Profiles of m^{6}A RNA methylation regulators for the prognosis of hepatocellular carcinoma

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Abstract. N6-methyladenosine (m^{6}A) RNA methylation, which is related to cancer initiation and progression, is dynamically regulated by the m^{6}A RNA methylation regulators (including 'writers', 'erasers' and 'readers'). However, the prognostic value of m^{6}A RNA methylation regulators involved in hepatocellular carcinoma (HCC) carcinogenesis and progression remains to be elucidated. The aim of the present study was to determine the prognostic score in predicting the prognosis of HCC patients based on these regulators. In The Cancer Genome Atlas, most of the 13 major m^{6}A RNA methylation regulators were found to be differentially expressed between HCC and normal samples (P<0.001). In addition, two subgroups (clusters 1/2) had also been identified by applying consensus clustering in the m^{6}A RNA methylation regulators. As compared with the cluster 1 subgroup, the cluster 2 subgroup was correlated with a poorer prognosis, as shown by the Kaplan-Meier method (P=6.197e-4). A risk signature was constructed based on these findings using six m^{6}A RNA methylation regulators, which could not only predict the clinicopathological features of HCCs, but also serve as an independent prognostic marker, as shown by Cox regression analysis (hazard ratio=1.219, 95% confidence interval: 1.143-1.299; P<0.001). Data from the International Cancer Genome Consortium were used for external validation. In addition, gene set enrichment analysis identified several pathways that m^{6}A RNA methylation regulators were closely associated with. In conclusion, the m^{6}A RNA methylation regulators are the crucial participants in the malignant progression of HCCs, which are potentially useful for prognosis stratification and therapeutic strategy development for HCC.

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Abbreviations: m^{6}A, N6-methyladenosine; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; ICGC, International Cancer Genome Consortium; NS, not significant; PCA, principal component analysis; OS, overall survival; HR, hazard ratio; LASSO, least absolute shrinkage and selection operator; ROC, receiver operating characteristic; AUC, area under the ROC curve; CI, confidence interval; KM, Kaplan-Meier; GSEA, gene set enrichment analysis

Key words: hepatocellular carcinoma, RNA methylation, prognosis, cluster analysis, gene set enrichment analysis

Introduction

Like histone and DNA, the epigenetic modification of RNA species has been extensively reported over the past few decades (1). Since the 1950s, over 100 chemical modification types have been described in RNA, particularly in rRNA and tRNA (2). Of note, any micro-event during base modification can result in potent influence on the metabolic pathways, as well as the resulting organism phenotype alterations. As a result, the abnormal alteration can result in the occurrence of abnormalities and disease initiation like tumors (3,4).

The m^{6}A modification has attracted wide attention in the field of epitranscriptomics, which is associated with the highest prevalence among transcripts (5,6). Thanks to the developments of recent technology, N6-methyladenosine (m^{6}A) modifications in mRNA have been identified (7,8). m^{6}A modification reveals an extensive, while rare, epitranscriptomic landscape, which participates in various physiological processes, including cancer (9).

There are 3 protein classes that can regulate m^{6}A modification, the 'reader' (m^{6}A-binding protein), 'eraser' (m^{6}A demethylating enzyme) and 'writer' (adenosine methyltransferase) (3,10,11). Specifically, m^{6}A modification can be subjected to reversible installment and removal by writers and erasers, separately. This process is dynamic and reversible. However, the deregulated m^{6}A modification, which is associated with abnormal expression levels or functions of the m^{6}A readers, erasers and writers, may result in cancer genesis and progression (12).

Hepatocellular carcinoma (HCC), one of the most frequently observed liver cancer types, is a severe worldwide

health problem (13,14). Nonetheless, no existing study has comprehensively analyzed the expression levels of m^{6}A RNA methylation regulators among HCCs that have various clinical and pathological features, or their role and prognosis significance in the malignant development of HCC. The present study carried out a systemic analysis on the expression levels of 13 extensively identified m^{6}A RNA regulators in HCCs, according to the RNA sequencing information extracted from The Cancer Genome Atlas (TCGA) (n=377) database. In addition, the expression profiles for all 13 m^{6}A modification regulators were provided based on various clinical and pathological characteristics. According to the present results, the expression levels of the m^{6}A RNA methylation regulators played an important role during HCC malignant development. A signature was also constructed using 6 screened m^{6}A RNA methylation regulators for HCC prognosis stratification. The constructed signature was further confirmed by the International Cancer Genome Consortium (ICGC) database.

Materials and methods

Data extraction. Data were downloaded from the TCGA database. Gene expression data and the clinical information of HCC patients (https://tcga-data.nci.nih.gov/) were downloaded using the Data Transfer Tool (provided by GDC Apps). A total of 374 tumor and 50 normal samples from 377 HCC patients were used in this study to analyze the differentially expressed m^{6}A RNA methylation regulators. Typically, the list of the 13 m^{6}A RNA methylation regulators was determined with reference to published literature (4). All data were publicly available and open-access; as a result, Ethics Committee approval was not required. Data were processed in accordance with the data access policies, as well as the TCGA Human Subject Protection system formulated by the National Institutes of Health (NIH; http://cancergenome.nih.gov/publications/publicationguidelines). The LIRI-JP project from the ICGC database was used as an independent validation cohort (n=237).

Bioinformatic analysis. First, the expression patterns of m^{6}A RNA methylation regulators were compared between tumors and normal samples, and Spearman's rank correlation coefficient was used for correlation analysis among the regulators. In addition, the interactions between m^{6}A RNA methylation regulators would be examined using the Search Tool for the Retrieval of Interacting Genes/Proteins database (http://www.string-db.org/). To investigate the function of m^{6}A RNA methylation regulators in HCCs, HCCs were clustered in various groups using the 'ConsensusClusterPlus' (http://www.bioconductor.org/). In addition, gene expression profiles of the various HCC groups were investigated using principal component analysis (PCA) as well as R package. Moreover, the c2.cp.kegg.v6.2.symbols were examined based on gene set enrichment analysis (GSEA) at 1,000 random sample permutations using JAVA procedure (http://software.broadinstitute.org/gsea/index.jsp).

Construction of a signature based on m^{6}A RNA methylation regulators. The association between each m^{6}A RNA methylation regulator and patient overall survival (OS) was calculated using the univariate Cox model. Subsequently, the thirteen m^{6}A RNA methylation regulators were screened and verified by least absolute shrinkage and selection operator (LASSO) regression using the 'glmnet' R software. Finally, the regulator-based prognostic risk score was constructed through linearly multiplying the expression level with the regression model (β) according to the following formula: Risk=β regulator$_1$ x regulator$_1$ expression + β regulator$_2$ x regulator$_2$ expression + $\cdot \cdots +$ β regulator$_n$ x regulator$_n$ expression (15,16).

Confirmation of the signature based on m^{6}A RNA methylation regulators. Patients, together with their survival information, were distributed according to risk score. Furthermore, patients were classified as high- or low-risk, according to their median risk score value. Next, survival curves were drawn according to the Kaplan-Meier method, which could predict the high or low risk of patients. Subsequently, the sensitivity and specificity of survival prediction were compared using risk score, and the time-dependent receiver operating characteristic (ROC) curves were employed to evaluate the accuracy of predicting the 5-year prognosis. In addition, one-way analysis of variance or t-test were carried out to compare risk scores among different cases stratified according to their clinical and molecular pathological features, in order to assess the signature risk score for HCC cases possessing various clinical and pathological features. Univariate and multivariate Cox proportional hazards regression analysis was then conducted to examine whether the risk was predicted independently from other clinical factors.

Statistical analysis. A two-sided P<0.05 was considered to indicate a statistically significant difference. Prism 7 (GraphPad Software Inc., La Jolla, CA, USA) and R software (version 3.4.1; R Foundation, Vienna, Austria), were employed for all analyses.

Results

Expression difference in the m^{6}A RNA methylation regulators between HCCs and normal tissues. The clinicopathological information of all patients is summarized in Table I. Considering the important biological functions of each m^{6}A RNA methylation regulator during tumorigenesis and development, the differences in all m^{6}A RNA methylation regulators between HCCs and normal samples were comprehensively examined. The expression level of each m^{6}A RNA methylation regulator is presented in heatmaps (Fig. 1A) and violin plots (Fig. 1B), which showed that the expression of most m^{6}A RNA methylation regulators was markedly upregulated in HCCs, namely ZC3H13 [not significant (NS)], METTL14 (NS), FTO (P<0.001), YTHDC2 (P<0.001), YTHDC1 (P<0.001), ALKBH5 (P=0.001), KIAA1429 (P<0.001), METTL3 (P<0.001), HNRNPC (P<0.001), RBM15 (P<0.001), YTHDF2 (P<0.001), WTAP (P<0.001) and YTHDF1 (P<0.001).

Regulator correlation and interaction. For a better understanding of interactions between these 13 m^{6}A RNA methylation regulators, the correlation (Fig. 2A) and interaction (Fig. 2B) among them was also analyzed. Clearly, ZC3H13

Table I. Baseline patient characteristics.

Characteristics	Number	Percentage
Total	377	100.0
Median follow-up, days (range)	557 (0-3,675)	
Age, years (mean ± SD)	59.5±13.5	
Sex		
Male	255	67.6
Female	122	32.4
Ethnicity		
White	235	62.3
Others	142	37.7
Grade		
I	55	14.6
II	180	47.7
III	124	32.9
IV	13	3.4
Unknown	5	1.3
Stage		
I	175	46.4
II	87	23.1
III	86	22.8
IV	5	1.3
Unknown	24	6.4
T stage		
I	185	49.1
II	95	25.2
III	81	21.5
IV	13	3.4
Unknown	3	0.8
N		
No	257	68.2
Yes	4	1.1
Unknown	116	30.8
M		
No	272	72.1
Yes	4	1.1
Unknown	101	26.8

and ALKBH5 were negatively correlated, while the other pairs were positively correlated. FTO, WTAP, YTHDC1, METTL3 and HNRNPC exhibited a significantly positive correlation with the other 12 regulators. Of note, the correlation between HNRNPC and METTL3 (0.72), YTHDC1 (0.67) and YTHDF1 (0.62), ranked top among all correlations. In Fig. 2B, the interactions between 2 regulators were supported by experimental determination (pink lines), the existing databases (blue lines), co-expression (black lines), or text mining (dark olive green lines). In addition, there was a pink line connected to neither two erasers (FTO and ALKBH5) nor two readers (YTHDF1 and YTHDF2), suggesting that more experiments should be carried out on these 4 regulators to examine their interactions with other regulators.

m^6A RNA methylation regulator cluster analysis. Based on the expression similarity of m^6A RNA methylation regulators, it appeared that k=2 was a sufficient value from the clustering stability range of k=2-10 in the TCGA datasets (Fig. 3A-L). Thereafter, patients were clustered into one of the two subgroups. Therefore, the clinical and pathological characteristics between the two subgroups classified based on k=2 (clusters 1/2) were compared (Fig. 4A). The cluster 1 subgroup was markedly correlated with late stage at diagnosis (P<0.05) and high frequency of grade III/IV (P<0.001). Furthermore, PCA was also employed for comparing transcriptional patterns between the two subgroups. Our findings indicated that these two subgroups were distinctly different (Fig. 4B). In addition, the cluster 1 subgroup had an evidently reduced OS, as compared with that in the cluster 2 subgroup (P=6.197e-4) (Fig. 4C).

Prognosis of m^6A RNA methylation regulator, as well as construction and validation of the risk signature. The prognostic value of the m^6A RNA methylation regulators in HCCs was also examined. Specifically, gene expression in TCGA datasets was analyzed using the univariate Cox regression model. According to the findings, 9/13 genes examined in this study exhibited a marked correlation with OS (P<0.05; Fig. 5). Among these 9 genes, YTHDF2, YTHDF1, METTL3, KIAA1429, HNRNPC, WTAP, YTHDC1 and RBM15 were the risk genes with a hazard ratio (HR) of >1, while ZC3H13 was the protective gene with a HR of <1.

For a more precise prediction of HCC prognosis using the m^6A RNA methylation regulators, the Cox regression algorithm LASSO was utilized (Fig. 6A and B). Six genes, including METTL3, KIAA1429, ZC3H13, YTHDF1, YTHDF2 and ALKBH5, were selected for the construction of a risk signature, according to the minimal standards. In addition, the associated coefficients were acquired based on the LASSO algorithm. Risk was formulated as follows: Risk=0.105*METTL3 expression + 0.041*KIAA1429 expression - 0.094*ZC3H13 expression + 0.025*YTHDF1 expression + 0.067*YTHDF2 expression - 0.005*ALKBH5 expression.

HCC cases obtained from TCGA datasets were classified as low- or high-risk, according to the median risk score value of 3.266, and the distinct heterogeneities with regard to OS were observed between these two subgroups, in order to examine the value of the as-constructed signature in predicting prognosis (P=1.062e-5; Fig. 6C). Furthermore, the ROC curves verified that, prognosis prediction using the risk signature could attain an area under the ROC curve (AUC) value of 0.774 (1 year), 0.732 (3 years) and 0.690 (5 years; Fig. 6D).

The risk signature showed a strong association between clinicopathological features and OS. The expression levels of 6 screened m^6A RNA methylation regulators in patients from the high- and low-risk groups within the TCGA dataset are presented in the heatmap (Fig. 7A). Clearly, differences in T stage (P<0.05), grade (P<0.001), status (P<0.05) and stage (P<0.01) were statistically significant between the two groups. Moreover, the association between risk score and every clinicopathological characteristic was examined, and it was found that differences in the risk scores among patients were associated with T stage, stage, grade, and status subgroups, but not age, gender, N stage and M stage (Fig. 7B-I).

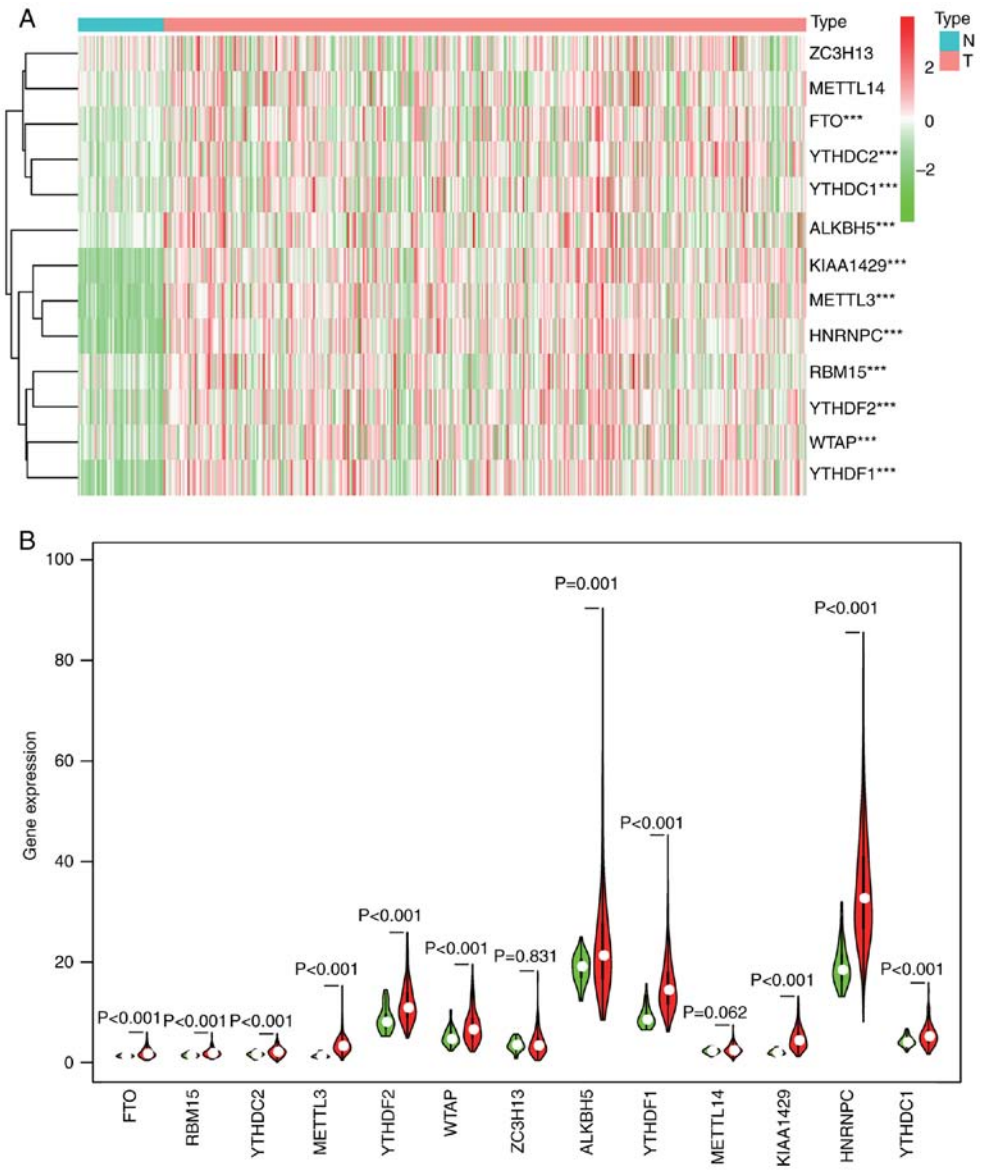

Figure 1. Differential expression of m^{6}A RNA methylation regulators between T and N tissues in hepatocellular carcinomas. (A) Heatmap. (B) Violin plot; red violins represent T and green violins N tissues. ***P<0.001 (normal vs. tumor tissues). N, normal; T, tumor.

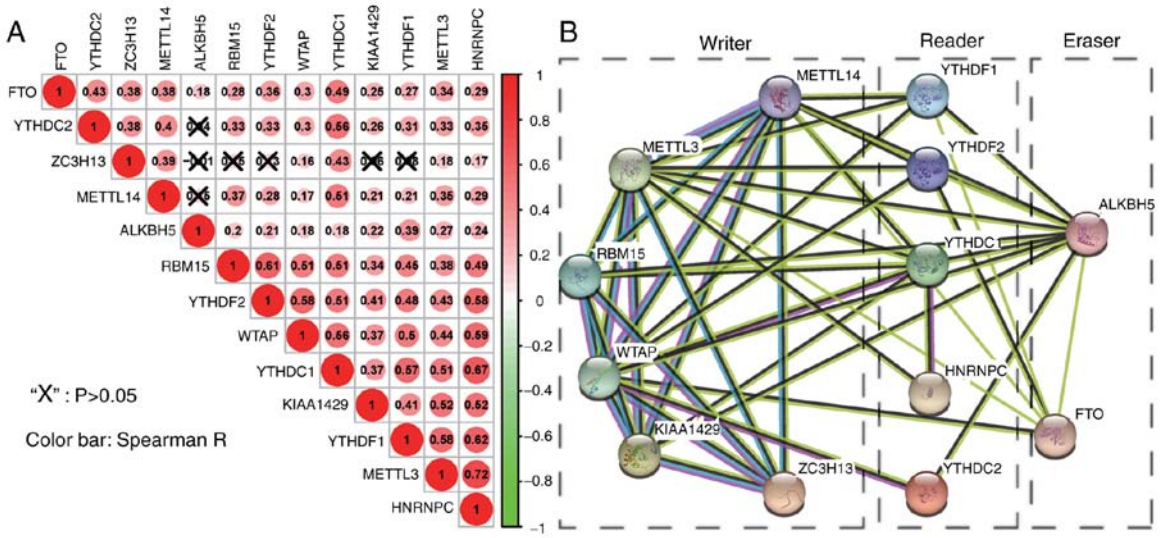

Figure 2. Correlation and interaction among m^{6}A RNA methylation regulators. (A) Spearman correlation analysis of the 13 m^{6}A modification regulators. (B) m^{6}A modification-related interactions among the 13 m^{6}A RNA methylation regulators.

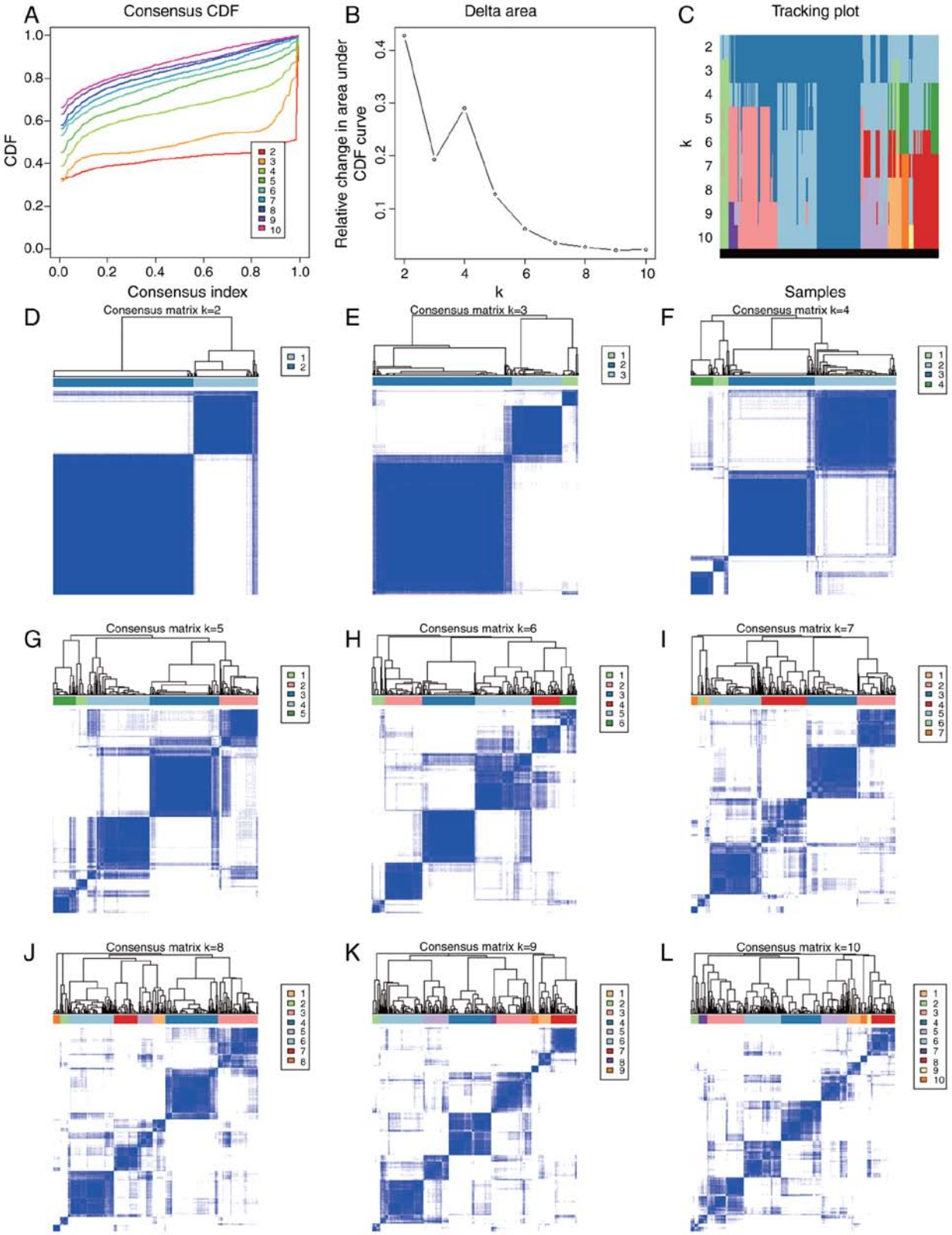

Figure 3. Two clusters of hepatocellular carcinomas with distinct m^{6}A RNA methylation regulator features were identified through consensus clustering. (A) CDF of consensus clustering at k=2-10. Numbers next to the colors represent cluster numbers. (B) Relative changes in the area under the CDF curve at k=2-10. (C) Tracking plot at k=2-10. (D-L) Consensus clustering matrix at k=2-10. (D) Two consensus clusters; (E) three consensus clusters; (F) four consensus clusters; (G) five consensus clusters; (H) six consensus clusters; (I) seven consensus clusters; (J) eight consensus clusters; (K) nine consensus clusters; and (L) ten consensus clusters. CDF, cumulative distribution function.

Meanwhile, the risk signature HR was 1.238 upon univariate Cox proportional hazards regression [95% confidence interval (CI): 1.168-1.313; P<0.001; Fig. 8A)]. In addition, the same results could be obtained by multivariate Cox proportional hazards regression analysis with adjusted clinical covariate (HR=1.219, 95% CI: 1.143-1.299; P<0.001; Fig. 8B).

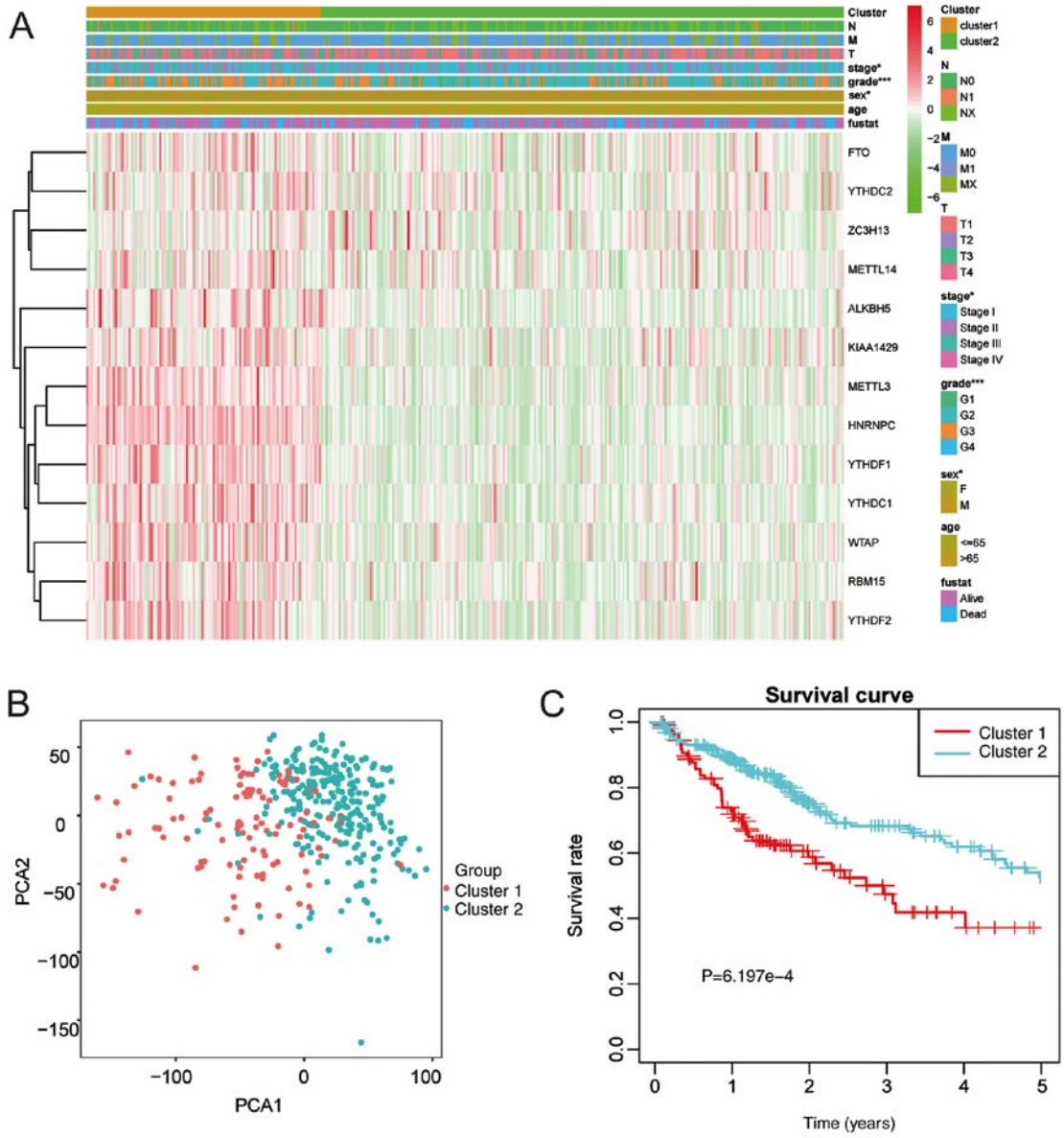

Figure 4. Different clinical and pathological characteristics and OS in the hepatocellular carcinomas between the cluster 1/2 subgroups. (A) Heatmap, together with the clinical and pathological characteristics for clusters 1/2, determined based on m^{6}A RNA methylation regulator consensus clustering. (B) Principal component analysis for total RNA expression pattern. Subgroups are marked with colors. (C) Kaplan-Meier OS curves for the two subgroups. *P<0.05 and ***P<0.001 (cluster 1 vs. cluster 2). OS, overall survival; PCA, principal component analysis.

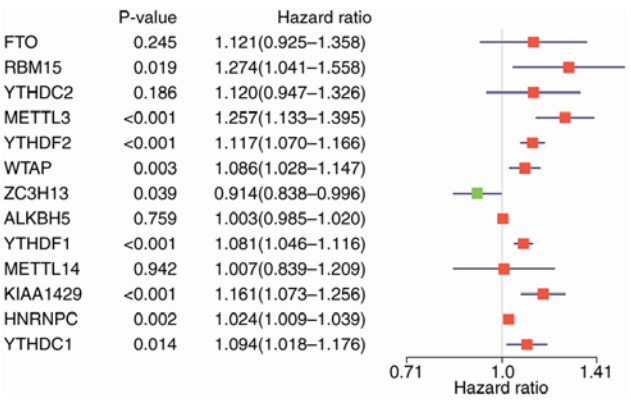

Figure 5. Univariate Cox regression analysis for OS-related m^{6}A RNA methylation regulators. Forest plots showing the associations between various regulators and OS, in which the unadjusted HRs and corresponding 95% confidence intervals are displayed. HR, hazard ratio; OS, overall survival.

The above findings suggested that risk scores determined based on the as-constructed signature were able to precisely estimate the prognosis and clinicopathological characteristics of HCC patients.

External validation of the prognostic signature in the ICGC cohort. To confirm the external validity, the prognostic signature was applied in the ICGC data. The expression levels of the 6 regulators were compared between the high- and low-risk groups, and the heatmap is presented in Fig. 9A. The high-risk group had a significantly shorter survival than the low-risk group in the ICGC cohorts (P=2.588e-3; Fig. 9B). ROC curve analysis showed that risk signature prognosis prediction could attain an AUC value of 0.693 (1 year), 0.723 (3 years) and 0.713 of (5 years; Fig. 9C). Using univariate (P=0.004) and multivariate (P=0.020) Cox regression analysis, the signature

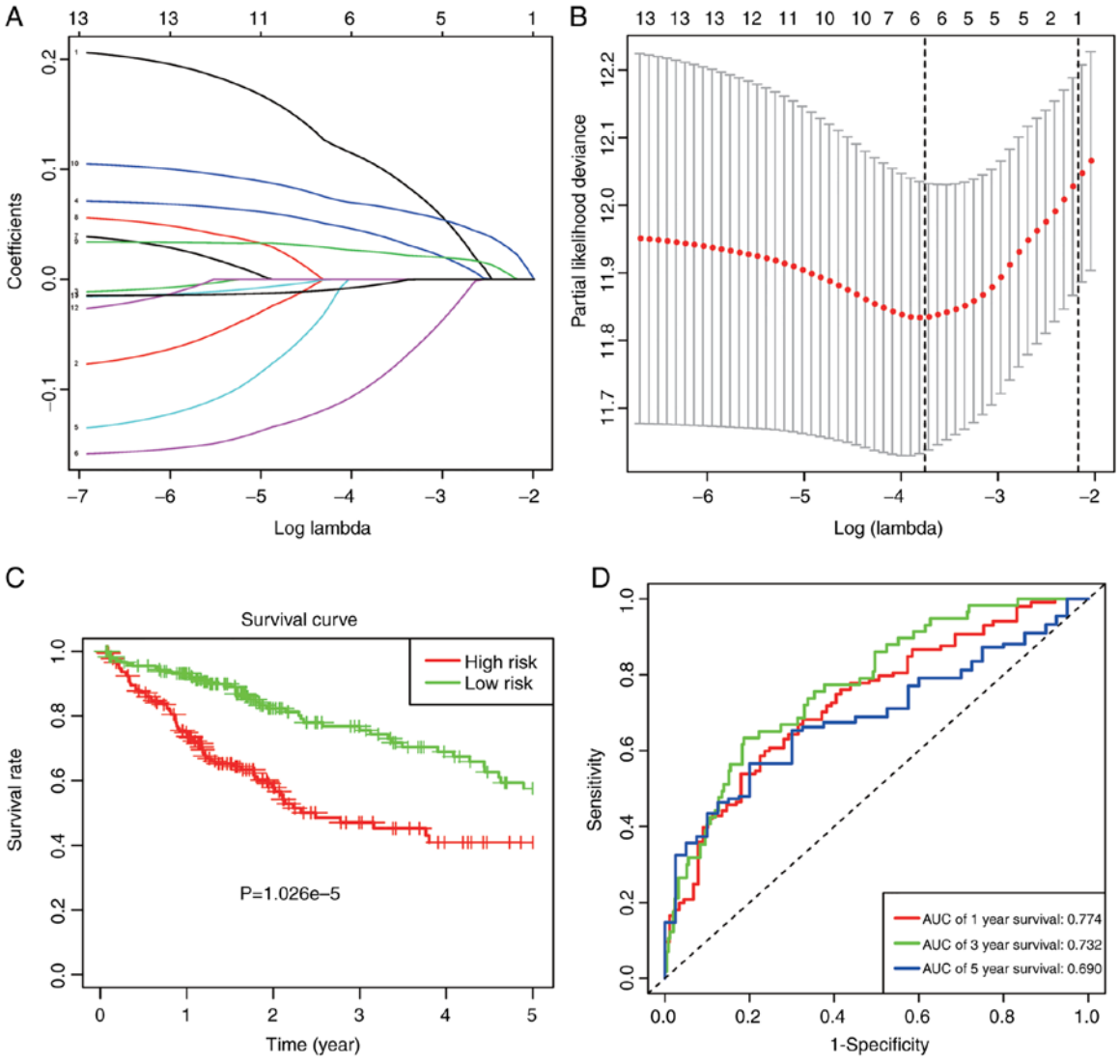

Figure 6. Risk signature to predict the prognosis of hepatocellular carcinoma. (A) LASSO was used to determine the coefficient profiles of 13 m^{6}A RNA methylation regulators. (B) 10-fold cross-validation was used to select parameters for the LASSO model, and 6 m^{6}A RNA methylation regulators were adopted for the LASSO model. (C) Kaplan-Meier curves of the OS of high- vs. low-risk groups. (D) Time-dependent risk receiver operating characteristic curves. The 1-, 3- and 5-year risk AUC were 0.774, 0.732 and 0.690, respectively. LASSO, least absolute shrinkage and selection operator; OS, overall survival; AUC, area under the receiver operating characteristic curve.

was further confirmed as an independent prognostic factor (Fig. 9D and E).

Functional analysis. mRNAs associated with the m^{6}A RNA methylation regulators were applied into the GSEA for enrichment analysis, in order to examine the potential biological functions. As indicated in Fig. 10, the top enrichments included ATM_PATHWAY, CCR5_PATHWAY, CXCR4_PATHWAY, IL6_PATHWAY, MCM_PATHWAY, NGF_PATHWAY, P53HYPOXIA_PATHWAY and TCR_PATHWAY.

Discussion

The present findings showed that the expression of m^{6}A RNA methylation regulators was closely associated with malignant grade and prognosis for HCCs. In addition, two HCC subgroups, namely cluster 1 and 2, were classified using consensus clustering on the basis of m^{6}A RNA methylation regulator expression levels. Specifically, the cluster 1/2 subgroups affected patient prognosis and exhibited a close correlation with clinicopathological features. Furthermore, a risk signature for prognosis was also constructed based on the 6 screened m^{6}A RNA methylation regulators, which could stratify patient OS into high- or low-risk subgroups.

The present study displayed obvious advantages. First, clustering analysis of m^{6}A modification regulators was carried out. Specifically, clusters were formed so that patients in the same cluster were similar, while patients in different clusters were distinct. Second, with regard to methodology, the application of the LASSO-penalized regression could boost the accuracy of the bioinformatics analysis. Different from the conventional stepwise regression used in prior research, the LASSO algorithm could analyze all independent factors simultaneously, identifying the most significant variables (17). Consequently, this formulation approach displayed a higher accuracy than stepwise regression using the multivariate Cox

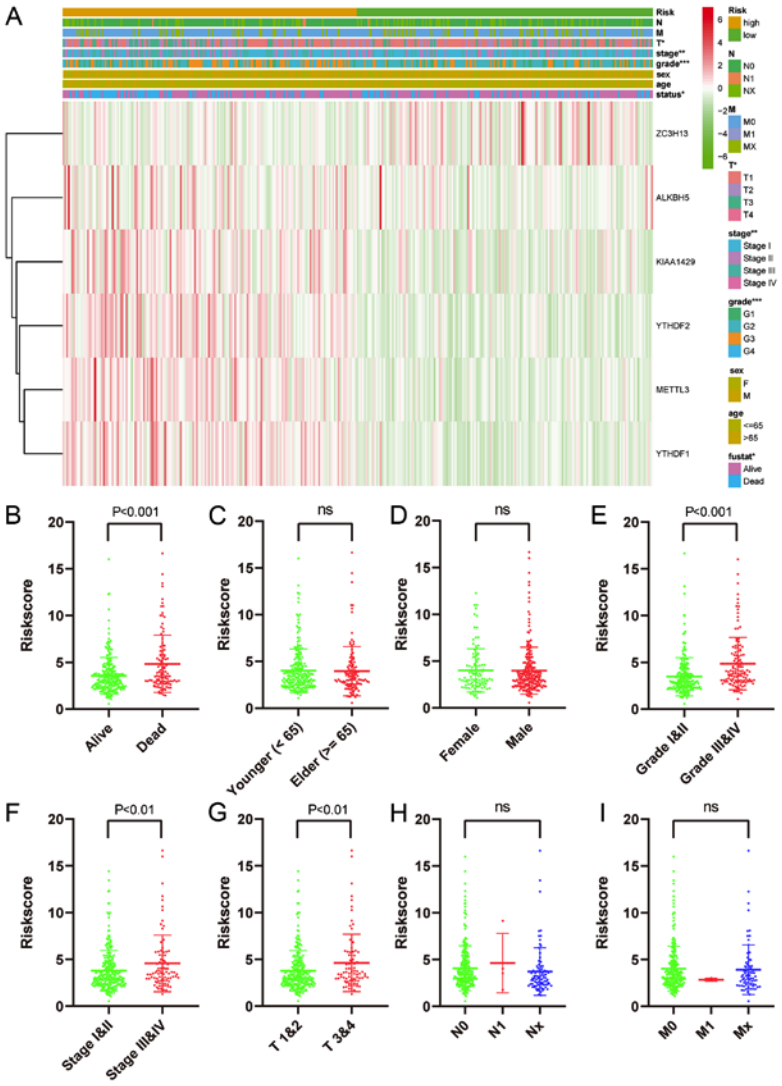

Figure 7. Association between risk score and clinicopathological features. (A) Heatmap showing the expression quantities for 6 screened m^{6}A RNA methylation regulators among the low- vs. high-risk hepatocellular carcinoma groups. Clinical and pathological characteristic distribution was examined between two groups. *P<0.05, **P<0.01 and ***P<0.001 (high risk vs. low risk). Distribution of risk scores stratified by (B) survival status, (C) age, (D) sex, (E) grade, (F) stage, (G) T stage, (H) N stage and (I) M stage. ns, not significant.

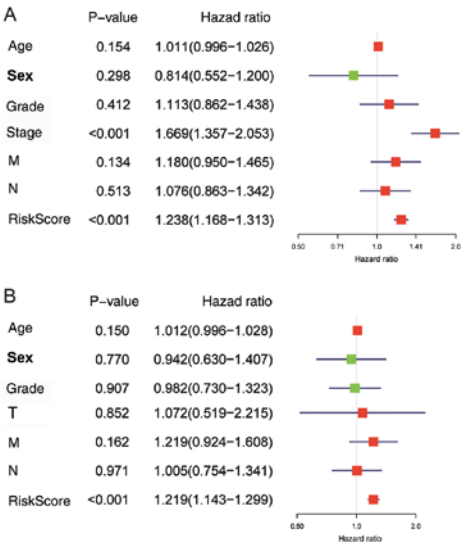

Figure 8. Cox regression analysis of the association between clinicopathological features and patient overall survival. (A) Univariate and (B) multivariate Cox regression analysis.

model, particularly in huge datasets, such as genomics (18). Thirdly, the results were validated in the ICGC dataset to check the general applicability. Next, we comprehensively analyzed 13 regulators simultaneously, while previous published studies usually focused on one regulator. Cheng *et al* (19) reported that KIAA1429 could regulate HCC invasion and migration by changing the m^{6}A modification in ID2 mRNA. In addition, Chen *et al* (20) reported that METTL3 expression was usually increased in human HCC, which contributed to the progression of HCC, while the SOCS2 level in HCC was repressed by a mechanism that depended on m^{6}A-YTHDF2. Zhao *et al* (21) discovered that YTHDF1 played a vital role in the regulation of HCC metabolism, as well as cell cycle development. Ma *et al* (22) reported that METTL14 could suppress the metastatic capacity of HCC cells by regulating the primary miRNA processing of m^{6}A-dependent tumor suppressors. In addition, it was found that YTHDF2 could modulate the m^{6}A level in HCC (23). However, the aforementioned studies only focused on one m^{6}A RNA methylation regulator. Recently, Zhou *et al* (24) reported the m^{6}A-related genes in HCC, and

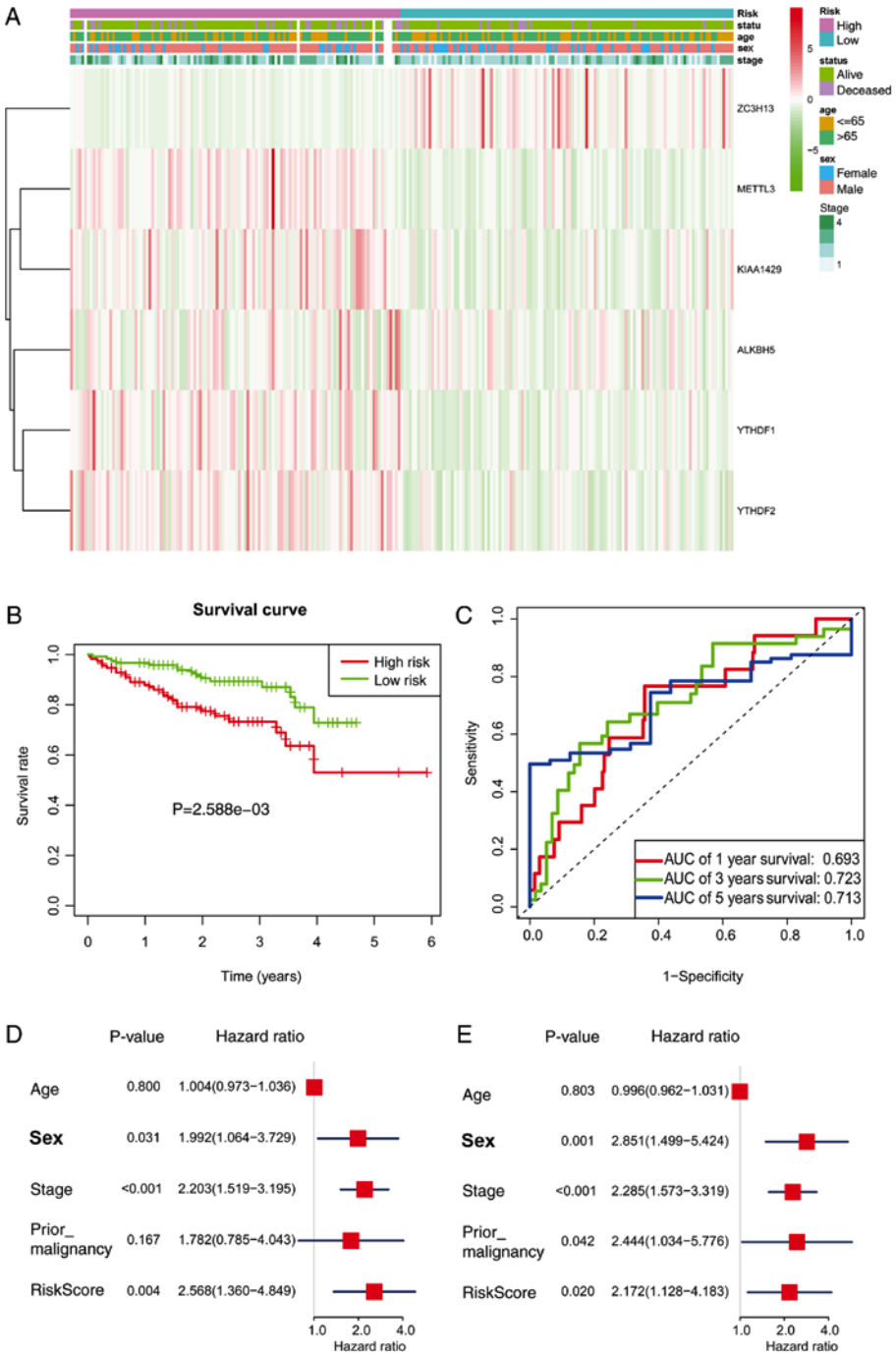

Figure 9. Validation of the m6A RNA methylation regulator signature in the International Cancer Genome Consortium cohort. (A) Heatmap showing the model related 6 m^{6}A RNA methylation regulator expression levels in the low- and high-risk groups. (B) Kaplan-Meier curves of overall survival. (C) Time-dependent receiver operating characteristic curves. (D) Univariate and (E) multivariate Cox regression analysis further confirmed the signature as an independent factor.

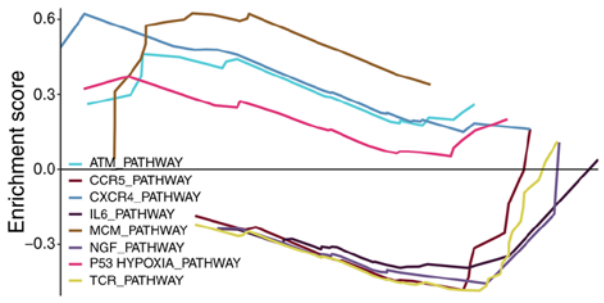

Figure 10. Gene set enrichment analysis of the established m^{6}A RNA methylation regulator signature-related genes.

confirmed the independent predictive value of both METTL3 and YTHDF1 in OS through multivariate Cox regression analysis; therefore, patients were further divided into three groups, based on METTL3 and YTHDF1 expression. Notably, no differential expression of ZC3H13 was observed between tumor and non-tumor samples (exact data not shown). However, ZC3H13 was a protective gene in univariate Cox regression analysis, and further investigations are needed.

The present study revealed that the m^{6}A RNA methylation regulators are correlated with biological processes during the malignant development of HCC. The RNA m^{6}A methylation

function within the tumor was recently confirmed, and certain biological processes were found to be affected by it, including tumor stem cell growth, tumorigenesis and self-renewal (25,26), as well as DNA damage response secondary to radiotherapy or chemotherapy (27,28). Herein, the expression levels of m^{6}A RNA methylation regulators in HCC were found to be correlated with HCC-related biological processes, such as ATM_PATHWAY (29), CXCR4_PATHWAY (30) and IL6_PATHWAY (31).

The present results showed that the expression levels of m^{6}A RNA methylation regulators could serve as prognostic markers. The overexpression of YTHDF1 was associated with poor prognosis, which was consistent with the results of Zhao *et al* (21). In this study YTHDF2 overexpression was correlated with poor prognosis however YTHDF2 suppressed cell proliferation and growth in the study by Zhong *et al* (32). More importantly, the as-constructed risk signature for the prognosis of HCC based on the 6 selected m6A RNA methylation regulators was proven valuable, and its significance in predicting the T stage, stage, grade and survival status was determined. However, no significant difference in risk score was identified between the N and M stages, which might be partially due to the small number of patients at these stages (Table I). Moreover, risk significance was finally verified by multivariate Cox analysis.

The study, however, had the following limitations: First, more data are necessary to confirm these findings. Second, these 13 regulators, as well as others, require further investigation. Third, consensus clustering analysis was conducted based on the m^{6}A RNA methylation regulator expression levels rather than writers, readers or erasers.

In conclusion, the present study comprehensively illustrated the expression patterns, possible role and prognostic significance of m^{6}A RNA methylation regulators in HCC. Typically, the expression levels of m^{6}A RNA methylation regulators exhibited a strong association with malignant clinical and pathological characteristics in HCCs, as well as with upregulated gene expression involved in biological processes to accelerate the malignant development of HCC. The present study provided critical support for future research into RNA m^{6}A methylation function in HCCs.

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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 on reasonable request.

Authors' contributions

WL, QFC, PW and ZLH conceived and designed the study. WL, QFC, TH, PW and LS analyzed the data. WL, TH, PW and LS wrote the paper. WL, QFC, PW and ZLH reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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