Uncovering of the Association Between m5C Regulator-Mediated Methylation Modification Patterns and Tumor Microenvironment Infiltration Characteristics in Hepatocellular Carcinoma

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Primary research

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Abstract

Background: 5-Methylcytosine (m^5C) plays essential roles in hepatocellular carcinoma (HCC), but the association between m^5C regulation and immune cell infiltration in HCC has not yet been clarified.

Methods: In this study, we analysed 371 patients with HCC from The Cancer Genome Atlas (TCGA) database, and the expression of 13 m^5C regulators was investigated. Additionally, gene set variation analysis (GSVA), unsupervised clustering analysis, single-sample gene set enrichment analysis (ssGSEA), correlation analysis, and immunohistochemical (IHC) staining were performed.

Results: Among the 371 patients, 41 had mutations in m5C regulators, the frequency of which was 11.26%. Then, we identified three m5C modification patterns that had obvious tumour microenvironment (TME) cell infiltration characteristics. Cluster-1 had an immune rejection phenotype; Cluster-2 had an immunoinflammatory phenotype; and Cluster-3 had an immune desert phenotype. In addition, we found that DNMT1 was highly expressed in tumour tissues compared with normal tissues in a tissue microarray (TMA) and that it was positively correlated with many TME-infiltrating immune cells. High expression of the m5C regulator DNMT1 was related to a poor prognosis in patients with HCC. Furthermore, we developed three Immu-clusters that were consistent with the immune characteristics of the m5C methylation modification patterns. We also discovered differences in the levels of immune cells and expression of chemokines and cytokines among the three Immu-clusters.

Conclusions: Our work revealed the association between m5C modification and immune regulators in the TME. These findings also suggest that DNMT1 has great potential as a prognostic biomarker and therapeutic target for HCC.

Background

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the fourth leading cause of cancer-related death worldwide [1]. Risk factors for HCC include hepatitis B virus (HBV), hepatitis C virus (HCV), non-alcoholic fatty liver disease, obesity with diabetes, etc. Patients who are infected with HCV can be treated with antiviral therapies, while patients who are infected with HBV remain infected throughout life [2]. The survival of patients is driven by tumour stage, with a 5-year survival rate exceeding 70% for those with early-stage HCC compared to a median survival time of 1-1.5 years for those with advanced-stage HCC [3]. Most HCC patients are diagnosed at advanced stages, and limited effective therapeutic strategies are available [4].

Tumour cells are the driving cause of tumour development and progression. However, without the tumour microenvironment (TME), tumour cells cannot act alone in the progression of cancer. The TME includes the surrounding blood vessels, fibroblasts, immune cells, extracellular matrix and signalling molecules. These elements contribute to the processes of carcinogenesis and progression, while it is still a major challenge to fully evaluate the complex TME [5].
More than 150 RNA modifications have been found, such as 5-methylcytosine (m\textsuperscript{5}C), N1-methyladenosine (m\textsuperscript{1}A), 5-hydroxymethylcytosine (hm5C), pseudouridine (Ψ), N6-methyladenosine (m\textsuperscript{6}A) and other types of RNA modifications [6, 7]. Previous studies have mainly focused on m6A modification in regulating coding and non-coding RNA processing and function [8]. Emerging evidence has revealed the important role of RNA m5C in posttranscriptional regulation [9]. In addition, m\textsuperscript{5}C modification was found to be abundant in mammalian cells, characterized by the addition of a methyl group at the carbon-5 position of the cytosine base [10]. m\textsuperscript{5}C is mainly distributed in GC-rich areas. Over 10000 potential sites of m\textsuperscript{5}C modification have been detected in the whole human transcriptome [11]. RNA m\textsuperscript{5}C is a dynamic process controlled by three major regulators, termed “writers” (add a special modification), “readers” (identify and bind modified nucleotides), and “erasers” (remove a special modification) [12].

Recently, targeting the TME has been an encouraging method for cancer treatment [13]. Some studies showed a correlation between RNA modification and TME-infiltrating immune cells [14–17]. However, due to technological limitations, the research above was restricted to one or two RNA modification regulators or cell types, while anti-tumour effects involve multiple tumour suppressors interacting in a vitally cooperative way. Hence, a deep understanding of TME cell infiltration mediated by several regulators of RNA modifications will help to enhance the perception of TME immune regulation, especially m\textsuperscript{5}C modification.

In this study, we analysed 371 patients with HCC from The Cancer Genome Atlas (TCGA) database, and the samples were integrated to evaluate m\textsuperscript{5}C modification patterns. Correlation analysis was performed between the m\textsuperscript{5}C modification pattern and TME cell infiltration characteristics. Three different m\textsuperscript{5}C modification patterns were discovered. Surprisingly, a high consistency was detected in TME characteristics under these three patterns, namely, the immune desert phenotype, immune-inflamed phenotype and immune-excluded phenotype, indicating that m\textsuperscript{5}C modification plays an essential role in forming an individual TME.

**Methods**

**HCC data source and preprocessing**

Gene expression and clinical annotation data were downloaded from the TCGA database. Patients without complete survival data were excluded. The TCGA-Liver Hepatocellular Carcinoma (TCGA-LIHC) dataset was used for further analysis. Finally, a total of 371 patient in the TCGA-LIHC cohort were selected for this study.

For the TCGA dataset, the R package TCGAbiolinks [18], which was developed to analyse Genomic Data Commons (GDC) data, was utilized to download the fragments per kilobase per million mapped reads (FPKM) values of gene expression from the GDC (https://portal.gdc.cancer.gov). FPKM values were further converted to transcripts per kilobase million (TPM) values. Batch effects generated by factors
unrelated to any biological variations were corrected for using the parametric and non-parametric empirical Bayes framework algorithm from the sva package. Data related to somatic mutations were downloaded from the TCGA database. R (3.6.1) together with Bioconductor packages were employed in the study.

**Unsupervised clustering analysis of m$^5$C regulators**

A total of 13 m$^5$C regulators were extracted from 371 patients in the TCGA-LIHC cohort: 11 writers (NOP2, NSUN2, NSUN3, NSUN4, NSUN5, NSUN6, NSUN7, DNMT1, TRDMT1, DNMT3A, DNMT3B), 1 eraser (TET2) and 1 reader (ALYREF). Unsupervised clustering analysis was employed to distinguish different m$^5$C modifications, after which the classification of patients was conducted for subsequent analysis.

A consensus clustering algorithm [19] was employed to assure the number of clusters and their stability. The ConsensusClusterPlus package was applied to execute the workflow mentioned above, and the stability of the classification was accomplished by conducting 1000 repetitions [20].

**Gene set variation analysis (GSVA) and functional annotation**

To explore the disparity of biological processes in m$^5$C modification patterns, the “GSVA” R package was used to perform GSVA. This package is based on a non-parametric and unsupervised algorithm and is widely used to estimate the variation in gene set enrichment in expression datasets [21]. GSVA was implemented with “c2.cp.kegg.v6.2.symbols” gene sets obtained from the Molecular Signatures Database (MSigDB). An adjusted P value of less than 0.05 was regarded as statistically significant. We applied the “ClusterProfiler” R package to functionally annotate m$^5$C-related genes under the false discovery rate (FDR) threshold of < 0.05.

**Single-sample gene set enrichment analysis (ssGSEA)**

The ssGSEA algorithm was used to determine the relative richness in cell infiltration in the TME. We obtained the gene set associated with each infiltrating immune cell type in the TME from Charoentong, who stores information on various human immune cells, including CD8 T cells, dendritic cells (DCs), natural killer (NK) T cells, macrophages, regulatory T cells, etc. [22, 23]. ssGSEA was employed to determine the enrichment scores and define the relative abundance of each TME-infiltrating cell type in the corresponding sample.

**Identification of differentially expressed genes (DEGs) among the m5C phenotypes**

With the aim of distinguishing m$^5$C-related genes, all the patients were divided into three m$^5$C modification patterns according to the expression of m$^5$C regulators. The empirical Bayesian algorithm under the limma package in R was used to assure DEGs in heterogeneous modification patterns.
Correlation between the m\textsuperscript{5}C gene signature and biological pathways

A set of genes was constructed by Mariathasan et al. [24–26], in which genes associated with certain biological processes are stored. Correlation analysis was employed to explore the association between the gene signature of m\textsuperscript{5}C and biological pathways.

Immunohistochemical (IHC) staining

Human HCC tissue arrays and normal tissues (catalogue number: HLivH180Su15) were purchased from Shanghai Outdo Biotech Co., Ltd. (Shanghai, P.R. China). The method of IHC staining has been reported previously. Briefly, antigen retrieval was performed by heating the tissue sections at 100°C for 30 min in target retrieval solution. Then, the tissue microarray (TMA) was incubated with a DNMT1 primary antibody ([EPR18453] (ab188453) Abcam, Cambridge, MA), followed by incubation with an anti-rabbit secondary antibody. Two independent pathologists blindly assessed the IHC results according to the staining area and intensity [27].

Statistical analysis

Spearman and distance correlation analyses were performed to obtain the correlation coefficients of the TME-infiltrating immune cells and the corresponding expression of m\textsuperscript{5}C regulators. One-way analysis of variance (ANOVA) and Kruskal-Wallis tests were performed to compare differences between three or more groups [28]. The Kaplan-Meier method was utilized to generate survival curves for the prognostic analysis, and the log-rank test was applied to identify significant differences. Univariate Cox regression was adopted to determine the hazard ratios of m\textsuperscript{5}C regulators and genes related to specific m\textsuperscript{5}C phenotypes. Multivariable Cox regression was utilized to identify independent prognostic risk factors. Patients with complete relevant data were subjected to further analysis with a multivariate model. The multivariate results were visualized with the forestplot package in R. Copy number variations (CNVs) in 13 m\textsuperscript{5}C regulators were plotted with the RCircos package [29]. All P values were two-sided, with p < 0.05 considered statistically significant. The analysis was accomplished in R 3.6.1 software.

Results

Landscape of genetic variations in m\textsuperscript{5}C regulators in HCC

Ultimately, 11 writers, 1 eraser and 1 reader were identified. First, the incidence of CNVs and somatic mutations in regulators in HCC were summarized. In 364 samples, 41 showed mutations in m\textsuperscript{5}C regulators, the occurrence of which was 11.26%. DNMT1 was found to be exposed to a higher frequency of mutations, followed by DNMT3A, while ALYREF, NSUN2, NSUN3, and NSUN5 were not (Fig. 1a). CNVs were also detected in 13 other regulators upon exploration of their modification frequencies. Most of the modifications involved a copy number expansion, but TET2, NOP2, and NSUN4 had a broad occurrence...
of deletions (Fig. 1b). The chromosome sites of the m\(^5\)C regulators are shown in Fig. 1c. Based on the expression of 13 m\(^5\)C regulators in HCC patients, HCC samples could be thoroughly differentiated from normal samples (Fig. 1d). To determine whether the expression of m\(^5\)C regulators was influenced by the genetic mutations mentioned above, the mRNA expression of regulators was explored. We found that a change in m\(^5\)C was an important factor leading to perturbations in the expression of m\(^5\)C regulators. Compared with normal hepatic tissues, the expression of m5C regulators with a CNV expansion was significantly higher than that in HCC tissues (e.g., ALYREF and NSUN2) (Fig. 1b and e). The analyses above showed that the genetic and expression alteration landscape of m5C regulators in normal tissues and HCC tissues is highly heterogeneous, suggesting that the expression imbalance of m\(^5\)C regulators plays an important role in HCC occurrence and progression.

m\(^5\)C methylation alteration patterns mediated by 13 regulators

Univariate Cox regression analysis showed that 13 m5C modulators have prognostic significance in HCC patients (Fig. 2a). The m5C regulator network revealed m5C modulator interactions, modulator connections and their prognostic significance for patients (Fig. 2b). The R package ConsensusClusterPlus was applied to classify patients with qualitatively different m\(^5\)C alteration patterns according to the expression of 13 m\(^5\)C regulators, and unsupervised clustering analysis was performed to identify a total of 3 different modification patterns (120 cases in modification pattern 1, 178 cases in modification pattern 2, and 73 cases in modification pattern 3; referred to as m5C Clusters 1–3, respectively) (Fig. 2c and Table S1). The prognostic analysis of the three major m5C modification subtypes showed that Cluster-2 had a clear survival advantage over the others (Fig. 2d). The above results indicate that the crosstalk between erasers, readers and writers may play an important role in m\(^5\)C alteration patterns and TME cell infiltration characteristics between individual tumours.

TME cell infiltration characteristics in different m\(^5\)C modification patterns

To investigate the biological actions associated with m\(^5\)C modification patterns, GSVA was conducted. As shown in Fig. 2e and Table S2, m\(^5\)C Cluster-2 was remarkably enriched in the activation of stroma and carcinogenesis pathways, such as the ERBB signalling pathway, cell cycle signalling pathway, and cell adhesion pathway. Cluster-1 was associated with enrichment pathways (Fig. 2e). Cluster-3 was highly associated with carcinogenic activation (Fig. 2f). Unexpectedly, further analysis of TME cell infiltration showed that Cluster-1 was significantly enriched in the infiltration of innate immune cells, including eosinophils, NK cells, macrophages, CD8 T cells, mast cells, and active B cells (Fig. 3a). Compared with patients in Cluster-3, patients in Cluster-2 showed a corresponding survival advantage (Fig. 2d). Prior research has shown that tumours with an immune rejection phenotype exhibit large amounts of immune cells, and these immune cells are in the matrix around the tumour cell nest instead of inside the tissue
GSVA showed that the modification of Cluster-1 was significantly related to matrix activation. Therefore, it was speculated that the Cluster-1 matrix serves as an activation inhibitor of the anti-tumour effect of immune cells. Further analysis showed that matrix activity was greatly upgraded, activating the angiogenesis pathway. These results supported our hypothesis (Fig. 3b). Based on the above analysis, we found that the 3 m^5C modification patterns had obvious TME cell infiltration characteristics. Cluster-1 had an immune rejection phenotype, featuring natural infiltrating immune cells and an activated matrix; Cluster-2 had an immunoinflammatory phenotype, featuring adaptively infiltrated immune cells and immune activation; and Cluster-3 had an immune desert phenotype, featuring immune suppression.

The m^5C regulator DNMT1 has a strong relationship with infiltrating immune cells

To further explore the role of each m5C regulator in the TME, Spearman correlation analysis was applied to examine the correlation between each TME-infiltrating cell type and m^5C regulators (Fig. 4a). An emphasis was placed on the regulator DNMT1, an m5C methyltransferase, and we revealed its positive relationship with the infiltration of many TME immune cells. An estimation method was applied to determine the expression of DNMT1 and the infiltration of immune cells. The results showed that higher DNMT1 expression was related to a higher immune score, which means that a TME with high DNMT1 expression has significantly high immune cell infiltration (Fig. 4b). Based on these results, the specific differences in 23 TME-infiltrating immune cells were explored between patients with high and low DNMT1 expression. We found that tumours exhibiting high DNMT1 expression had markedly more infiltration of 13 TME immune cells than those exhibiting low expression (Fig. 4c). Recently, attention was drawn to the regulatory mechanisms of m^5C modification on the activation of DCs, which are the bridge connecting innate immunity with adaptive immunity, the activation of which depends on upregulating the expression of MHC molecules, adhesion molecules and costimulatory molecules (Fig. 4d). As expected, subsequent enrichment analysis showed that tumours with high DNMT1 expression showed remarkable enrichment in immune activation pathways (Fig. 4e). Therefore, it was speculated that m^5C methylation modification mediated by DNMT1 may contribute to activated DCs in the TME, thus promoting the anti-tumour immune response in HCC.

High expression of the m5C regulator DNMT1 in tumour tissues is related to a poor prognosis in patients with HCC

IHC staining was used to determine the expression pattern of DNMT1 on a TMA consisting of 90 pairs of HCC tissues and adjacent tissues. Representative micrographs illustrate the various degrees of DNMT1 expression (Fig. 5a and b). The expression of DNMT1 was higher in tumour tissues than in control tissues (Fig. 5c), which was consistent with the findings in the TCGA-LIHC cohort (Fig. 1e). The correlation of DNMT1 expression with the clinicopathological characteristics of patients with HCC is shown in Table S3. In addition, Kaplan-Meier curve analysis showed that patients with high DNMT1 expression had shorter overall survival (OS) than those with low DNMT1 expression (Fig. 5d). Univariable
and multivariable Cox regression analyses were used to determine whether the expression of DNMT1 was an independent risk factor. The univariable analysis revealed that DNMT1 expression was associated with tumour size and TB, AFP, and PD-L1 levels (P < 0.05, Table S4). Further analysis demonstrated that DNMT1 might serve as a prognostic predictor for HCC.

**Generation of the m⁵C gene signature and functional annotation**

For subsequent exploration of the biological behaviour of each m⁵C modification pattern, we ascertained 307 m⁵C phenotype-related DEGs with the limma package (Fig. 6a). clusterProfiler was employed to implement enrichment analysis on the DEGs. Table S5 summarizes the significantly enriched pathways. As expected, we detected enrichment in biological processes that are notably related to m⁵C modification and immunity, which verified the important role that m⁵C modification plays in immune regulation in the TME (Fig. 6b).

To further explain the association, we performed unsupervised clustering analysis to classify 307 m⁵C phenotype-related genes and extracting 27 immune-related genes: VIPR2, CCL7, RBP2, SLC10A2, FGF5, DEFA5, HTR3A, TRH, LCN15, AMBN, ADIPOQ, FGF3, CCK, NTF4, NDP, FGF9, PF4, CMA1, SFTPA2, CGB8, DEFA6, PF4V1, IL25, GH2, FGF8, SST and IAPP. Furthermore, we performed unsupervised clustering analysis based on these genes to categorize patients into different subtypes (Figure S1a-d). In line with the clustering analysis of m⁵C modification patterns, unsupervised clustering analysis revealed three different m⁵C-modified phenotypes termed Immu-clusters 1–3, respectively. Thus, there are 3 different distinct immune-related m⁵C methylation patterns. We observed that tumours in Immu-clusters 2 and 3 were associated with poor differentiation and enriched in diffuse histological subtypes. The opposite pattern was observed in Immu-Cluster 1. Patients whose survival status was known were mainly concentrated in Immu-cluster 1, while patients in clinical stage IV or with a high TNM grade were mainly concentrated in Immu-cluster 2 (Fig. 6c). The analysis also showed that three different gene clusters had different feature genes (Fig. 6c). In total, 114 of the 317 HCC patients clustered in Immu-cluster 1, which was associated with a better prognosis. The prognosis of patients in Immu-cluster 1 (110 patients) and Immu-cluster 3 (93 patients) was poor (Fig. 6d). In the three immune clusters, a significant distinction in the expression of m5C regulatory factors emerged. This result was consistent with the m5C methylation modification patterns (Fig. 6e).

**Clinical and transcriptional features of the m⁵C-related phenotypes**

To further explain the role that m5C-related phenotypes play in TME immune regulation, the levels of immune cells and expression of chemokines and cytokines in the three Immu-clusters were examined. The chosen cytokines and chemokines were taken from previously existing studies. Our analysis showed that activated CT4 T cells, immature B cells, regulatory T cells, NK cells, macrophages, mast cells,
myeloid-derived suppressor cells (MDSCs), monocytes, neutrophils and plasmacytoid DCs were significantly different among the Immu-clusters (Fig. 7a). Tumour necrosis factor, interferon, CD8A, CXCL9, CXCL10, GZMA, GZMB, PRF1 and TBX2 were associated with immune activation transcription (Fig. 7b) [23, 31]. PD-L1, CD80, CD86, CTLA-4, HAVCR2, etc., were thought to be related to the transcription of immune checkpoints. We compared the transcription of these immune checkpoint genes in the three Immu-clusters (Fig. 7c). ACTA2, CLDN3, VIM, COL4A1, SMAD9, TWIST1, TGFBR2, TGRB1 and ZEB1 are related to the transcription of growth factor β/EMT pathway transformation and exhibited significant differences between the three Immu-clusters (Fig. 7d). We found that mRNAs related to the TGF-β/EMT pathway were significantly upregulated in Immu-cluster 2 and Immu-cluster 3, indicating that this cluster is the matrix-activated group. Immu-cluster 1 showed elevated expression of mRNAs related to activated immune transcription. The above results indicated that Immu-cluster 1 could be categorized into an immune-activated group. The above results also showed that m5C modification has a non-negligible regulatory effect on the formation of different TMEs.

Discussion

According to previous reports, tumours, including HCC, are mainly driven by genetic mutations. In recent years, epigenetic modifications have been found to play a critical role in the carcinogenesis and molecular pathogenesis of HCC [8, 9]. m5C is the most preventative and best understood DNA modification in eukaryotes [32]. In recent years, emerging evidence has revealed the important role of RNA m5C in posttranscriptional regulation. Several studies have revealed that m5C regulators and m5C methylation play essential roles in different cancer types, including HCC. He et al. [33] found that ALYREF and NSUN4 could be promising targets for HCC therapies. In addition, studies showed the map of m5C methylation based on HCC tissues and paired non-tumour tissues at the mRNA, lncRNA, and circRNA levels [34–36]. Recent studies showed that NSUN2 could promote tumour progression in HCC [37] and gastric cancer [38]. Similar to our findings, Xue et al. [39, 40] found that DNMT1 played important roles in head and neck squamous cell carcinoma.

Recently, increasing evidence has shown interactions between the tumour immune-microenvironment (TIME) and RNA modifications. Yi et al. [17] reported that copy number alterations in m6A methylation regulators affected immune cell infiltration in head and neck squamous cell carcinoma. Lin and colleagues also attempted to explore the relationship between m6A regulators and tumour-infiltrating immune cells by ssGSEA in glioma. Shen et al. [16] found that m6A modification patterns were correlated with immune regulation in HCC and might provide novel immune therapeutic targets. However, as an important posttranscriptional modification, the role of RNA m5C methylation in the immune regulation of HCC is still unclear. Here, we described the TME cell infiltration characteristics in different m5C modification patterns. Furthermore, we identified 3 distinct immune-related m5C methylation subtypes and investigated the levels of immune cells and expression of chemokines and cytokines in the three Immu-clusters. All the results indicate that the generation of immune-related m5C methylation subtypes contribute to understanding the molecular mechanisms of HCC and provide novel clues for predicting the prognosis of patients with HCC.
It has been demonstrated that DNMT1 is an essential methyltransferase for the maintenance of DNA methylation. Previous evidence has shown that DNMT1 is overexpressed in breast cancer [41], thyroid cancer cells [42], and pancreatic cancer [43]. Furthermore, high DNMT1 expression is significantly associated with a poor prognosis [44, 45]. Consistent with our results, we found that DNMT1 expression was increased in tumour tissues compared with normal tissues in the TMA and TCGA cohort. In our study, Kaplan-Meier curve analysis and univariable and multivariable Cox regression analysis further demonstrated that the expression of DNMT1 is an independent risk factor for HCC. Therefore, DNMT1 might serve as a promising prognostic predictor and therapeutic target for HCC.

**Conclusions**

Taken together, our results showed the association between m5C modification and distinct immune phenotypes. Moreover, we found a key m5C modification regulator, DNMT1, which has great potential as a prognostic biomarker and therapeutic target for HCC.

**Abbreviations**

m5C, 5-Methylcytosine; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; GSVA, gene set variation analysis; ssGSEA, single-sample gene set enrichment analysis; IHC, immunohistochemical; TME, tumour microenvironment; TMA, tissue microarray; HBV, hepatitis B virus; HCV, hepatitis C virus; m1A, N1-methyladenosine; hm5C, 5-hydroxymethylcytosine; Ψ, pseudouridine; m6A, N6-methyladenosine; GDC, Genomic Data Commons; FPKM, fragments per kilobase per million mapped reads; TPM, transcripts per kilobase million; GSVA, Gene set variation analysis; MSigDB, Molecular Signatures Database; FDR, false discovery rate; DCs, dendritic cells; NK, natural killer; DEGs, differentially expressed genes; ANOVA, one-way analysis of variance; CNVs, copy number variations; OS, overall survival; MDSCs, myeloid-derived suppressor cells; TIME, tumour immune-microenvironment.

**Declarations**

**Ethical approval and consent to participate**

The study has been approved by the institutional ethical committee and all patients have signed the informed consent.

**Consent for publication**

Consent to publish was obtained from all authors.

**Availability of data and materials**

All data in our study are available upon request.

**Competing interests**
The authors declare no competing interests.

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**Authors’ Contributions**

XG, HZ and QC have equal contributions to this study. XG and HZ designed the whole study. QZ and JW conducted the statistical analysis. XG and QC draft the manuscript. QC made the relevant edits to the manuscript. XG and HZ revised the manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

CNVs and somatic mutations in 13 m5C regulators in HCC. a. Mutation frequencies of the top 9 m5C regulators. b. CNV alterations among the 13 regulators. c. Locations of mutations in the m5C regulators at the chromosome level. d. Principal component analysis was used to distinguish tumour tissues and normal tissues based on the expression of m5C regulators. e. The expression profiles of m5C regulator genes in tumour tissues and adjacent normal tissues.
Figure 2

m5C methylation alteration patterns and related biological characteristics. a. Univariate Cox regression analysis of the 13 m5C regulators in patients with HCC. b. The network of m5C regulators and their prognostic significance for HCC patients. c. Unsupervised clustering analysis of 13 m5C regulators in HCC. d. Survival analysis of HCC patients in the TCGA-LIHC cohort according to the three m5C clusters. e-f. A heatmap of GSVA results shows the representative hallmark pathways associated with distinct m5C modification patterns.
Figure 3

TME characteristics in different m5C modification patterns. a Comparison of the abundance of immune infiltrating cells in three clusters. b. Differences in cellular biological pathways among the three clusters.
Figure 4

Association of TME-infiltrating cells with the m5C regulator of DNMT1. a. Correlation between m5C regulators and different immune cells using Spearman analysis. b. Immune scores of the low DNMT1 group and the high DNMT1 group. c. Comparison of the abundance of immune-infiltrating cells in the low DNMT1 group and high DNMT1 group. d. Correlation between m5C regulators and the activation of dendritic cells. E. High DNMT1 expression shows significant enhancement of the immune-activated pathway.
Figure 5

Expression of DNMT1 in human HCC tumour tissues and control tissues. a. Panoramic scanning of DNMT1 by IHC staining. b. Representative IHC staining of DNMT1 in samples. c. The expression of DNMT1 is higher in HCC tissues than in normal tissues. d. Kaplan–Meier analysis showed that patients with higher levels of DNMT1 had shorter OS times than those with low levels of DNMT1.
Identification of distinct Immu-clusters based on immune-related DEGs in m5C modification patterns.

a. A total of 307 m5C-related DEGs between three m5C clusters were identified, as shown in the Venn diagram.

b. Enrichment of biological processes significantly related to DEGs.

c. The selected genes were used to classify patients into different genomic subtypes by unsupervised clustering analysis.

d. Kaplan–Meier curves indicated that the genomic subtypes were correlated with the prognosis of patients with HCC.

e. Significant differences in the expression of m5C regulators.

Figure 6
Figure 7

Association between the expression of m5C regulators and immunoregulation in the TME. a. Differences in immune cell infiltration in the three Immu-clusters. b. Comparison of immune-related cytokine expression in the three Immu-clusters. c. Comparison of the transcription of immune checkpoint genes in the three Immu-clusters. d. Immu-clusters involved in the transcription of the TGF-β/EMT pathway.

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