Construction and validation of an m6A RNA methylation regulator prognostic model for early-stage clear cell renal cell carcinoma

Yushi Zhang (✉ beijingzhangyushi@126.com)

Chinese Academy of Medical Science and Peking Union Medical College

Zhan Wang

Chinese Academy of Medical Science and Peking Union Medical College

Mingxin Zhang

The Affiliated Hospital of Qingdao University

Samuel Seery

Lancaster University

Guoyang Zheng

Chinese Academy of Medical Science and Peking Union Medical College

Wenda Wang

Chinese Academy of Medical Science and Peking Union Medical College

Yang Zhao

Chinese Academy of Medical Science and Peking Union Medical College

Xu Wang

Chinese Academy of Medical Science and Peking Union Medical College

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Abstract

Background: N6-methyladenosine (m6A) is the most common type of RNA methylation and is thought to participate in various biological and pathological processes, specifically in the regulation of tumorigenesis and metastasis. However, the exact prognostic role of m6A methylation regulators in early-stage clear cell renal cell carcinoma (ccRCC) is currently unknown.

Purposes: To construct a prognostic model consisting of m6A RNA methylation regulators in early stage ccRCC and assess the reliability of the signature by proteomics. Additionally, we also explored the relationship between the prognostic model and tumor infiltrating immune cells within the tumor microenvironment.

Materials and methods: Gene mutation and RNA sequencing data of 19 m6A methylation regulators for early-stage ccRCC patients were extracted from The Cancer Genome Atlas (TCGA) database with the corresponding clinical information. Univariate and multivariate Cox regression analysis were applied to construct a prognostic model and the proteomic data was used to validate the result. The correlations with the prognostic model and tumor infiltrating immune cells were assessed using Spearman rank correlation analysis.

Results: 192 early stage ccRCC gene mutation data as well as 261 RNA sequencing data with relative clinical data were extracted from the TCGA. The overall mutation frequency of the 19 m6A RNA methylation regulators was relatively low with 4.69%. The transcriptome data showed that 11 genes were differentially expressed between cancer tissues and relatively normal tissues. Survival analysis highlighted four specific genes as having a significant influence on overall survival. An established model with four genes demonstrated the best predictability for early-stage ccRCC. After integrating clinical characteristics into the multivariate analysis, the model remained effective at predicting ccRCC prognosis. Spearman rank analysis suggested several tumor infiltrating immune cells like dendritic cells, CD4+ cells, CD8+ T cells and macrophages were significantly correlated with the model. Proteomic data analysis showed that all the genes used to construct the model were differentially expressed in paired ccRCC tissues.

Conclusion: A novel m6A methylation regulators-based prognostic signature was established and validated using a proteomic approach. In addition, the model was significantly correlated with multiple infiltrating immune cells in tumor microenvironment.

Introduction

Renal cell carcinoma is one of the most common malignant tumors in urinary system, third only to prostate cancer and bladder cancer [1]. According to the latest survey, there were approximately 76,000 new cases in the United States, including 48,780 men and 27,300 women, which accounts for between 3–5% of all malignant tumors [1; 2]. Prognosis for renal cell carcinoma (RCC) is relatively favorable when identified at an early stage with five-year survival rates for localized and locally advanced RCC ranging

from 70–90% [1] and minimally invasive surgery including robotic assisted partial nephrectomy [3] is regarded as gold standard for treatment. However, once distant metastasis takes place, the five-year survival rate decreases dramatically and can be as little as 10% [1]. It is therefore necessary to investigate all possible methods to stratify the RCC patients upon disease initiation and make more individualized follow-up strategies.

Clear cell renal cell carcinoma (ccRCC) is the most common pathological type and accounts for between 60–80% of all types [4]. With radiographic imaging becoming common place in routine diagnostics, we have also witnessed a dramatic rise in the number of small and early-stage ccRCC cases. Although other algorithms have been developed to predict the urological survival outcomes [5; 6; 7; 8; 9; 10], the Tumor, Node, Metastasis (TNM) staging system is still the most commonly used method for determining prognosis and the demand for surgical intervention. However, for early-stage RCC, the TNM staging system alone is insufficient to determine postoperative treatment and survival prognostics for its lacks of sensitivity and specificity. It remains of the utmost importance to construct and validate a prognostic model for early-stage RCC.

Recently, accumulative evidence has suggested that as the most common type of RNA modification, N6-methyladenosine (m6A) affects tumorigenesis in various types of cancers and could even hold a function in regulating the tumor immune microenvironment [11; 12; 13; 14; 15; 16]. In addition, they could also serve as diagnostic and prognostic biomarkers [17]. Thus, we combined the gene mutation data, transcriptional sequencing data of TCGA database with our proteomic data detected by high-performance liquid chromatography-mass spectrometry (UPLC-MS) to construct a prognostic model made up of m6A methylation regulators for early-stage renal cell carcinoma, and evaluated the relationship between the model and tumor infiltrating immune cells, so as to preciously stratify the ccRCC patients and provide new ideas for the inner mechanism related to renal cell carcinoma.

Materials And Methods

The data collection and analyzation of 19 m6A methylation regulators from the TCGA database

The gene mutation files and RNA sequencing data (count value) with the corresponding clinical information from ccRCC patients were downloaded from TCGA database ([https://portal.gdc.cancer.](https://portal.gdc.cancer.gov/repository)

[gov/repository](https://portal.gdc.cancer.gov/repository)). Based on the clinical data, we selected the 192 somatic mutation data and 261 RNA transcriptome with early-stage ccRCC (pT1) for inclusion. VarScan2 was used to analyze the somatic mutation data and calculate the tumor mutation burden (TMB). The result was displayed with the R packages “maftools” [18]. RNA sequencing counts in tumor tissues and adjacent relatively normal tissues were standardized and analyzed using the “Desq2” package [19].

Proteomic examination of 27 paired early-stage ccRCC tissues

To assess the reliability of the m6A methylation regulator expression across protein levels, 27 paired early-stage ccRCC tissues were then collected and analyzed by means of high-performance liquid chromatography-mass spectrometry (HPLC-MS).

This study was approved by the Institutional Review Board (IRB) of Peking Union Medical College Hospital, Chinese Academy of Medical Science and Peking Union Medical College. Written informed consent was requested as a matter of course and signed by everyone to signify approval to participate. All the patients received the partial or radical nephrectomy and the ccRCC diagnosis was determined by at least two independent specialists for pathological analysis in our center. The UPLC-MS process could be divided into the following steps:

• sample collection and preparation

A total of 27 paired ccRCC tissues, including 27 paired tumor and non-cancerous tissues (NATs), were collected for further analysis. All tissue samples were collected from the operation room and transferred directly into the -80 °C refrigerator for storage until analysis took place.

Approximately 25-120 mg of each cryopulverized renal tumor tissue or NATs were homogenized separately in an appropriate volume of lysis buffer (i.e. 2% SDS, 20mM Tris, Cocktail [1:100 dilution], DNase [1:100 dilution], RNase [1:1000 dilution]) by repeated vortexing. Protein concentration was determined using Pierce™ BCA assay protein assay kit (Thermofisher). 100 mg of protein was reduced with 20 mM dithiothreitol (DTT) for 5 mins at 95 °C and subsequently alkylated with 50 mM iodoacetamide for 45 min at room temperature in the dark. Protein digestion was carried out using a filter-aided sample preparation technique (FASP) method. Proteins were loaded onto 30-kDa filter devices (Pall, Port Washington, NY, USA). Trypsin (Trypsin Gold, mass spec grade, Promega, WI, USA) was added at an enzyme to protein ratio of 1:50, and samples were incubated at 37 °C overnight.

• ESI-LC-MS/MS for proteome library generation

The pooled peptide samples of each group were separated by high-pH RPLC columns (4.6 mm × 250 mm, C18, 3 μm; Waters, USA). Each pooled sample was loaded onto a column in buffer A1 (H₂O, pH 10). The elution gradient was between 5–30% buffer B1 (90% ACN, pH 10; flow rate, 1 mL/min) for 30 mins. Eluted peptides were collected at one fraction per minute. After lyophilisation, 30 fractions were re-suspended in 0.1% formic acid, before being concatenated into 10 fractions by combining fractions 1, 11, 21, etc.

In order to generate a spectral library, fractions from RPLC were analyzed in the DDA mode. Parameters were set as follows: the MS was recorded at 350–1500 m/z at a resolution of 60,000 m/z; the maximum injection time was 50 ms, the auto gain control (AGC) was 1e6, and the cycle time was 3 sec. MS/MS

scans were performed at a resolution of 15,000 with an isolation window of 1.6 Da and a collision energy at 32% (HCD); the AGC target was 50,000, and the maximum injection time was 30 ms.

• ESI-LC-MS/MS for proteome data-independent acquisition analysis

Digested peptides were dissolved in 0.1% formic acid and separated on an RP C18 self-packing capillary LC column (75 μm ×150 mm, 3 μm). The eluted gradient was 5–30% buffer B2 (0.1% formic acid, 99.9% ACN; flow rate, 0.3 $\mu\text{L}/\text{min}$) for 60 mins. For MS acquisition, the variable isolation window DIA method with 38 windows was developed. The specific window lists were constructed based on the DDA experiment of the pooled sample. The full scan was set at a resolution of 120,000 over the m/z range of 400 to 900, followed by DIA scans with a resolution of 30,000; the HCD collision energy was 32%, the AGC target was 1E6, and the maximal injection time was 50 ms.

• Spectral library generation

To generate a comprehensive spectral library, a pooled sample from each group was processed. DDA data were processed using Proteome Discoverer software (Thermo Scientific, Germany) and searched against the human UniProt database (<https://sparql.uniprot.org/>) appended with the iRT fusion protein sequence (Biognosys).

A maximum of two missed cleavages for trypsin were used, cysteine carbamidomethylation was set as a fixed modification, and methionine oxidation deamination and +43 on Kn (Carbamyl) were used as variable modifications. Parent and fragment ion mass tolerances were set to 10 ppm and 0.02 Da, respectively. The applied false discovery rate (FDR) cutoff was 0.01 at the protein level. The results were then imported to Spectronaut Pulsar software (Biognosys, Switzerland) to generate the spectral library.

Additionally, DIA data were imported into Spectronaut Pulsar software and searched against the human UniProt database to generate DIA library. The final library was generated by combining DDA and DIA libraries of ccRCC and controls.

• Data analysis

DIA–MS data were analyzed using Spectronaut Pulsar (Biognosys, Switzerland) with default settings. All results were filtered with a Q-value cutoff of 0.01 which corresponded to an FDR of 1%. Proteins identified in more than 50% of the samples in each group were retained for further analysis. Missing values were imputed based on the k-nearest neighbour method.

Raw proteomics data were transformed using log2 and then centralized. Wilcoxon signed rank test was implemented with the software R (version 4.1.1).

Selection of m6A RNA methylation regulators

After integrating RNA sequencing and proteomic data, 19 m6A RNA methylation regulators were included for further analysis and included: ALKBH5, CBLL1, ELAVL1, FMR1, FTO, FXR2, HNRNPA2B1, HNRNPC, METTL 14, METTL 3, RBM15, RBM15B, RBMX, RBMXL1, WTAP, YTHDC1, YTHDC2, YTHDF2 and YTHDF3.

Uni-variate survival analysis and establishment of prognostic models based on the 19 m6A methylation regulators

To assess the survival impact of every single m6A methylation regulators on early stage ccRCC, univariate survival analysis was applied. Firstly, pROC package [20] was applied to determine the optimal threshold of every single gene expression and then all the ccRCC patients were divided into two subgroups, namely high and low subgroup. Then Kaplan-Meier survival analysis with log rank test was calculated and displayed with package “survminer” [21].

Aiming to construct a comprehensive prognostic model, all 19 m6A methylation regulators were included into multivariate Cox’s regression analysis with risk scores for each sample being calculated. Prognostic risk scores for ccRCC patients were calculated using the following formula:

$$\text{Risk score} = \sum \beta_i \times \text{Exp } i$$

with i representing the number of prognostic m6A expressions.

According to the median of risk score, all samples were divided into two subgroups: high risk and low risk, and survival curves and time-dependent ROC curves were generated.

Multivariate Cox analysis and nomogram development for survival predictions

In order to assess the independence of the diagnostic model, some critical clinical parameters of the patients were included into the multivariate analysis, including gender, age, tumor side, pathological T stage, N stage and tissue grade. Furthermore, these factors were applied using the “rms” package [22] to develop a survival time nomogram.

Correlations between the prognostic model and tumor infiltrating immune cells

To assess the relationship between prognostic model and tumor purity, R package “estimate” [23] was used to calculate tumor purity, the scores of stromal cells and the infiltration level of immune cells. To further explore the immune cell composition within the tumor microenvironment, Tumor Immune Estimation Resource (TIMER, <http://timer.cistrome.org/>) was used to compute the proportion of six kinds of immune cells. Spearman correlation analysis was used to investigate correlations between risk score and immune infiltrating cells. In addition, associations between clinical characteristics, tumor purity and risk scores were analyzed using the standard Kruskal-Wallis test.

Statistical Analysis

All the data analysis and the figures were completed by R (version 4.1.1) as well as corresponding R packages illustrated in the Methods. The non-parametric Spearman rank correlation analysis was used to calculate the correlation coefficient. All the tests were two-sided, and $p < 0.05$ was regarded as significance.

Results

Baseline information

Clinical characteristics for 261 TCGA participants and 27 paired proteomic samples have been provided in Table 1. At the end of the following-up period, 44 of 261 patients had died while none of the 27 patients had experienced tumor recurrence or died. The number of men in this cohort was slight greater than women with a ratio of 158:103 for TCGA and 20:7 in the proteomic sample. In addition, the average age within the two datasets was similar at 59 years for the TCGA group and 57 years for the proteomic sample.

Table 1

The baseline information of enrolled patients from TCGA database and our cohort

Items	subtypes	TCGA (n=261)	Our paired sample (n=27)
Survival status	Alive: Dead	217: 44	27: 0
Gender	Male: Female	158: 103	20: 7
Age		59 (26-90)	57 (24-71)
Laterality	Left: Right	116: 145	10: 7
Pathological Stage	G1: G2: G3: G4	13: 155: 86: 7	4: 19: 4: 0
T stage	T1a: T1b: T1	135: 105: 21	13: 14: 0
N stage	NX: N0	159: 102	0: 27
Neoadjuvant therapy	Yes: No	7: 254	0: 27

Expression levels of 19 m6A RNA methylation regulators in genome, transcriptome and proteome

Firstly, we examined the gene mutation pattern of the 19 m6A methylation regulator genes and the result was demonstrated in Figure 1A. From the result, it's evident that the mutation frequency is relatively low with a rate of 4.69%. Among the 9 mutated samples, YTHDC2 demonstrated the highest mutation frequency at 2% and followed by WTAP with 1%. Furthermore, missense mutation is the most common mutation type with a rate of 3.6%.

Expression levels for RNA sequencing and proteomic data are presented in Figure 1B and 1C, respectively. From Figure 1B, it shows that there existed 11 differentially expressed genes, including 4 upregulated and 7 downregulated m6A regulators. From Figure 1C, according to the level of proteins, 15 aberrantly expressed m6A regulators were detected, except for FMR1, FTO, METTL14 and METTL3.

Identification of survival-related m6A methylation regulators

In order to identify survival-related m6A methylation regulators, we performed single-variate

Cox analysis and the results were provided in a forest plot (Figure 2A). From the plot, one can clearly see that the expression of FMR 1, METTL 14, RBMXL1 and YTHDF 2 were significantly associated with early-stage ccRCC survival. Interestingly, all 4 regulators were negatively associated with ccRCC overall survival having hazard ratios (HR) of less than 1. After calculating the best cut-off value of each gene with ROC curves, we plotted the survival curves for m6A regulators. The results suggested that 8 gene

expressions, namely RBMXL1, YTHDF3, FMR1, METTLE14, YTHDF2, ELAVL1, FXR2 and RBM 15 were associated with ccRCC patient survival and the top three were depicted in Figures 2B-D.

Construction of m6A methylation regulators based prognostic signature

In order to comprehensively describe the predictability of m6A methylation regulators, we included all 19 m6A regulators to construct a prognostic model. After applying multivariate Cox regression analysis, we established a risk score algorithm:

$$\text{Risk score} = (-0.54) * \text{expr (YTHDF2)} + 0.67 * \text{expr (YTHDC2)} + (-1.05) * \text{expr (FMR1)} + 1.43 * \text{expr (ELAVL1)}$$

Then, all 261 patients were classified into two subgroups according to the median risk score, namely high risk and low risk. Through survival analysis plots, we can clearly see that a higher risk score was significantly associated with poorer ccRCC survival. Please see Figure 3A for further details.

To further assess the accuracy of the prognostic model, an ROC curve was generated with an AUC was 65.46% (Figure 3B). To examine the independency of the model, we performed multivariate Cox analysis with several confirmed critical clinical characteristics and the results showed that the model remained an independent indicator of the survival risk (depicted in Figure 3C).

In addition, we also assessed the association between risk scores and clinical characteristics and the results have been provided in Figure 4A-F. This also suggests that risk scores are significantly associated with survival status. A cluster heat map was generated to show RNA expression level for all regulators included in the model (Figure 5).

Establishing a prognostic nomogram for early-stage clear cell renal cell carcinoma

To predict survival status more comprehensively and easily, a nomogram was constructed after integrating prognostic risk score and clinical information such as gender, age, laterality, pathological grade, T stage and N stage. Please see Figure 6 for further details. From the figure, it's evident that the nomogram is suitable for predicting 1-year, 2-year and 3-year survival for ccRCC patients.

Correlations between risk scores and immune cells in tumor microenvironment

Accumulating evidence suggests that m6A methylation regulators participate in the regulation of tumor microenvironment immune status during tumorigenesis. After comparing the tumor purity within different risk subgroups, we found that the higher risk group was significantly associated with higher immune score ($p < 0.001$) and lower tumor purity ($p = 0.0066$). However, the stromal score within high and low risk groups didn't show significant difference ($p = 0.68$). The result was depicted in Figure 7A-C.

To further confirm which kind of immune cells maybe associated with the prognostic model, Spearman correlation analysis was implemented to assess the association between risk scores and immune cell composition (Figure 7). The results turned out that risk scores were statistically associated with various immune cell, including dendritic cells, CD 4+ T cells, CD8+ T cells and macrophages as opposed to B cells and neutrophils.

Discussion

As the most common type of renal cancer, the prognosis of ccRCC remains poor with both a high incidence and mortality. Epigenetic modifications, especially RNA modifications have proven to play various functions in the bioprocess of tumorigenesis and therefore produce a frame of tumor biomarkers [24]. This should not be overlooked as a key element in the quest to identify elements which will enable clinicians to identify cases early and to determine those who are more likely to require swift interventions. As the most prevalent type of RNA post-translational modification, m6A has drawn an increasing attention since the identification of the first RNA demethylase in 2011, the fat mass and obesity-associated protein (FTO), revealing that the RNA methylation is a reversible process [25].

In this study, we applied multi-omics to reveal the prognostic role of m6A methylation regulators in early-stage clear cell renal cell carcinoma. As far as we know, our study is the first one to suggest that m6A could predict the prognosis for pT1 stage ccRCC patients. From the 19 m6A methylation regulators, the expression of four genes were significantly associated with overall survival for ccRCC patients, namely; FMR1, METTL14, RBMXL1 and YTHDF2. To further predict survival, a mode consisting of YTHDF2, YTHDC2, FMR1 and ELAVL1 was constructed based on Cox's regression analysis. Additionally, multivariate Cox analysis revealed that the signature was an independent predictor of ccRCC. In order to make our model more applicable, we established a nomogram that could be more easily adopted into clinical practice although this is also only an initial study. Previous researchers have found that ccRCC is an immune-rich tumor since multiple kinds of immune-infiltrating cells exist in the tumor microenvironment[15; 26; 27]. Therefore, we also assessed the relationship between the generated prognostic model and immune cells with the help of online TIMER software and "ESTIMATE" package. The results suggest that the m6A methylation prognostic model was significantly associated with dendritic cells, CD 4 + T cells, CD8 + T cells and macrophages. This may provide new evidence for the role of m6A methylation in regulating tumor immune-infiltrating microenvironment; however, of course further interdisciplinary research is required.

In our analysis and relative articles[28], the molecule YTHDF2 appears to play a critical role since this was not only associated with survival status but also made up a proportion of the prognostic signature. Additionally, protein expression for YTHDF2 between cancer and non-cancerous normal tissues remains differential in the samples examined here. As a member of m6A-binding proteins (i.e. readers), YTHDF2 possesses a YTH binding capability which may interact with m6A modifications for specific RNAs. Therefore, this ability may play a critical role in improving target mRNA translation efficiency [29].

Previous research has reported that YTHDF2 may participate in the progression of various malignant tumors. For example, Zhang et al. found that by maintaining m6A methylation of the OCT4 mRNA, YTHDF2 enhances its protein expression and therefore promotes liver cancer stem cell phenotype [30]. This appears to suggest that YTHDF2 acts as an oncogene in the progression of hepatic cell carcinoma (HCC). However conversely, other research has demonstrated that YTHDF2 is a novel tumor suppressor for HCC since it mechanistically suppresses STAT3 phosphorylation and tumor growth by degrading IL-6 family cytokine encoded mRNAs [31]. Likewise in glioma studies, YTHDF2 has been found to downregulate the expression of LXR α and HIVEP2 through m6A-dependent mRNA decay and therefore may impact on the survival of glioma patients [32]. Xie et al. found that METTL3/YTHDF2 m6A axis may directly degrade mRNAs of tumor suppressors such as SETD7 and KLF4 which can lead to carcinogenesis and progression of bladder cancer [33].

Another important molecule in the prognostic model is FMR1, which has been found to be involved in the negative regulation of mRNA translation. Recently, the FMR1 was identified as a putative m6A reader which means that it binds to m6A containing transcripts. For example, Edens et al. showed that FMRP, the protein encoded by FMR1, preferentially binds m6A-modified mRNAs and thereby promotes their nuclear export through CMR1, thus regulating neural differentiation [34; 35]. Interestingly, FMRP could also bind to m6A sites in targets and interact with YTHDF2, which is yet another 'm6A reader', in an RNA-independent manner. The results suggested that the function of FMR1 was maintaining the stability of its mRNA targets while YTHDF2 focused on the degradation of these transcripts [36].

As an m6A writer, METTL14 mainly performed its function through combining with METTL3 and forming an m6A-METTL complex (MAC). Contrary to the evidenced catalytic function of METTL3, METTL14 appears to be mainly responsible for maintaining MAC integrity and mediating RNA bind [37]. Although more recently, METTL14 has also been reported to participate in crosstalk between histone modification and RNA methylation. METTL14 thereby could recognize H3K36me3 and facilitate binding between m6A MTC and the adjacent RNA polymerase II thereby writing m6A into actively transcribed nascent RNAs [38]. Zhang et al. reported that METTL14-mediated m6A modification negatively regulates mRNA stability of bromodomain PHD finger transcription factor (BPTF), therefore facilitating the tumor metastasis and reprogramming glycolysis of renal cell carcinoma [39]. This suggests that the functions of METTL14-mediated m6A are likely to be multifaceted and demand further research.

Another reader, ELAVL1 also participated in the composition of the prognostic model. Embryonic lethal abnormal visual protein 1 (ELAVL1), known as RNA binding proteins, also called human antigen R (HuR)

has been reported to participate in various processes, including nervous system development, cellular proliferation and migration [40]. Through binding to specific RNAs, ELAVL1 could increase mRNA stability of target genes. Ronkainen et al. reported that ELAVL1 was associated with reduced RCC-specific survival and inner mechanism study revealed that the HuR protein could regulate the expression of COX-2 in RCC cells, which maybe a putative mechanism of action for the progression of RCC [41]. In addition, Danilin et al. found that knockdown of HuR in renal cell carcinoma cell lines could inhibit proliferation and induce apoptosis, the process of which was mediated by inhibiting the PI3K/Akt and MAPK oncogenic signaling pathways [42; 43]. All the evidences suggested that the molecular 'HuR' may be potential drug target for clear cell renal cell carcinoma.

As a retrospective study, it is necessary to discuss the limitations involved in this study. Firstly, the sample size for this study was relatively small which inhibits our ability to generalize findings to a broader community. We initially enrolled 261 cases with early-stage clear cell renal clear cell carcinoma from the TCGA database and all the subsequent analysis was based on this data. Even though the data is reliable the sample size remains small for high-throughput omics research. Secondly, until now, the prognosis of patients with early-stage renal clear cell carcinoma we detected was favorable. There is still no recurrence or metastasis across this cohort, which means that we are unable to verify findings from a proteomic perspective. To sum up, a larger cohort with much longer follow-up period is required. Finally, all our analysis was based on the multi-omics data. In order to explore the concrete effect of critical m6A RNA methylation regulators on ccRCC tumorigenesis, more in vitro and vivo experiments are required to validate the result in the future.

In conclusion, we established a prognostic model of early-stage clear cell renal cell carcinoma with m6A methylation regulators. The evidence-based model could predict survival for early stage ccRCC patients and is closely related to various tumor infiltrating immune cells.

Declarations

• Ethics approval and consent to participate

This study was approved by the Institutional Review Board (IRB) of Peking Union Medical College Hospital, Chinese Academy of Medical Science and Peking Union Medical College and was conducted in accordance with Declaration of Helsinki. Written informed consent was signed by every participant before the experiments.

• Consent for publication

Not applicable.

• Availability of data and materials

The data have been provided in the article. To protect the patients' privacy, other information could be only acquired with the permission of the corresponding author.

•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.

•Funding

Not applicable.

•Authors' contributions

ZW & YZ paper design, manuscript writing and revision. ZW & MZ experiments completion, manuscript writing and data collection. GZ, WW, YZ, SS & XW data collection and paper revision. All authors have read and approved the manuscript.

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Figures

Figure 1

The gene mutation pattern, transcriptome and proteome expression level of m6A methylation regulators in early stage ccRCC. (A) The gene mutation pattern of the m6A molecules in the TCGA database. (B) The RNA expression level of the m6A molecules in the TCGA database. (C) The protein expression level in our cohort. (TMB: tumor mutation burden; NS: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$).

Figure 2

Single variate COX analysis and survival analysis of all the 19 m6A methylation regulators. (A) the forest plot of single variate analysis based on every m6A methylation regulators. (B) The survival curve of RBMXL1. (C) The survival curve of YTHDF3. (D) The survival curve of FMR1.

Figure 3

The survival analysis of the m6A methylation regulators based on the prognostic model. (A) The survival curve of the prognostic model. (B) The ROC curve of the prognostic model. (C) The forest plot of multi-variate analysis with prognostic model and critical clinical parameters included.

Figure 4

The relationship between the risk score and the clinical characteristics of ccRCC.

Figure 5

The clustering heat map of the m6A methylation regulators included in the prognostic signature.

Figure 6

The prognostic nomogram based on the prognostic model and clinical characteristics.

Figure 7

The relationship between prognostic model and the immune cell compositions within the tumor microenvironment. (A-C): The relationship of immune cell scores, stromal cell scores and tumor purity within different risk groups. (D-I): The correlation between immune cell composition with the risk score of the prognostic model using the Spearman analysis.