular carcinoma, *METTL14* expression is reduced by regulating pri-mir126 expression, while *METTL3* expression lacks correlation with poor prognosis and tumor metastasis[49]. Wang et al[50]'s findings suggest that *METTL14* may be a favorable prognostic factor for ccRCC, aligning with our research. Moreover, the *Wnt, mTOR, MAPK, TGF-β,* and *ERBB* pathways might be the primary regulatory pathways for *METTL14*.

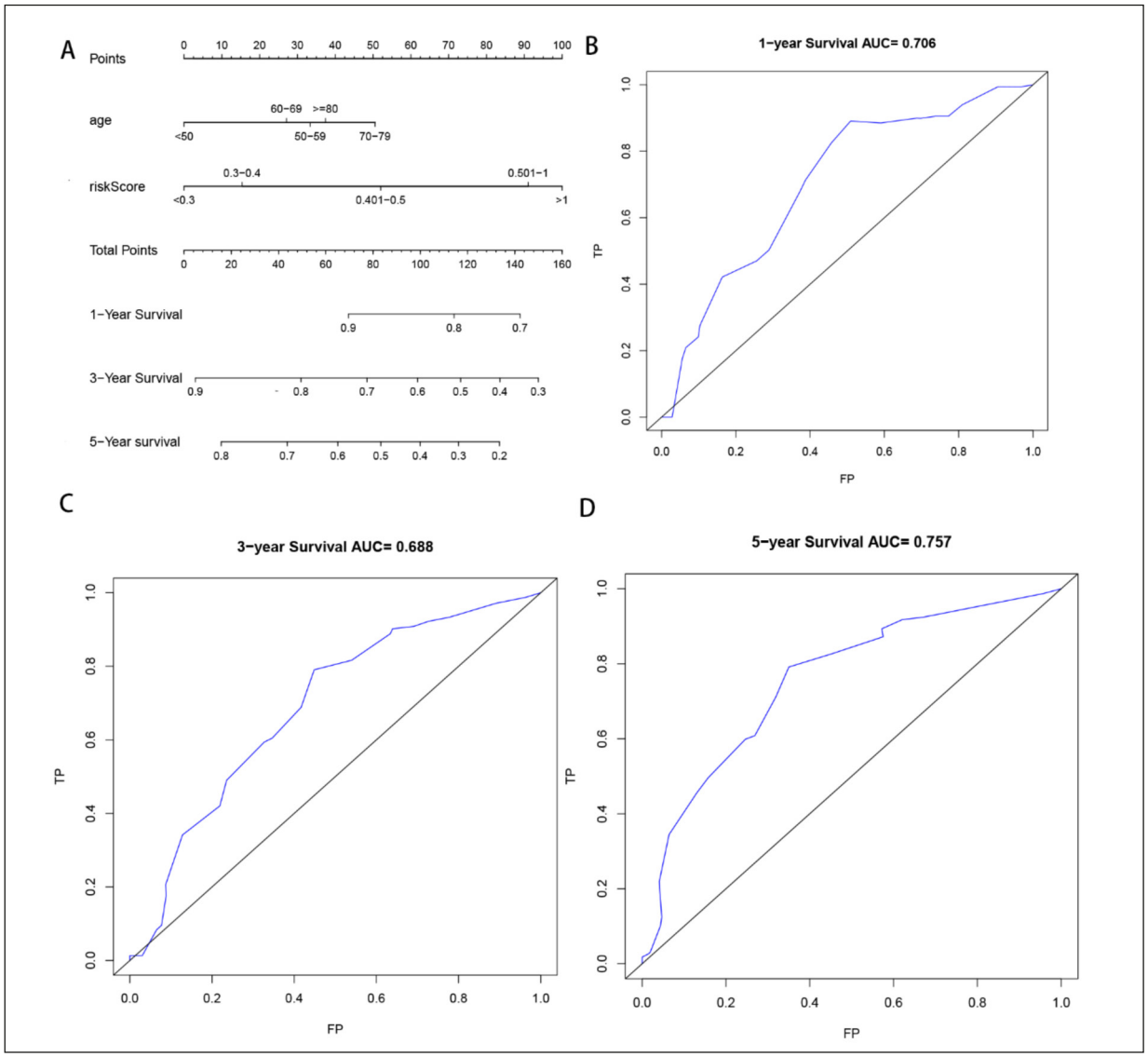

Figure 8. Construction, performance and validation of the radiomics nomogram. **A**, Nomogram to predict 3- and 5-year survival for RCC patients. Receiver operating characteristic (ROC) analysis to assess 1-year (**B**) 3-year (**C**) and 5-year (**D**) survival for RCC patients.

Limitations

This study also has certain limitations and has not been validated through experiments. Relevant clinical studies should be included for further validation in subsequent works to clarify effective biomarkers and reveal the molecular mechanisms underlying RCC progression[51].

Conclusions

The expression of m6A methylation-related genes was closely associated with the clinical characteristics of RCC, which can guide clinical individualized treatment and predict prognosis. This research presented important evidence to detect the function of m6A methylation-related genes in RCC in the future.

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Authors' Contributions
GCG designed the research, and downloaded and analyzed the data. CHW wrote the manuscript. All authors approved the manuscript.

Ethics Approval
Not applicable.

Informed Consent
Not applicable.

Conflict of Interest
The authors declare that there is no competing of interest.

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