RESEARCH ARTICLE

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m5C-Related Regulators Define Tumor Microenvironment and Predict Prognosis in Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is a highly lethal cancer and a leading cause of cancer-related deaths globally. RNA 5-methylcytosine (m5C) modification plays a vital role in epigenetic regulation, yet its impact on prognosis and the tumor immune microenvironment (TIME) in HCC remains unclear. **Materials and methods:** RNA sequencing and clinical data were obtained from the Cancer Genome Atlas (TCGA) database. We applied an unsupervised clustering algorithm for the cluster analysis of m5C RNA methylation regulators, and then performed survival analyses to determine the best prognosis for HCC samples. Univariate and multivariate Cox regression analyses were conducted to construct a prognostic model. HCC patients were classified into high- and low-risk groups based on risk scores. Model performance was evaluated using ROC curves and validated with the ICGC cohort. Immune infiltration, clinicopathological features, and functional enrichment analyses were also performed. **Result:** We analyzed the differential expression patterns of the m5C-related regulators between HCC and normal tissue samples. Based on consensus clustering of these regulators, three distinct molecular subgroups were identified, each associated with differences in patient survival and immune cell infiltration. Furthermore, we developed a prognostic signature comprising *NSUN3, NSUN5*, and *YBX1*, and stratified HCC patients into low- and high-risk groups. Patients in the low-risk group exhibited significantly better overall survival (OS) than those in the high-risk group. The robustness of this risk model was validated using the ICGC database. When integrated with clinicopathological characteristics, the risk score emerged as an independent prognostic factor. Additionally, we performed functional annotation and enrichment analyses based on differentially expressed genes (DEGs) between the two risk subgroups to explore potential underlying biological mechanisms. **Conclusion:** Our study revealed the potential roles of these m5C-related regulators in TIME and identified their prognosis value and therapeutic potential for HCC patients.

Keywords: hepatocellular carcinoma- m5C methylation- tumor microenvironment- prognostic signature

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Introduction

According to the estimates of cancer incidence and mortality provided by the American Cancer Society, hepatocellular carcinoma (HCC) is a relatively lethal type of cancer, and ranks among the top seven most frequent cause of cancer-related deaths worldwide [1]. Despite great improvements in treatment, such as molecular-targeted therapy and immunotherapy, the overall prognosis of patients with HCC remains far from satisfactory [2]. Considering the heterogeneity and complexity of various pathogenic signaling pathways of HCC, a similar treatment regimen for patients with HCC would not achieve the same therapeutic effect and may possibly even exacerbate symptoms. Therefore, it is critical to clarify the molecular mechanisms of HCC to develop novel therapeutic targets and formulate personalized treatments.

Recently, an increasing number of studies have shown that RNA methylation is an important mechanism involved in epigenetic regulation of posttranscriptional RNA and plays a key role in the onset and development of diseases [3, 4]. Due to the different RNA methylation sites, RNA methylation includes m6A, m5C, m1A, m7G, and 2-O-methylation modifications, that affects the splicing, export, translation, stability, miRNA biogenesis and immunogenicity of RNA [5]. Among these RNA methylations, m6A and m5C modification are the two most prominent form of RNA modifications [6]. Modification of m5C is conducted by three types of factors: methyltransferases ("writers"), m5C-binding proteins ("readers"), and demethylases ("erasers") [7]. Similar to the function of m6A modification, the aberrant expression of m5C regulators are associated with the occurrence and

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development of various tumors [8-10]. For instance, the m^{5}C modification of ALYREF in PKM2 mRNA promoted the glucose metabolism, promoting the progression of bladder cancer [11]. RNA methyltransferase NSUN2 promoted gastric cancer development by repressing p57Kip2 in an m^{5}C-dependent manner [12]. Furthermore, NSUN2-mediated m^{5}C methylation promoted the occurrence and progression of esophageal squamous cell carcinoma via LIN28B dependent stabilization of GRB2 transcript [13]. However, the gene expression patterns and prognosis values of m^{5}C-related regulators in HCC remain ambiguity and need in-depth study.

In addition, recent studies have shown that m^{5}C-related regulators were highly associated with the microenvironment of tumors and immune cells [14-16]. Many studies have demonstrated that infiltrating immune cells play key roles in cancer development and the therapeutic response to immunotherapy [17]. Therefore, m^{5}C methylation regulated HCC progression by regulating the immune system, which might be a therapeutic target for HCC. To demonstrate this hypothesis, we explored the data from The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) databases, and systematically evaluated the expression patterns and prognostic values of 15 widely studied m^{5}C-related regulators. Then, we identified three different HCC subtypes that had distinct clinicopathological parameters, immune infiltration and prognostic values. Next, we constructed a risk model for m^{5}C-related regulators to explore the therapeutic target and improve the prognostic prediction for HCC. Next, we fully elucidated the correlations between the risk model and clinicopathological parameters, immune cell infiltration, immune checkpoints, signaling pathways, and biological functions to examine the effects of m^{5}C-related regulators on the prognosis and immune microenvironment of HCC. Finally, these results confirmed the ability of m^{5}C-related regulators in predicting the prognosis and therapeutic sensitivity.

Materials and Methods

Data Collection

The RNA sequencing data and corresponding clinical data were downloaded from the Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/) [18], which consisted of 371 HCC samples. The RNA-seq data and survival data of another 231 HCC samples were obtained from the International Cancer Genome Consortium (ICGC) database (https://dcc.icgc.org/projects/LIRI-JP) [19]. Patients with missing status, TMN stage, and time data of over survival (OS) were excluded. Data were analyzed using the statistical software packages R (version 4.1.1) and R Bioconductor.

Identification and Cluster Analysis of m^{5}C-Related Regulators

An unsupervised clustering algorithm was applied to the cluster analysis of m^{5}C RNA methylation regulators in HCC. A total of 15 m^{5}C-related regulators, including 11 writers (*NOP2, NSUN2, NSUN3, NSUN4, NSUN5, NSUN6, NSUN7, DNMT1, DNMT3A, DNMT3B* and *TRDMT1*), 2 erasers (*TET2* and *TET3*), and 2 readers (ALYREF and YBX1) were retrieved from the previous published literature. The correlation between the 15 m^{5}C-related regulators and the prognostic effect on HCC was calculated. Next, we performed unsupervised cluster analysis using the R package "ConsensusClusterPlus" [20]. Additionally, survival analysis was conducted among the three clusters to identify which subgroup exhibited the most favorable prognosis in HCC patients.

Construction and Validation of the Prognostic Prediction Model

The risk model of 3 m^{5}C-related regulators was constructed using univariate and multivariate Cox regression analysis. The coefficients were achieved from the multivariate regression analysis. The risk score was calculated with the formula:

$$\text{risk score} = \sum_{i=1}^{n} coefi \times \text{mRNAi}$$

Next, HCC patients were stratified into low-risk and high-risk subgroups based on the median risk score.

Functional Annotations Analysis and GSEA

To identify the biological function of the two risk subgroups, we used the "Limma" R package for differentially expressed genes (DEGs) analysis. Q-value < 0.05 and $|\log2 \text{ fold change (FC)}| \geq 1$ were set as the cutoff. Enrichment analysis and functional annotation of differentially expressed genes (DEGs) were performed using the R package "clusterProfiler" [21]. The "org.Hs.eg.db" database was employed as the annotation reference for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Additionally, Gene Set Enrichment Analysis (GSEA) (version 4.2.3) was conducted to compare gene set differences between the two risk subgroups. A $p < 0.05$ and a false discovery rate (FDR) q-value < 0.25 were considered statistically significant.

Immune Cell Infiltration Estimation and TIME

The immune cell infiltration levels were evaluated using the Tumor Immune Estimation Resource (TIMER), which comprises six immune cell types [22]. Additionally, we used single-sample gene set enrichment analysis (ssGSEA) to calculate individual enrichment scores for each sample based on the gene expression matrix and the human immune cell subtypes gene set [23].

Statistical Analysis

Statistical analysis was conducted using R software (4.1.1). The student's t-test and one-way ANOVA were used to perform the group comparisons, and the Kruskal-Wallis and Wilcoxon tests were used to compare the differences. The differences in OS were analyzed using the log-rank test with Kaplan-Meier estimation. Spearman's correlation coefficient was used to calculate the correlation between the m^{5}C-related regulators and the risk score for immune genes. All p values were two-sided, and $p < 0.05$ was considered statistically significant.

Results

Expression of m5C-Related Regulators in HCC

The overall workflow used for the research strategies and m5C-related regulators in HCC prognosis is shown in Figure 1. To elucidate the expression and biological function of m5C-related regulators in HCC, we first downloaded the efficacious gene expression and clinicopathological data from the TCGA database. We began by comparing the expression levels of m5C-related regulators between HCC and normal tissue samples. The results showed that, with the exception of *NSUN6* and *NSUN7*, most m5C-related regulators were significantly upregulated in HCC. However, no significant difference was observed in the expression of *TET2* (Figure 2A). In addition, the correlation between the 15 m5C-related regulators and the prognostic effect on HCC was calculated. As shown in Figure 2B, there were positive pairwise correlations between the expression levels of all m5C-related regulators. Notably, nearly all of these regulators, except for *NSUN6*, were associated with poorer prognosis in HCC patients. In summary, these findings demonstrated that m5C-related regulators had distinct changes in expression and exerted their effects on prognosis in HCC.

The Association of Three Clusters of HCC Samples with Survival Status, Clinicopathological Characteristics, and Immune Cell Infiltration

Consensus clustering based on the expression profiles of the 15 m5C-related regulators was used to elucidate the biological discrepancies among subgroups. After unsupervised clustering, k = 3 was ultimately identified (Figure 3A-D). Then, the HCC samples were categorized into three different subtypes. The overall survival (OS) of the patients with HCC in Cluster 3 was shorter than those in Cluster 1 and 2 ($p < 0.001$) (Figure 3E). In addition, the clinicopathological characteristics were compared among the three subgroups. As shown in the heatmap (Figure 3F), significant difference was present in T, stage and survival status ($p < 0.01$), while no evident differences were observed in other characteristics, such as M, N, gender, age, and grade. These results imply that the expression of m5C-related regulators was associated with the T classification, stage and survival status of HCC patients.

To analyze the differences in the infiltration of immune cells among the three subgroups, we used the ssGSEA method and found that the infiltration by activated CD4+T cell, activated dendritic cell, CD56dim natural killer cell, immature dendritic cell, T follicular helper cell, and type 2 T helper cells was higher in Cluster 3. The infiltration of eosinophil, monocyte, natural killer T cell, and neutrophil

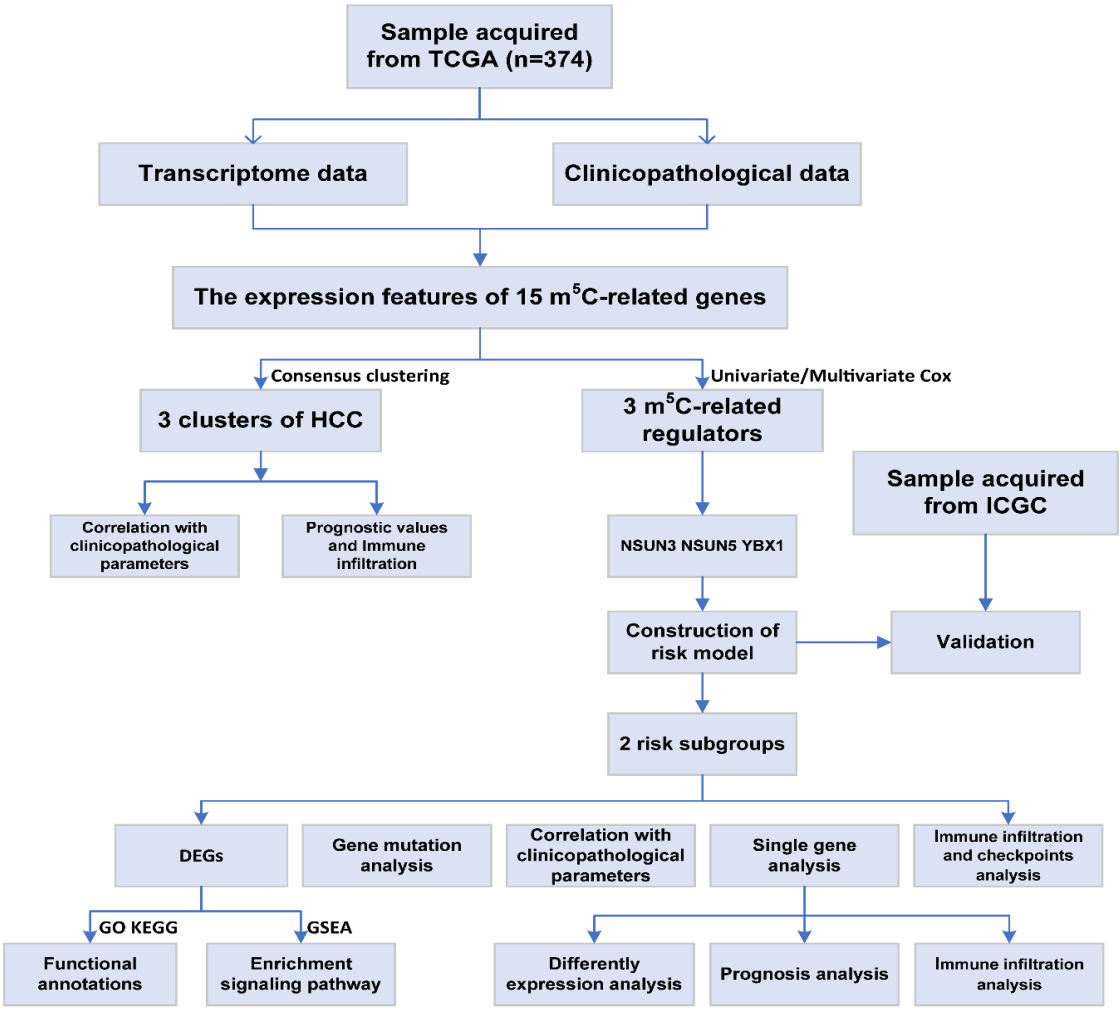

Figure 1. Workflow of the Study and Different Analytic Strategies in the Study

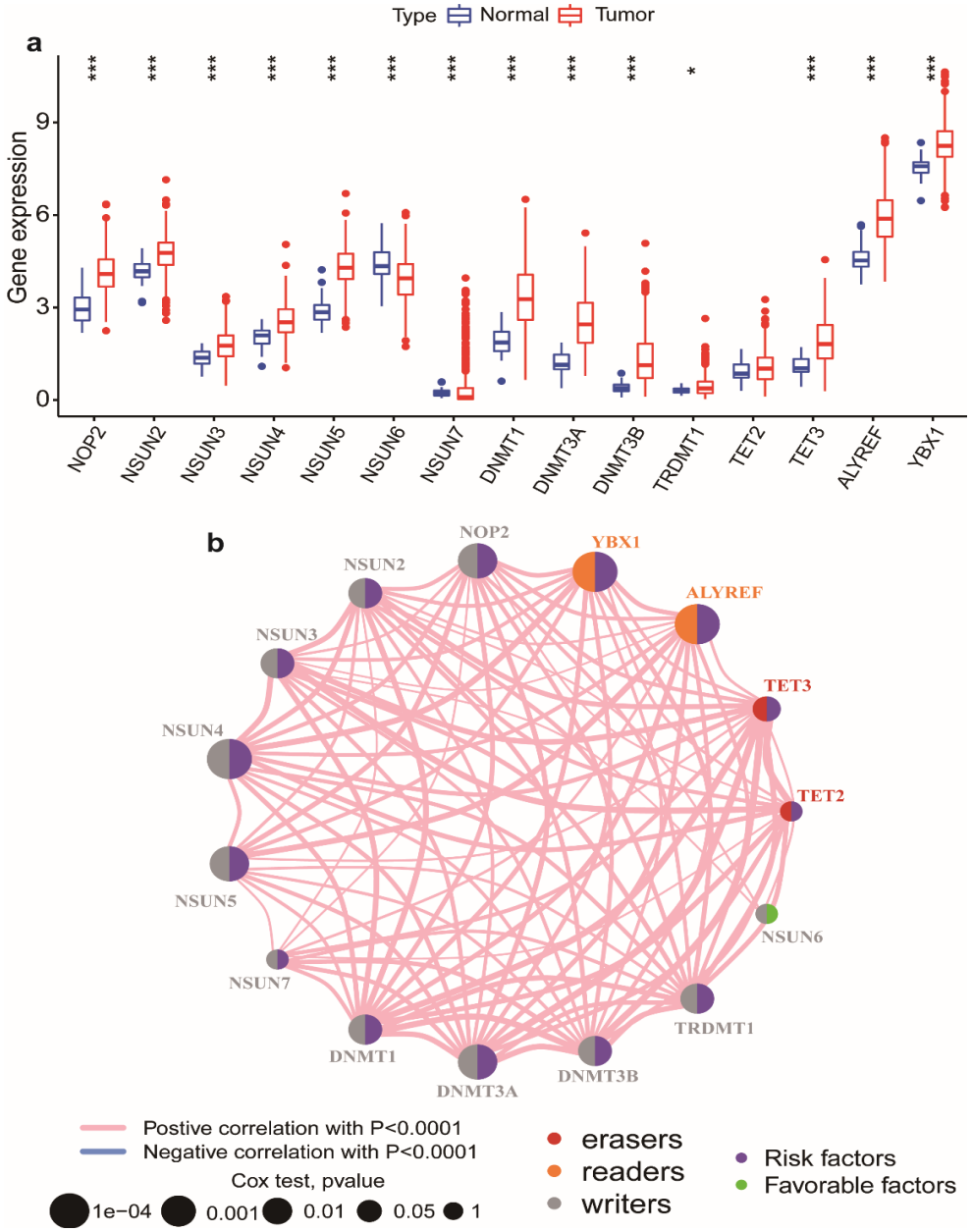

Figure 2. Expression and Inner Crosslink of the 15 m^{5}C-related Regulators and Prognostic Effect on HCC. (a) Box plots showing the differential expression of m^{5}C-related regulators between normal and tumor tissue samples. (b) The PPI network of m^{5}C-related regulators shows the interaction and prognostic effect on HCC. *p < 0.05, ***p < 0.001.

was higher in Cluster 1 (Figure 4). These results suggest that m^{5}C-related regulators affect the degree of infiltration of immune cell types.

Construction of an m^{5}C-Related Regulators Signature with Distinct Prognostic Value

To further characterize the functional role of the three m^{5}C-related regulators in HCC, univariate Cox regression analysis was conducted to the 15 m^{5}C-related regulators expression profiles. As shown in Figure 5A, 12 m^{5}C-related regulators (*TRDMT1, DNMT3B, NSUN2, NSUN4, NSUN5, YBX1, TET3, ALYREF, DNMT3A, NSUN3, NOP2*, and *DNMT1*) exerted a significant prognostic value ($p < 0.05$). Next, we further applied multivariate Cox regression analysis to build a risk model. As a consequence, 3 m^{5}C-related regulators (*NSUN3*, *NSUN5*, and *YBX1*) were identified (Figure 5B). the risk score of each HCC sample was calculated as follows: risk score = (0.588795*expression of *NSUN3*) + (0.294523*expression of *NSUN5*) + (0.708092*expression of *YBX1*). Subsequently, 370 HCC patients were divided into two subgroups based on their median risk score: low-risk and high-risk. It was recognized that patients in the low-risk group had a better survival rate than those in the high-risk group ($p < 0.001$) (Figure 5C-E). The heatmap further demonstrated that the expression levels of the three m^{5}C-related regulators were significantly different between the two risk subgroups (Figure 5F). In addition, the ROC analysis was performed to evaluate the accuracy of the risk model. The area under the curve (AUC) for risk model was as high as 0.755, which was better than other clinicopathological parameters such as age (AUC = 0.511), gender (AUC = 0.504), grade (AUC = 0.478), AJCC stage (AUC = 0.703), T stage (AUC =

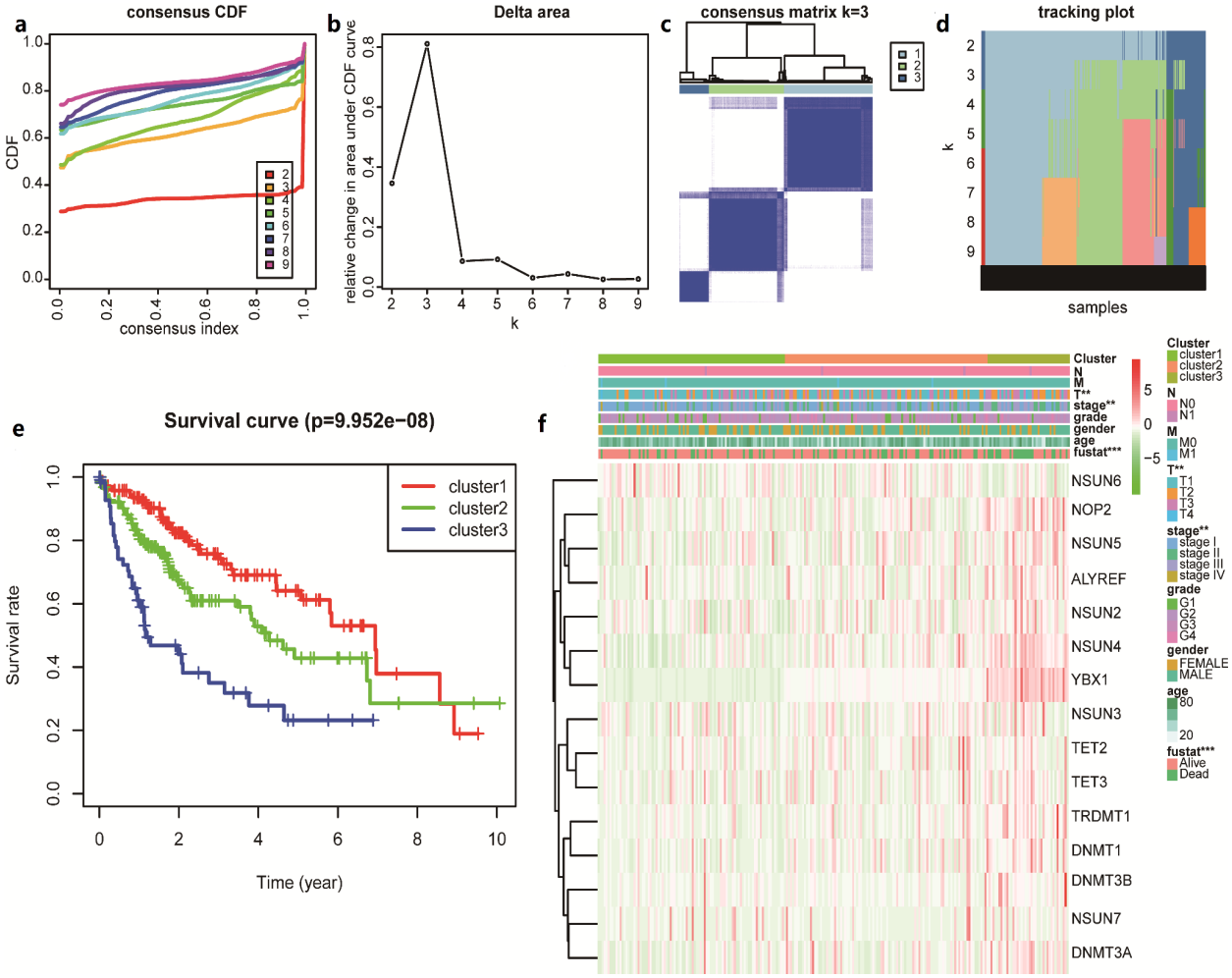

Figure 3. Patterns of Expression, Clinicopathological Characteristics, and Survival of HCC in Three Clusters in TCGA. (a) Distribution function (CDF) and (b) relative changing area under the CDF curve from k = 2 to k = 9 in Consensus clustering. (c) Consensus clustering matrix for k = 3 in HCC. (d) The tracking plot of the HCC patients. (e) Kaplan-Meier curve of overall survival among three clusters. (f) Heatmap of correlation of m5C-related regulators with clinicopathological characteristics of HCC patients. **p < 0.01, ***p < 0.001.

0.708), N stage (AUC = 0.508), and M stage (AUC = 0.508) (Figure 5G).

Subsequently, to validate the robustness of the risk model in an independent cohort, we selected the ICGC database as the validation set. Consistent with the findings from the TCGA dataset, HCC patients in the low-risk group exhibited significantly better overall survival than those in the high-risk group (P < 0.01). The area under the curve (AUC) for the risk model in the ICGC cohort was 0.724 (Supplementary Figure 1), further supporting its predictive accuracy. Collectively, these results indicate that the risk model based on the three m^{5}C-related regulators demonstrates reliable prognostic value for HCC patients across different datasets.

The Association of Risk Score with Clinicopathological Characteristics in HCC

To confirm the independent prognostic value of the risk score in HCC, univariate and multivariate Cox regression analyses were performed. The results demonstrated that the risk score (p < 0.001, HR = 1.642, 95% CI = 1.365-1.976), was an independent prognostic factor for overall survival (Supplementary Figures 2A and 2 B). Next, we further performed stratified survival analysis by age, gender, N stage, and T stage. As shown in Supplementary Figure 2C-I, the patients in the low-risk group had a better survival rate than those in the high-risk group in each stratified group (p < 0.001).

Next, to evaluate the association of the three selected m^{5}C-related regulators with the clinicopathological characteristics, the heatmap showed that the expression of *NSUN3, NSUN5*, and *YBX1* was high in the high-risk group. Significant differences were observed in tumor stage and survival status, whereas no notable differences were found in T stage, M stage, N stage, gender, age, or tumor grade (Supplementary Figure 3A). Furthermore, we evaluated the distribution of risk scores across stratified groups based on age and tumor grade. The risk scores were significantly higher in the younger age group and in patients with grade G3–4 tumors, compared to their respective counterparts (Supplementary Figure 3B).

The Association of Risk Score with Immune Characteristics in HCC and Single Gene Analysis

To demonstrate the effect of risk score on the TIME in HCC, we investigated the relationship of risk score

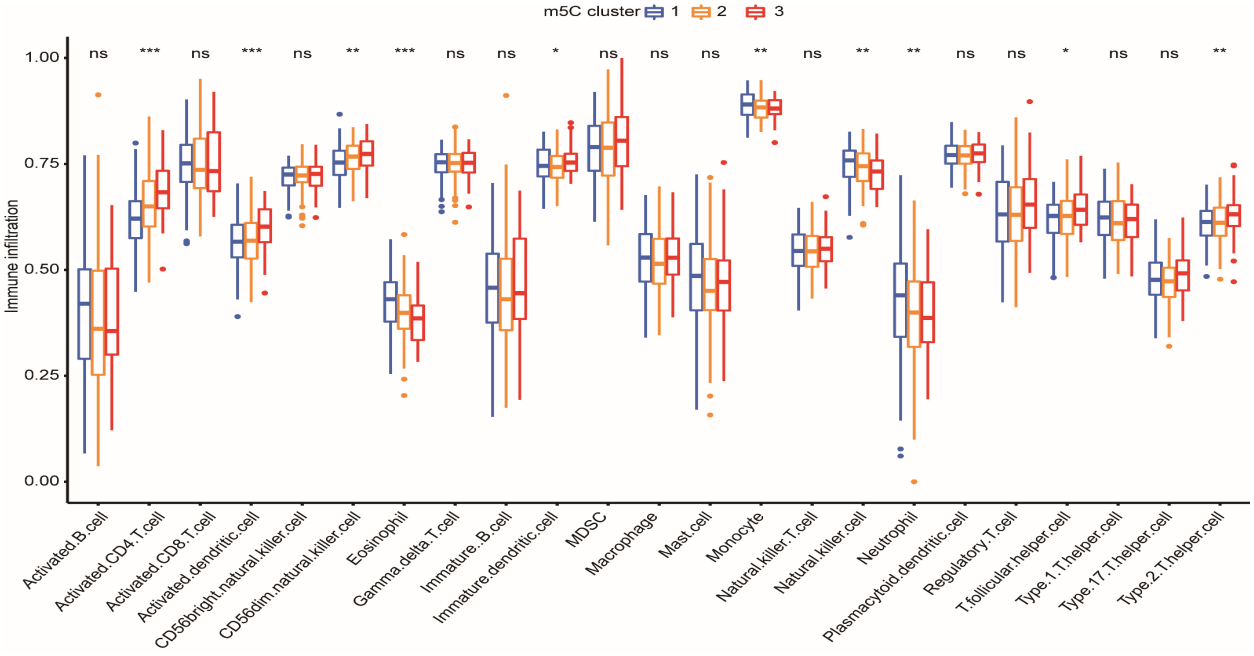

Figure 4. Comparison in the Relative Abundance of Immune Cell Infiltration between Three m^5C modification Clusters. *p < 0.05, **p < 0.01, ***p < 0.001.

with the infiltration levels of 6 immune cell types (B cell, CD4+ T cell, CD8+ T cell, dendritic, macrophage, and neutrophil) and found that the risk score was significantly positively correlated with the infiltration of all 6 immune cells (Supplementary Figure 4A-F). Next, we evaluated the expression levels of immune checkpoints (*PD-L1, CTLA4, HAVCR2, IDO1, LAG3,* and *PDCD1*) between the two risk subgroups. It was recognized that the expressions of all the immune checkpoints in high-risk group were higher than those in low-risk group (Supplementary Figure 4G-L). These results revealed that the risk score is associated with the TIME and a tumor response to immunotherapy for HCC patients.

Next, we analyzed the expression, overall survival, the immune cell infiltration levels of the 3 m^5C-related regulators. We found that the expression levels of *NSUN3, NSUN5*, and *YBX1* were higher in tumor tissues than those in normal tissues (Supplementary Figure 5A). In addition, patients with low expression of the 3 m^5C-related regulators had better survival rates (Supplementary Figure 5B). Then, we further explored the effects of the 3 m^5C-related regulators on immune cell infiltration. As shown in Supplementary Figure 5C, the expression levels of the 3 m^5C-related regulators had a significant effect on the immune cell infiltration levels in HCC. Collectively, these results indicated that these m^5C-related regulators significantly affect the survival time and tumor immune microenvironment for HCC patients.

Function Annotations and Enrichment Signaling Pathway

To explore the potential biological functions in the two subgroups, we identified 3176 DEGs, with 3061 being upregulated and 115 being downregulated ($p < 0.05$, $|\log_2 FC| > 1$), by "Limma" R package and performed GSEA analysis (Supplementary Figure 6A). As shown in Supplementary Figure 6B, we found that the Cell cycle signaling pathway was activated in the high-risk subgroup according to the screening criteria. In addition, we performed GO and KEGG enrichment analysis in the low- and high- risk subgroup. The leading highly GO enrichment biological processes were "humoral immune response mediated by circulating immunoglobulin", "complement activation, classical pathway", "phagocytosis", "lymphocyte mediated immunity", "complement activation" in the high- risk subgroup. Additionally, KEGG analysis revealed that the potential pathways were enriched in "Cell adhesion molecules", "Cytokine-cytokine receptor interaction", "ECM-receptor interaction", and so on (Supplementary Figure 7B, D). On the other hand, the principal GO enrichment biological process was "metabolic process" in the low-risk subgroup. Furthermore, KEGG analysis showed that "Retinol metabolism", "Histidine metabolism", "Chemical carcinogenesis- DNA adducts", "Drug metabolism- cytochrome P450" were enriched in low-risk subgroup (Supplementary Figure 7A, C).

Discussion

In recent years, previous studies have reported the interaction of proteins and related to the metastasis pathway in HCC mechanism [24]. Similarly, increasing evidence has also showed that RNA modifications play key roles in the carcinogenesis and physiopathology for HCC and are identified to be promising therapeutic targets and novel strategies [25]. 5-methylcytosine (m^5C), as an abundant modification, is participated in a broad variety of RNA types including rRNAs, tRNAs, mRNAs, eRNAs, and non-coding RNAs, and is also associated with various diseases [26]. Particularly, m^5C modification has been

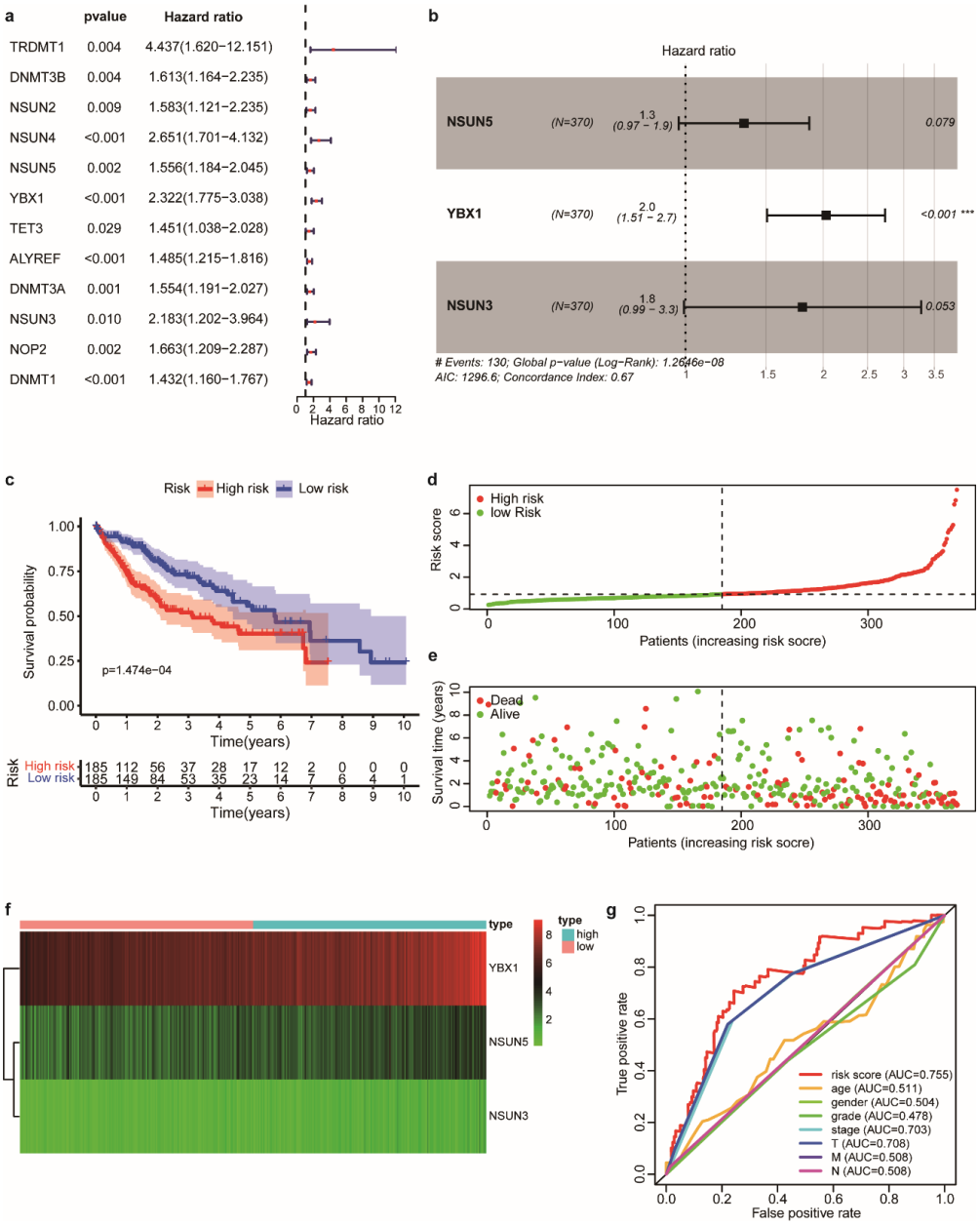

Figure 5. Construction of a Prognostic Risk Signature with three m5C-related Regulators. (a) Univariate Cox regression and (b) multivariate Cox regression analysis of the 15 m5C-related regulators. (c) Kaplan–Meier survival curves comparing overall survival between low- and high-risk subgroups. (d) The distribution of risk score, (e) survival time and status of HCC patients. (f) Heatmap of the identified regulator in two groups. (g) ROC curve analysis in the TCGA cohort.

involved in onset and development of many tumors [10, 27, 28]. Although, existing studies have reported that some m5C-related regulators could predict the prognosis of HCC patients [29, 30], the specific role of the m5C-related regulators in HCC is still obscure, especially in immune microenvironment of HCC. Therefore, in this study, we systematically evaluated the expression patterns and prognostic values of 15 m5C-related regulators and constructed a risk model for m5C-related regulators to explore the therapeutic target and improve the prognostic prediction for HCC.

Similar to m6A methylation, m5C modification is conducted by different m5C-related regulators, which were classified into methyltransferases (“writers”), m5C-binding proteins (“readers”), and demethylases (“erasers”). Among these regulators, *NSUN2*, as an m5C writer, has been demonstrated to be associated with poor prognosis in various tumors, such as gallbladder carcinoma [31], gastric cancer [32], sophageal squamous cell carcinoma [13]. Moreover, *TET2*, a member of m5C erases, has been recognized to be contributed to the development of hematological malignancies [33]. Cui et al. [34] revealed that overexpressed *YBX1* increased the development of non-small cell lung cancer (NSCLC) and decreased the sensitivity to cisplatin by targeting p110β and beclin1. Except for the role of methylation, Deng et al. [35] revealed that LINC00472 inhibited the invasion and metastasis of lung adenocarcinoma by binding to *YBX1*.

In this study, we found that the expression levels of m^{5}C-related, except for *NSUN6, NSUN7* and *TET2,* were highly upregulated in patients with HCC compared with normal samples. And there were positive pairwise correlations between the expression of all m^{5}C-related regulators, and almost all m^{5}C-related regulators, except for *NSUN6*, exerted negative effects on prognosis in HCC. Therefore, it is valuable to further investigate the biological function of m^{5}C-related regulators in HCC. We identified three different subtypes of HCC based on 15 m^{5}C-related regulators and found that the three cluster subtypes had different OS, immune cell infiltration levels, and clinicopathological characteristics. The Cluster 3 subtype had a significantly shorter survival rate and higher stage than Clusters 1 and 2. Previous studies have demonstrated that the TCGA HCC cohort could divided into different subgroups based on the m6A regulators and showed the significantly different OS and tumor stage among the identified subgroups [36, 37]. Collectively, these results indicated that RNA methylation might have an effect on the prognosis of HCC.

Subsequently, an m^{5}C-related regulators signature, consisted of *NSUN3*, NSUN5, and *YBX1*, was constructed, which effectively categorized the HCC patients into low- and high- risk subgroup. We observed that patients in low-risk group had a better OS than those in high-risk group and proved the accuracy of risk model. The ICGC database also confirmed the reliable predictive value for HCC patients. Moreover, the risk score was determined to be the independent prognostic factor by univariate and multivariate Cox regression analysis. While as for *NSUN3, NSUN5*, and *YBX1*, previous study has reported that *NSUN5* promoted the development of colorectal cancer via cell cycle regulation [38]. *YBX1* is known to activate *PI3K/AKT* and mTOR signaling, pathways crucial to immune evasion and HCC progression, also severed as an oncogene factor [34]. However, the study of *NSUN3* has been reported rarely. Though *NSUN3* has been less studied, recent evidence suggests its involvement in mitochondrial tRNA methylation, which may affect energy metabolism in tumor cells. Taken together, the risk model provides a solid foundation for the studies of pathogenesis, and for the development of novel therapeutic targets and predicting of prognosis for HCC. Furthermore, we further evaluated the effect of the risk score on the TIME, and demonstrated that the risk score was evidently associated with the expression levels of immune cell infiltration and immune checkpoints, such as *PD-1* and *CTLA4*. These results showed that the dysregulation of m^{5}C-related regulators plays a key role in tumor progression by influencing TIME in HCC.

On this basis, we further investigated the signaling pathways and biological functions between the two subgroups and found that the Cell cycle signaling pathway was activated in the high-risk subgroup, which was ascertained the carcinogenic effect for various tumors [39, 40]. In addition, GO enrichment analysis revealed that the top terms were "immune regulation" in high-risk subgroup, and KEGG analysis implied that the potential pathways were enriched in "Cell adhesion molecules", "Cytokine-cytokine receptor interaction", "ECM-receptor interaction". However, the principal GO and KEGG enrichment biological process was "metabolic". These might be the causes of the difference in prognosis between the low- and high- risk subgroup.

However, it is undeniable that our study has some limitations. First, the data for the study are obtained from TCGA and ICGC datasets. Therefore, further validation from other external cohorts and clinical sample data are warranted to test our findings. Additionally, these findings of the study need further experimental verification. Lastly, the regulatory mechanisms of m^{5}C-related regulators in TIME require further study to enhance the efficacy of immunotherapy in HCC. In conclusion, systematic and integrated analysis of 15 m^{5}C-related regulators in HCC revealed a regulatory mechanism which affects HCC prognosis and the tumor microenvironment. We constructed a risk model to elaborate the roles of the m^{5}C-related regulators and identify their predicting value in prognosis. This work highlights the molecular mechanisms of HCC and will aid in developing novel therapeutic targets and formulate personalized treatments.

Author Contribution Statement

Xiang-Qian Gu and Ning Wang designed the study. Xiang-Qian Gu, Bin Li, and Cheng-Yu Gu extracted and analyzed the data. Xiang-Qian Gu, Bin Li, Ming-Yu Wu, and Ning Wang performed the data mining, data sorting and screening, and statistical analysis; Xiang-Qian Gu wrote the manuscript and Ning Wang revised the manuscript. All authors reviewed the manuscript.

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Ethical Declaration

This study was exempt from ethical review and approval, as it involved only bioinformatics analyses based on publicly available datasets.

Availability of Data

All data used in this study are publicly available. The datasets supporting the findings can be accessed through platforms such as TCGA and ICGC.

Study Registration

As a bioinformatics analysis based entirely on publicly available data, this study did not involve primary data collection, clinical trials, or meta-analyses, and therefore did not require formal registration in a research database.

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