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Research Paper

# Distinct Diagnostic and Prognostic Values of Minichromosome Maintenance Gene Expression in Patients with Hepatocellular Carcinoma

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## Abstract

**Background:** The aim of the present study was to identify diagnostic and prognostic values of minichromosome maintenance (MCM) gene expression in patients with hepatocellular carcinoma (HCC).

**Methods**: The biological function of the MCM genes were investigated by bioinformatics analysis. The diagnostic and prognostic values of the MCM genes were investigated by using the data of HCC patients from the GSE14520 and The Cancer Genome Atlas (TCGA) databases.

**Results**: Bioinformatics analysis of the MCM genes substantiated that MCM2–7 genes were significantly enriched in DNA replication and cell cycle, and co-expressed with each other. These genes also co-expressed in HCC tumor tissue in both the GSE14520 and TCGA cohort. We also observed that the expression of the MCM2–7 genes was increased in tumor tissue, and diagnostic receiver operating characteristic analysis of MCM2–7 indicated that these genes could serve as sensitive diagnostic markers in HCC. Survival analysis in the GSE14520 cohort suggested that expression of *MCM2*, *MCM4*, *MCM5*, and *MCM6* were significantly associated with hepatitis B virus-related HCC overall survival (OS). However, none of the MCM genes were associated with recurrence-free survival in the GSE14520 cohort. The validation cohort of TCGA suggested that the expression of *MCM2*, *MCM6*, and *MCM7* were significantly correlated with HCC OS.

**Conclusion**: Our study indicated that MCM2–7 genes may be potential diagnostic biomarkers in patients with HCC. Among them, *MCM2* and *MCM6* may serve as potential prognostic biomarkers for HCC.

Key words: minichromosome maintenance, mRNA, hepatocellular carcinoma, prognosis, diagnosis

## Introduction

Liver cancer is more common in males than females and has become the second leading cause of cancer-related death worldwide and in developing countries in 2012 [1]. Approximately half of the new cases and deaths involving liver cancer worldwide occurred in China in 2012. Moreover, liver cancer was the third leading cause of cancer-related death in China in 2015 [1, 2]. Therefore, the early detection and

management of liver cancer would be valuable. Most liver cancers are diagnosed as hepatocellular carcinoma (HCC) [3]. As with other cancers, hepatocarcinogenesis is also derived from genetic and environmental factors. Furthermore, genes that are dysregulated between tumors and normal tissues are the most promising source of diagnostic and prognostic biomarkers [4-6].

Minichromosome maintenance (MCM) genes play an essential role in DNA replication and include six highly related MCM genes (*MCM2, MCM3, MCM4, MCM5, MCM6,* and *MCM7*) [7, 8]. Numerous studies have demonstrated that MCM genes play essential roles in various cancers, especially in cancer diagnosis and prognosis prediction [9-13]. However, a comprehensive analysis of the diagnostic and prognostic values of MCM genes in HCC still needs further in-depth investigation. The aim of the present study was to identify the diagnostic and prognostic values of MCM gene expression in patients with HCC based on information from public databases and bioinformatics analysis.

## Materials and Methods

### Bioinformatics analysis of MCM genes

In order to investigate the biological functions and pathways involving the MCM genes, gene function enrichment analysis of MCM genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/home.jsp, accessed December 15, 2017) version 6.8[14, 15]. An enrichment *P*-value <0.05 was considered statistically significant. We also investigated the Gene Ontology (GO) terms of MCM genes by using the Biological Networks Gene Ontology tool (BiNGO) in Cytoscape_version 3.4.0[16]. Investigation of gene-gene and protein-protein interactions of MCM genes were performed by GeneMANIA (http://www.genemania.org/, accessed December 15, 2017) [17, 18] and the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, https://string-db.org/, accessed December 15, 2017) [19, 20], respectively.

### Data source

The GSE14520 dataset of MCM gene expression and corresponding clinical data of hepatitis B virus (HBV)-related HCC were downloaded from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14520, accessed December 15, 2017) [21, 22]. To validate the results obtained from GSE14520 and generalize these results to HCC, a gene expression dataset from HCC patients was obtained from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/, accessed December 15, 2017) and used as the verification cohort [23]. The corresponding clinical information of TCGA HCC patients was downloaded from the University of California, Santa Cruz Xena browser (UCSC Xena: http://xena.ucsc.edu/, accessed December 15, 2017). The datasets included in the current study were downloaded from public databases, therefore there was no need for the study to be approved by an additional ethics committee.

### Association analysis and diagnostic value assessment

The comparison between HCC tumor tissues and adjacent normal liver tissues were evaluated by the Student's *t*-test. Pearson's correlation coefficient was used to evaluate correlations among genes in co-expression analysis and visualized by the *corrplot* package in the R platform. The additional analysis of MCM mRNA expression between normal liver tissue and primary liver cancer tissue was performed by Metabolic gEne RApid Visualizer (MERAV, http://merav.wi.mit.edu/, accessed December 15, 2017) [24]. Diagnostic values of the MCM genes in distinguishing HCC tumors from adjacent normal liver tissue were performed using the receiver operating characteristic (ROC) curve calculated using SPSS software.

### Survival analysis

All patients were divided into two groups according to the median value of gene expression levels in tumor tissues for survival analysis. Based on the survival analysis results of a single MCM gene, we also investigated the joint effects survival analysis of the MCM genes that were significantly correlated to HCC prognosis.

### Prognostic signature construction

We investigated a prognostic model based on the expression of prognostic MCM genes. A prognosis risk score was established on the basis of a linear combination of gene expression levels multiplied by a regression coefficient(β) as the weight that was derived from a multivariate Cox proportional hazards regression model with the prognostic genes fitting the multivariate Cox regression model with OS as a dependent variable. The risk score formula was as follows: Risk score=expression of $gene_1$ × $\beta_1 gene_1$ + expression of $gene_2 \times \beta_2 gene_2$+…expression of $Gene_n \times \beta_n Gene_n$ [25-28]. Patients were divided into high and low risk groups according to the risk score median values. In order to evaluate the predictive accuracy of this gene expression-based prognostic signature in HCC outcome, a time-dependent ROC curve was constructed using the *survivalROC* package in the R platform [29].

### Gene set enrichment analysis

To investigate the difference of biological functions and pathways between high and low expression groups of these prognostic MCM genes in HCC survival, gene set enrichment analysis (GSEA, http://software.broadinstitute.org/gsea/index.jsp, accessed December 15, 2017) [30, 31] was used to investigate potential mechanisms in the Molecular Signatures Database (MSigDB) of c2(c2.all.v6.1.symbols) and c5 (c5.all.v6.1.symbols) [32]. The enrichment gene sets in GSEA that reached a nominal *P*-value <0.05 and false discovery rate (FDR) <0.25 were considered statistically significant.

### Statistical analysis

FDRs in the GSEA were adjusted for multiple testing with the Benjamini–Hochberg procedure to control FDR [33-35]. Univariate survival analysis of clinical features and MCM genes were compared using the log-rank test; those clinicopathological parameters significantly associated with OS ($P < 0.05$) were entered into the multivariate Cox proportional hazards regression model for adjustment, whereas, hazard ratios (HRs) and 95% confidence intervals (CIs) were used to assess the relative risk in different HCC patients that were stratified by the expression of the MCM genes. Co-expression relationships between MCM genes were assessed by the Pearson's correlation coefficient. All statistical analyses were conducted with SPSS software, version 20.0 (IBM Corporation, Armonk, NY, USA) and R 3.3.0. A *P*-value <0.05 was considered statistically significant.

## Results

### Bioinformatics analysis of the MCM genes

GO term enrichment analysis of the MCM genes, performed using DAVID, suggested that MCM genes were significantly enriched in DNA replication-related biological processes and the G1/S transition of the mitotic cell cycle (**Figure 1A**). However, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using DAVID indicated that all of the MCM genes were significantly associated with DNA replication and the cell cycle signaling pathway (**Figure 1B, Figure S1 and S2**). The directed acyclic graph of MCM genes that was constructed by BiNGO in Cytoscape also suggested that the most significant biological function of these genes was in DNA replication (**Figure S3**). Gene-gene and protein-protein interaction networks substantiated that the MCM genes had a strong protein homology and co-expression with each other at both the gene and protein levels (**Figure 2A and 2B**).

### Data source

In order to avoid the batch effect of microarray data in GSE14520, only the dataset of Affymetrix HT Human Genome U133A Array of GSE14520 was included in the current study. Because most of the patients in GSE14520 were HBV-related HCC, we excluded those patients without HBV infection reports and survival information. As a result, there were 212 HBV-related HCC tumor tissues and 204

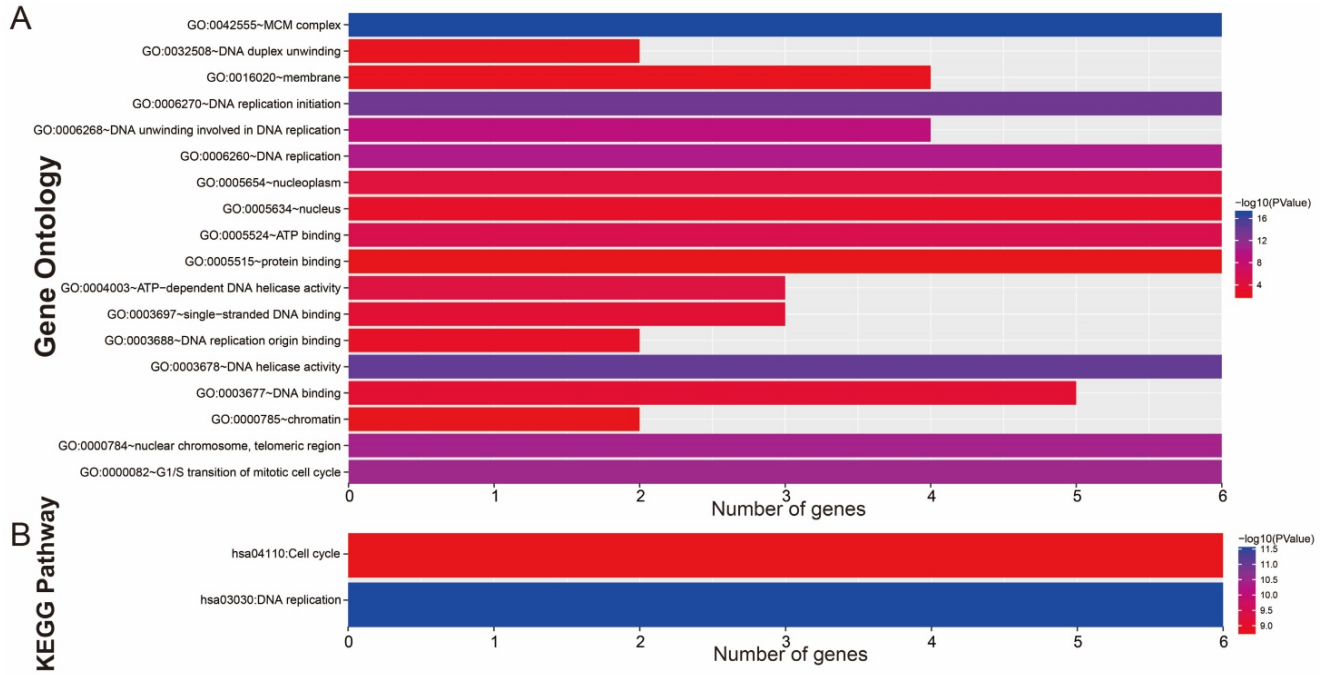


**Figure 1.** GO term and KEGG analysis of MCM2–7 genes. (A) GO term enrichments of MCM2–7 genes. (**B**) KEGG enrichments of MCM2–7 genes.

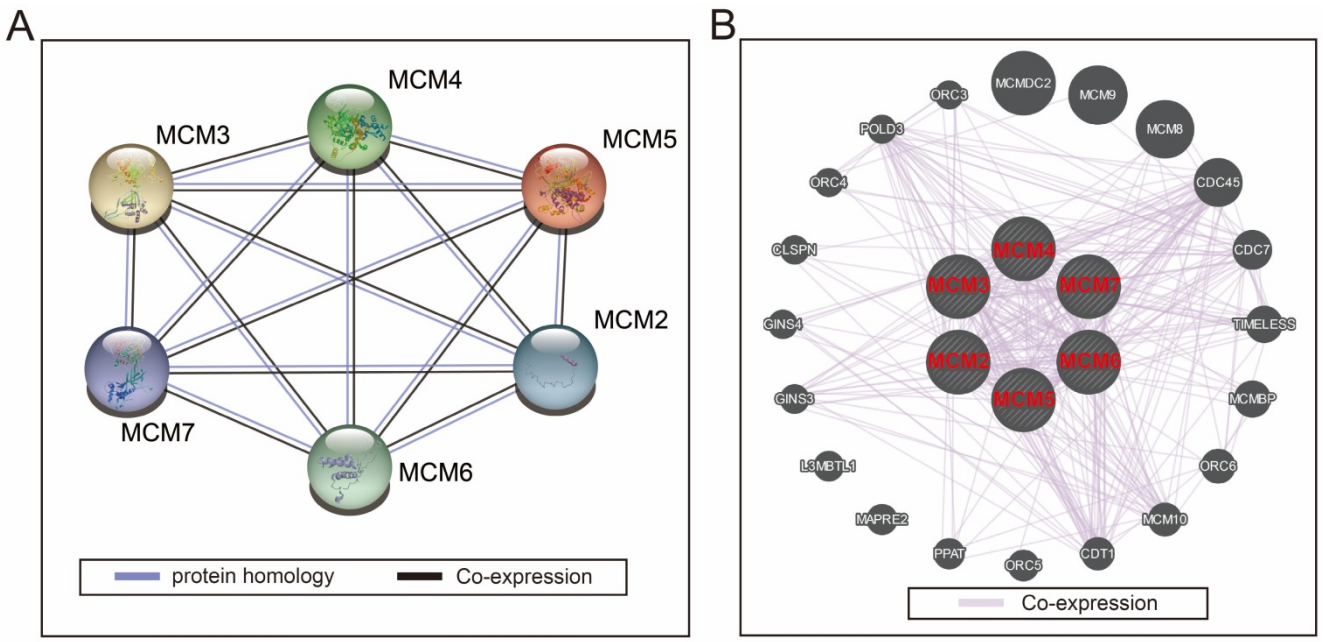


**Figure 2.** Protein-protein and gene-gene interaction networks of MCM2–7 genes. (A) Protein–protein interaction networks; (B) GeneMANIA interaction networks.

adjacent normal liver tissues included in the current study, and all of the 212 HBV-related HCC patients had prognosis information. The raw data of the GSE14520 genome-wide expression profile were processed according to the manufacturer's guidelines (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM362959). For multiple probe sets, the average value corresponding to the same gene was regarded as the gene's expression value and normalized by the *limma* package in R platform. In the validation cohort of HCC patients from TCGA, there were 371 primary tumor tissues and 50 adjacent normal liver tissues that were included in the current study. Of these, 370 HCC patients with prognosis information were used in the survival analysis. The RNA sequencing data of TCGA HCC genome-wide expression profile datasets were normalized by the *DESeq* package in the R platform.

## Association analysis and diagnostic value assessment

Co-expression analysis of MCM genes in HCC tumor tissues was assessed by Pearson's correlation coefficient. All the MCM genes were co-expressed strongly with each other in both the GSE14520 and TCGA cohort (**Figure 3A and 3B**). When comparing the expression of MCM genes between tumor tissues and adjacent normal liver tissues, we observed that all MCM genes were significantly upregulated in HCC tumor tissue in both the GSE14520 and TCGA cohorts (**Figure 3C and 3D**). Additional comparison of the MCM genes expression between normal liver tissue and primary liver cancer tissue was performed by MERAV. We observed a marked increase of expression in all the MCM genes in liver tumor tissue (**Figure S4**).

Because the MCM genes were significantly upregulated in HCC tumor tissue, the potential application of MCM genes in distinguishing HCC tumor tissues and adjacent normal liver tissues was also explored. The ROC analysis of MCM genes in the GSE14520 HBV-related HCC cohort indicated that all the MCM genes had high accuracy in distinguishing tumor tissues and adjacent normal liver tissues (the area under the curve [AUC] of the ROC curves of all MCM genes was >0.90, **Figure 4A–F**). The MCM genes of the TCGA HCC cohort showed a high accuracy in distinguishing tumor tissues and adjacent normal liver tissues (the AUC of the ROC curves of all MCM genes was >0.88; **Figure 5A–F**).

## Survival analysis

In the GSE14520 HBV-related HCC cohort, we observed that patients with advanced BCLC stage and cirrhosis were at significantly increased risk of HBV-related HCC death and recurrence (**Table 1**). Male patients also have a high risk of recurrence in HBV-related HCC, whereas, patients with tumor sizes >5 cm and serum α-fetoprotein (AFP) >300 ng/ml also had a significantly increased risk of death (**Table 1**). The other clinical features in the GSE14520 cohort do not show a significant association with HBV-related HCC recurrence-free survival (RFS) and overall survival (OS).

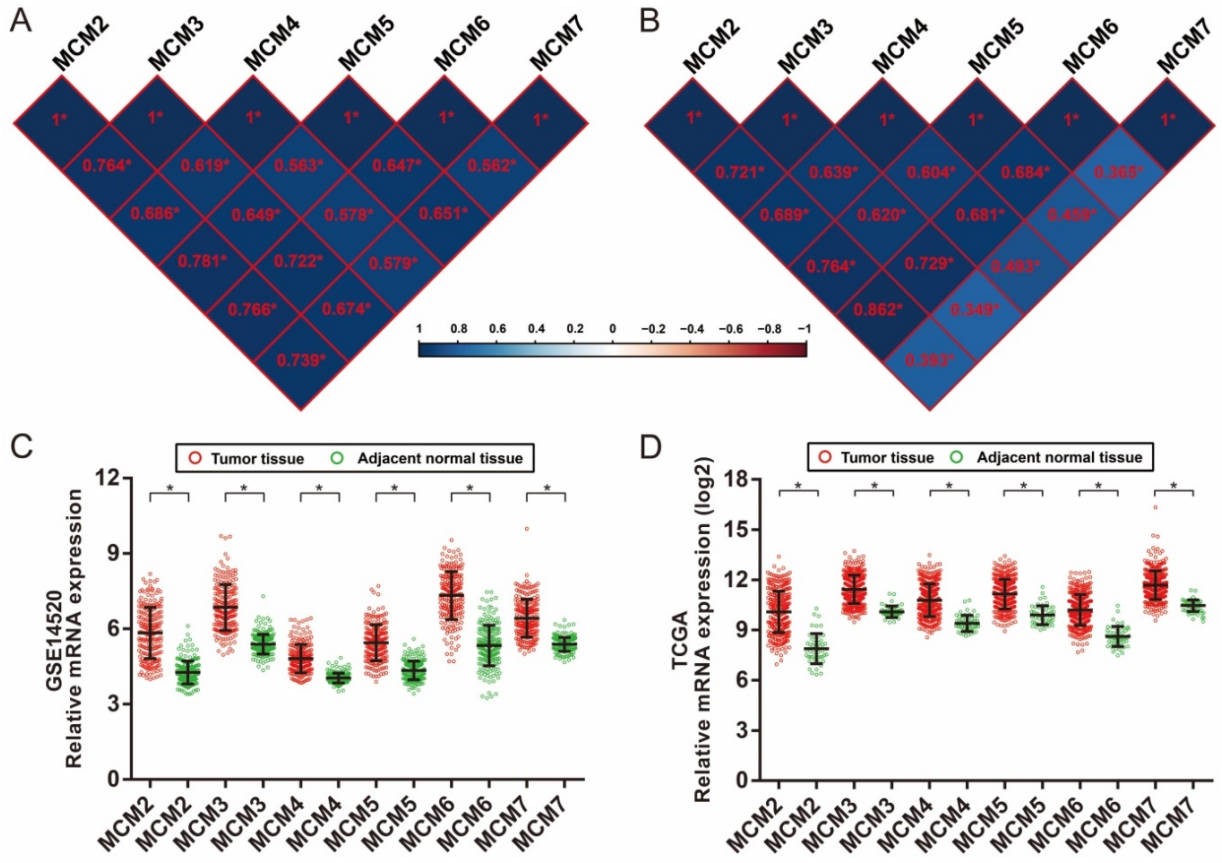


**Figure 3.** Co-expression heat map and gene expression distribution of MCM genes in GSE14520 and TCGA cohort.(A) co-expression heat map of MCM genes in GSE14520; (B) co-expression heat map of MCM genes in TCGA; (C) gene expression distribution of MCM genes in GSE14520; (D) gene expression distribution of MCM genes in TCGA.* $P < 0.0001$.

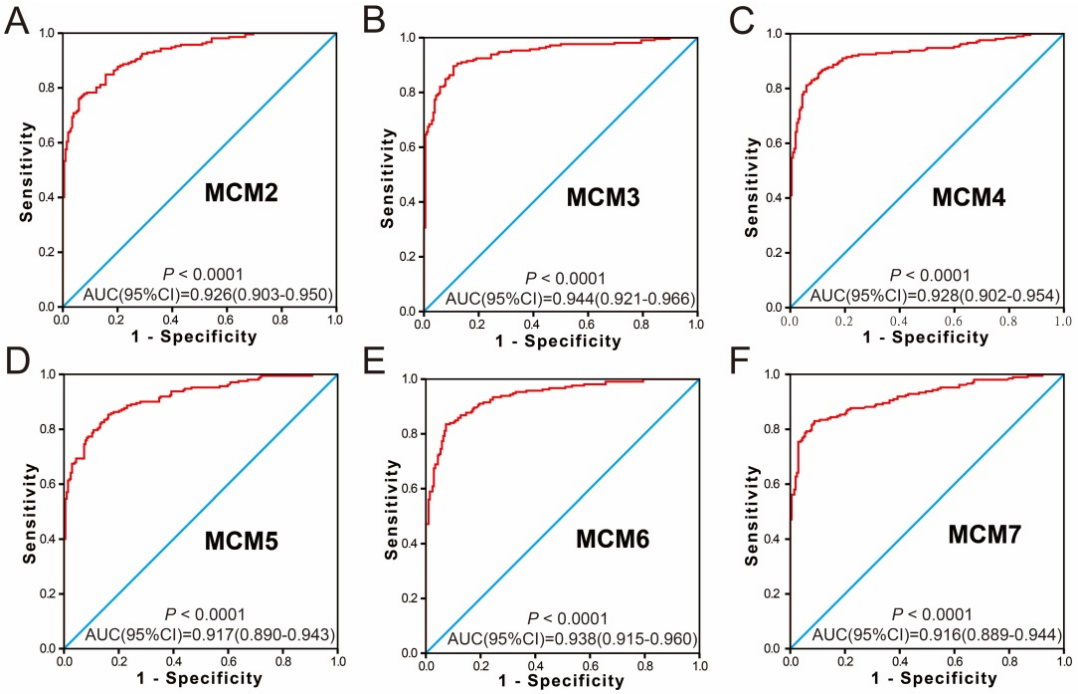


**Figure 4.** The ROC curves of MCM gens in distinguish HBV-related HCC tumor tissue and adjacent normal tissues in GSE14520 cohort. ROC curves of *MCM2* (A); *MCM3* (B); *MCM4* (C); *MCM5* (D); *MCM6* (E); *MCM7* (F).

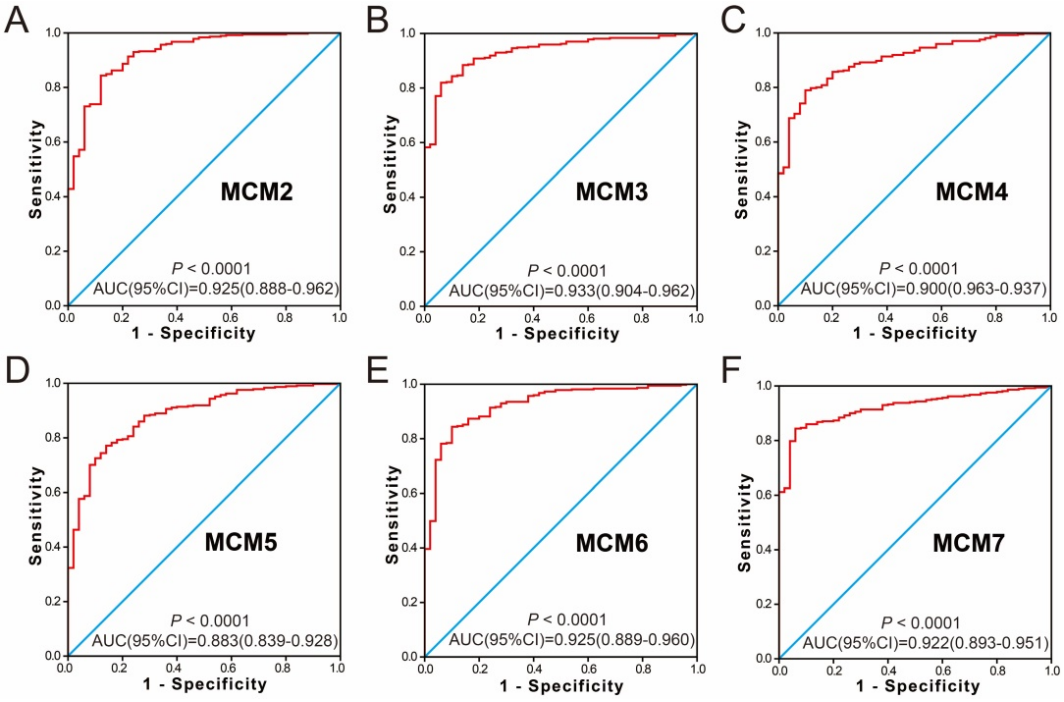


**Figure 5.** The ROC curves of MCM gens in distinguish HCC tumor tissue and adjacent normal tissues in TCGA cohort. ROC curves of *MCM2* (A), *MCM3* (B), *MCM4* (C), *MCM5* (D), *MCM6* (E), and *MCM7* (F).

**Table 1.** Clinical characteristics of HBV-related HCC patients in GSE14520 cohort

| Variables | Patients (n=212) | RFS | | | | OS | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | No. of events | MRT (months) | HR (95% CI) | P | No. of events | MST (months) | HR (95% CI) | P |
| **Age(years)** | | | | | | | | | |
| **≤60** | 175 | 96 | 45 | 1 | | 69 | NA | 1 | |
| **>60** | 37 | 20 | 48 | 0.974(0.602-1.578) | 0.916 | 13 | NA | 0.864(0.478-1.564) | 0.63 |
| **Gender** | | | | | | | | | |
| **Female** | 29 | 10 | NA | 1 | | 8 | NA | 1 | |
| **Male** | 183 | 106 | 40 | 2.143(1.120-4.100) | 0.021 | 74 | NA | 1.704(0.821-3.534) | 0.152 |
| **Multinodular** | | | | | | | | | |
| **Single** | 167 | 90 | 49 | 1 | | 59 | NA | 1 | |
| **Multiple** | 45 | 26 | 28 | 1.216(0.785-1.883) | 0.382 | 23 | 47 | 1.607(0.992-2.604) | 0.054 |
| **Tumor Size&** | | | | | | | | | |
| **≤5 cm** | 137 | 73 | 51 | 1 | | 46 | NA | 1 | |
| **>5 cm** | 74 | 43 | 28 | 1.409(0.966-2.056) | 0.075 | 36 | 53 | 1.975(1.274-3.060) | 0.002 |
| **Cirrhosis** | | | | | | | | | |
| **NO** | 17 | 5 | NA | 1 | | 2 | NA | 1 | |
| **Yes** | 195 | 111 | 37 | 2.612(1.066-6.402) | 0.036 | 80 | NA | 4.335(1.065-17.638) | 0.041 |
| **BCLC stage** | | | | | | | | | |
| **0** | 20 | 6 | NA | 1 | | 2 | NA | 1 | |
| **A** | 143 | 74 | 51 | 2.050(2.892-4.711) | 0.091 | 48 | NA | 4.119(1.001-16.951) | 0.05 |
| **B** | 22 | 15 | 26 | 4.019(1.550-10.421) | 0.004 | 12 | 46 | 8.992(2.005-40.320) | 0.004 |
| **C** | 27 | 21 | 8 | 6.163(2.477-15.333) | <0.001 | 20 | 13 | 18.993(4.419-81.632) | <0.001 |
| **Serum AFPφ** | | | | | | | | | |
| **≤300 ng/ml** | 115 | 62 | 48 | 1 | | 39 | NA | 1 | |
| **>300 ng/ml** | 94 | 54 | 35 | 1.200(0.833-1.728) | 0.328 | 43 | NA | 1.546(1.002-2.385) | 0.049 |

**Notes**: &Information of tumor size was unavailable in 1 patients; φ Information of serum AFP was unavailable in 3 patients. HBV, hepatitis B virus; HCC, hepatocellular carcinoma; BCLC, Barcelona Clinic Liver Cancer; AFP, α-fetoprotein; MRT, median recurrence time; MST, median survival time; RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; NA, not available.

The survival analysis of MCM genes are shown in **Figure 6A–L** and **Table 2**, suggesting that patients with a high expression of MCM genes in the GSE14520 cohort seem to have a longer RFS in HBV-related HCC (**Table 2, Figure 6A–F**) compared to patients with a low expression, however, the *P* values did not reach statistical significance. Patients with high expression of *MCM2* (adjusted *P* =0.043; adjusted HR=1.587; 95% CI=1.016–2.480; **Table 2; Figure 6G**), *MCM4* (adjusted *P* =0.043; adjusted HR=1.577; 95%CI=1.014–2.543; **Table 2; Figure 6I**), *MCM5* (adjusted *P* =0.003; adjusted HR=1.991;

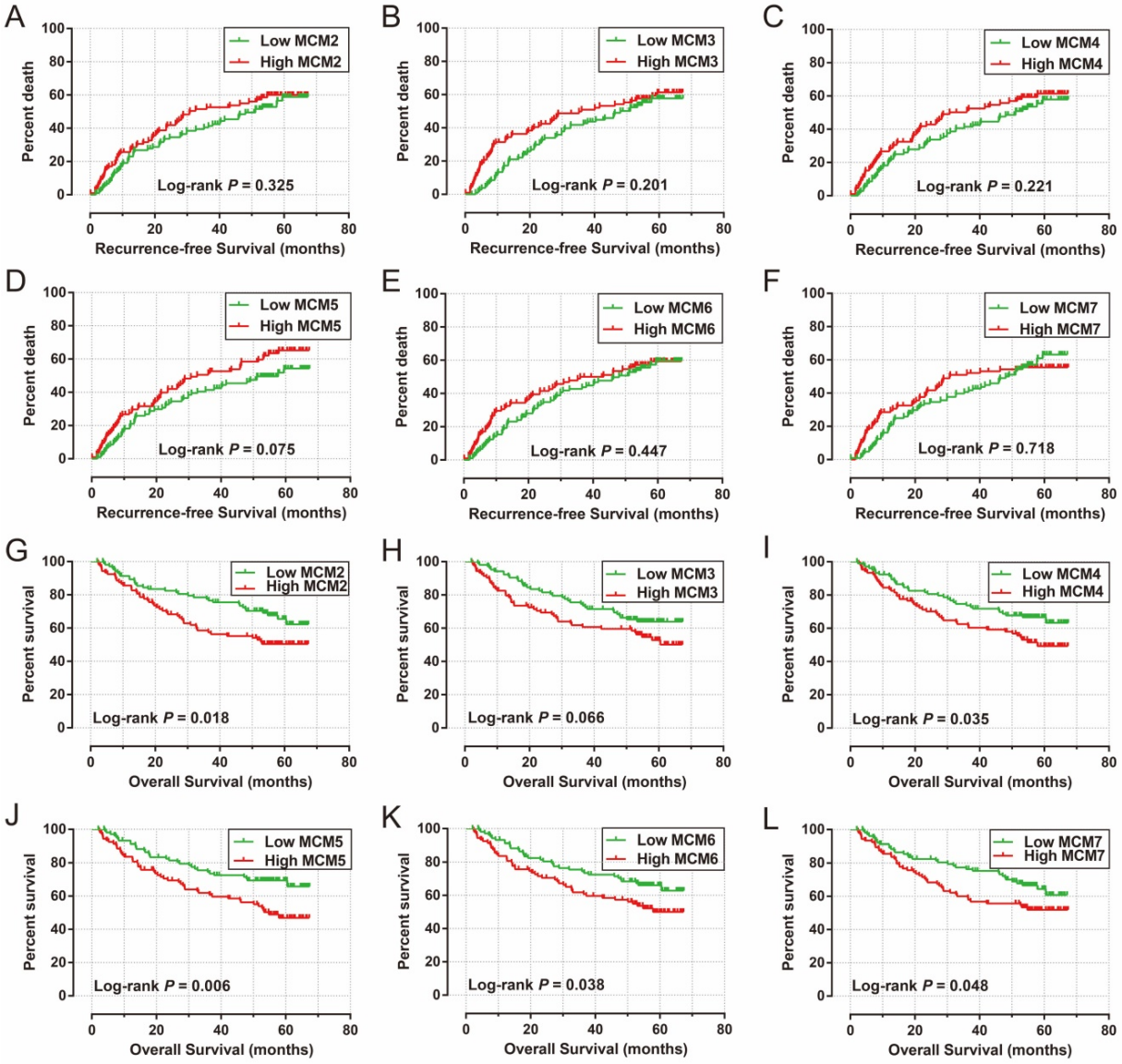


**Figure 6.** Kaplan–Meier survival curves for MCM gens in HBV-related HCC of GSE14520 cohort. RFS stratified by *MCM2* (A), *MCM3* (B), *MCM4* (C), *MCM5* (D), *MCM6* (E), and *MCM7* (F). OS stratified by *MCM2* (G), *MCM3* (H), *MCM4* (I), *MCM5* (J), *MCM6* (K), and *MCM7* (L).

95%CI=1.272–3.117; **Table 2; Figure 6J**), and *MCM6* (adjusted *P*=0.046; adjusted HR=1.572; 95% CI= 1.008–2.452; **Table 2; Figure 6K**) were significantly associated with OS in HBV-related HCC, after adjusting for tumor size, cirrhosis, and BCLC stage.

To verify and generalize the results obtained from the GSE14520 cohort, we also assessed the prognostic values of MCM genes expression in HCC OS prediction in the HCC patients from the TCGA cohort. The clinical characteristics of HCC patients in the TCGA cohort are summarized in **Table 3**. Patients with tumor stage III/IV (*P* <0.0001; HR=2.764; 95% CI=1.823–4.190; **Table 3**) and without radical resection (*P* =0.007; HR=2.030; 95% CI=1.213–3.395; **Table 3**) had a significantly increased risk of death from HCC, and this data was adjusted in the multivariate Cox proportional hazards model. Survival analysis of MCM genes in TCGA HCC patients are shown in **Table 4** and **Figure 7A–F**. The mRNA expression of *MCM2* (adjusted *P* =0.02; adjusted HR=1.574; 95% CI=1.073–2.309; **Table 4; Figure 7A**), *MCM6* (adjusted *P* =0.015; adjusted HR=1.603; 95% CI=1.094–2.350; **Table 4; Figure 7E**), and *MCM7* (adjusted *P* =0.003; adjusted HR=1.793; 95% CI=1.222–2.630; **Table 4; Figure 7F**) were significantly associated with HCC OS in the TCGA cohort.

After performing survival analysis in both the GSE14520 and TCGA cohorts, we found that both the expression of *MCM2* and *MCM6* genes were significantly associated with HCC OS in these two cohorts. Therefore, we investigated the joint effects of *MCM2* and *MCM6* expression in the OS of HCC patients. In the GSE14520 cohort, patients with both low expression of *MCM2* and *MCM6* had a significa-

ntly decreased risk of death in HBV-related HCC (adjusted *P* =0.025; adjusted HR=0.562; 95% CI= 0.339–0.929; **Table 5; Figure 8A**), compared to patients with high expression of both *MCM2* and *MCM6*. Similar results were found in the TCGA cohort (both the low *MCM2* and *MCM6* groups vs. both the high *MCM2* and *MCM6* groups, adjusted *P* =0.01; adjusted HR=0.584; 95% CI=0.388–0.881; **Table 5; Figure 8B**). In addition, we observed that patients with high *MCM2* and low *MCM6* had a significantly decreased risk of death in the TCGA HCC cohort (adjusted *P* =0.044; adjusted HR=0.444; 95% CI=0.202–0.977; **Table 5; Figure 8B**), compared to the patients with a high expression of both *MCM2* and *MCM6*.

**Table 2.** Prognostic values of MCM genes expression in HBV-related HCC of GSE14520 cohort

| Gene expression | Patients (n=212) | RFS | | | | | | OS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | NO. of event | MRT (months) | Crude HR (95% CI) | Crude P | Adjusted HR (95% CI) | Adjusted P § | NO. of event | MST (months) | Crude HR (95% CI) | Crude P | Adjusted HR (95% CI) | Adjusted P § |
| MCM2 | | | | | | | | | | | | | |
| Low | 106 | 57 | 51 | 1 | | 1 | | 34 | NA | 1 | | 1 | |
| High | 106 | 59 | 30 | 1.200(0.834-1.728) | 0.326 | 1.125(0.776-1.629) | 0.534 | 48 | NA | 1.693(1.090-2.629) | 0.019 | 1.587(1.016-2.480) | 0.043 |
| MCM3 | | | | | | | | | | | | | |
| Low | 106 | 56 | 48 | 1 | | 1 | | 36 | NA | 1 | | 1 | |
| High | 106 | 60 | 36 | 1.268(0.880-1.826) | 0.202 | 1.306(0.905-1.885) | 0.154 | 46 | NA | 1.502(0.971-2.324) | 0.068 | 1.516(0.976-2.354) | 0.064 |
| MCM4 | | | | | | | | | | | | | |
| Low | 106 | 56 | 51 | 1 | | 1 | | 35 | NA | 1 | | 1 | |
| High | 106 | 60 | 30 | 1.255(0.872-1.807) | 0.222 | 1.285(0.891-1.854) | 0.179 | 47 | 57 | 1.596(1.030-2.474) | 0.037 | 1.577(1.014-2.543) | 0.043 |
| MCM5 | | | | | | | | | | | | | |
| Low | 106 | 53 | 57 | 1 | | 1 | | 32 | NA | 1 | | 1 | |
| High | 106 | 63 | 32 | 1.392(0.966-2.007) | 0.076 | 1.427(0.985-2.066) | 0.06 | 50 | 54 | 1.857(1.191-2.895) | 0.006 | 1.991(1.272-3.117) | 0.003 |
| MCM6 | | | | | | | | | | | | | |
| Low | 106 | 58 | 48 | 1 | | 1 | | 35 | NA | 1 | | 1 | |
| High | 106 | 58 | 36 | 1.152(0.800-1.657) | 0.448 | 1.111(0.765-1.613) | 0.58 | 47 | 57 | 1.584(1.022-2.455) | 0.04 | 1.572(1.008-2.452) | 0.046 |
| MCM7 | | | | | | | | | | | | | |
| Low | 106 | 60 | 48 | 1 | | 1 | | 35 | NA | 1 | | 1 | |
| High | 106 | 56 | 30 | 1.069(0.743-1.539) | 0.719 | 0.987(0.677-1.437) | 0.945 | 47 | NA | 1.549(1.000-2.401) | 0.05 | 1.387(0.885-2.174) | 0.154 |

**Notes**: §Adjusted for tumor size, cirrhosis, BCLC stage; MCM, minichromosome maintenance; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; MRT, median recurrence time; MST, median survival time; RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; NA, not available.

**Table 3.** Clinical characteristics of HCC patients in TCGA cohort

| Variables | Patients (n=370) | No. of events | MST (days) | Crude HR (95% CI) | P |
|---|---|---|---|---|---|
| **Age(years)** | | | | | |
| **≤60** | 177 | 55 | 2532 | 1 | |
| **>60** | 193 | 75 | 1622 | 1.246(0.879-1.766) | 0.217 |
| **Sex** | | | | | |
| **female** | 121 | 51 | 1490 | 1 | |
| **male** | 249 | 79 | 2486 | 0.817(0.573-1.164) | 0.262 |
| **Alcohol consumption a** | | | | | |
| **NO** | 234 | 84 | 1694 | 1 | |
| **YES** | 117 | 40 | 1624 | 1.026(0.703-1.496) | 0.896 |
| **Ishak fibrosis score b** | | | | | |
| **0 - No Fibrosis** | 74 | 30 | 2131 | 1 | |
| **1,2 - Portal Fibrosis** | 31 | 9 | 1372 | 0.917(0.429-1.962) | 0.823 |
| **3,4 - Fibrous Speta** | 28 | 6 | NA | 0.682(0.281-1.654) | 0.397 |
| **5 - Nodular Formation and Incomplete Cirrhosis** | 9 | 2 | 1386 | 0.750(0.177-3.167) | 0.695 |
| **6 - Established Cirrhosis** | 69 | 17 | NA | 0.766(0.418-1.403) | 0.388 |
| **Tumor Stage c** | | | | | |
| **I** | 171 | 42 | 2532 | 1 | |
| **II** | 85 | 26 | 1852 | 1.427(0.874-2.330) | 0.155 |
| **III/IV** | 90 | 48 | 770 | 2.764(1.823-4.190) | <0.0001 |
| **Histologic Grade d** | | | | | |
| **G1** | 55 | 18 | 2116 | 1 | |
| **G2** | 177 | 60 | 1685 | 1.181(0.697-2.000) | 0.537 |
| **G3** | 121 | 43 | 1622 | 1.233(0.711-2.140) | 0.456 |
| **G4** | 12 | 5 | NA | 1.693(0.626-4.584) | 0.3 |
| **Serum AFP e** | | | | | |
| **≤400 ng/ml** | 213 | 62 | 2456 | 1 | |

| Variables | Patients (n=370) | No. of events | MST (days) | Crude HR (95% CI) | P |
|---|---|---|---|---|---|
| >400 ng/ml | 64 | 22 | 2486 | 1.055(0.645-1.724) | 0.832 |
| Radical resection f | | | | | |
| R0 | 323 | 110 | 1852 | 1 | |
| R1/R2/RX | 40 | 17 | 837 | 2.030(1.213-3.395) | 0.007 |
| Micro Vascular Invasion g | | | | | |
| NO | 206 | 60 | 2131 | 1 | |
| YES | 108 | 36 | 2486 | 1.351(0.892-2.047) | 0.155 |
| Child-Pugh score h | | | | | |
| A | 216 | 59 | 2542 | 1 | |
| B/C | 22 | 9 | 1005 | 1.614(0.796-3.270) | 0.184 |

**Notes**: [a] Information of alcohol consumption was unavailable in 19 patients; [b] Information of ishak fibrosis score was unavailable in 159 patients; [c] Information of tumor stage was unavailable in 24 patients; [d] Information of histologic grade was unavailable in 5 patients; [e] Information of serum AFP was unavailable in 93 patients; [f] Information of radical resection was unavailable in 7 patients; [g] Information of micro vascular invasion was unavailable in 56 patients; [h] Information of Child-Pugh score was unavailable in 132 patients; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; OS, overall survival; MST, median survival time; HR, hazard ratio; CI, confidence interval; AFP, α-fetoprotein; NA, not available.

**Table 4.** Prognostic values of MCM genes expression in HCC OS of TCGA cohort

| Gene expression | Patients(n=370) | NO. of event | MST (days) | Crude HR (95% CI) | Crude P | Adjusted HR (95% CI) | Adjusted P § |
|---|---|---|---|---|---|---|---|
| MCM2 | | | | | | | |
| Low | 185 | 54 | 2116 | 1 | | 1 | |
| High | 185 | 76 | 1397 | 1.782(1.256-2.529) | 0.001 | 1.574(1.073-2.309) | 0.02 |
| MCM3 | | | | | | | |
| Low | 185 | 60 | 2116 | 1 | | 1 | |
| High | 185 | 70 | 1372 | 1.580(1.114-2.242) | 0.010 | 1.456(0.992-2.136) | 0.055 |
| MCM4 | | | | | | | |
| Low | 185 | 62 | 1791 | 1 | | 1 | |
| High | 185 | 68 | 1397 | 1.408(0.997-1.990) | 0.052 | 1.302(0.895-1.894) | 0.167 |
| MCM5 | | | | | | | |
| Low | 185 | 59 | 1.791 | 1 | | 1 | |
| High | 185 | 71 | 1622 | 1.386(0.980-1.960) | 0.065 | 1.299(0.893-1.891) | 0.172 |
| MCM6 | | | | | | | |
| Low | 185 | 54 | 2131 | 1 | | 1 | |
| High | 185 | 76 | 1372 | 1.842(1.297-2.615) | 0.001 | 1.603(1.094-2.350) | 0.015 |
| MCM7 | | | | | | | |
| Low | 185 | 53 | 2131 | 1 | | 1 | |
| High | 185 | 77 | 1149 | 1.852(1.304-2.632) | 0.001 | 1.793(1.222-2.630) | 0.003 |

**Notes**: §Adjusted for tumor stage and radical resection. MCM, minichromosome maintenance; HCC, hepatocellular carcinoma; OS, overall survival; MST, median survival time; HR, hazard ratio; CI, confidence interval; TCGA, The Cancer Genome Atlas.

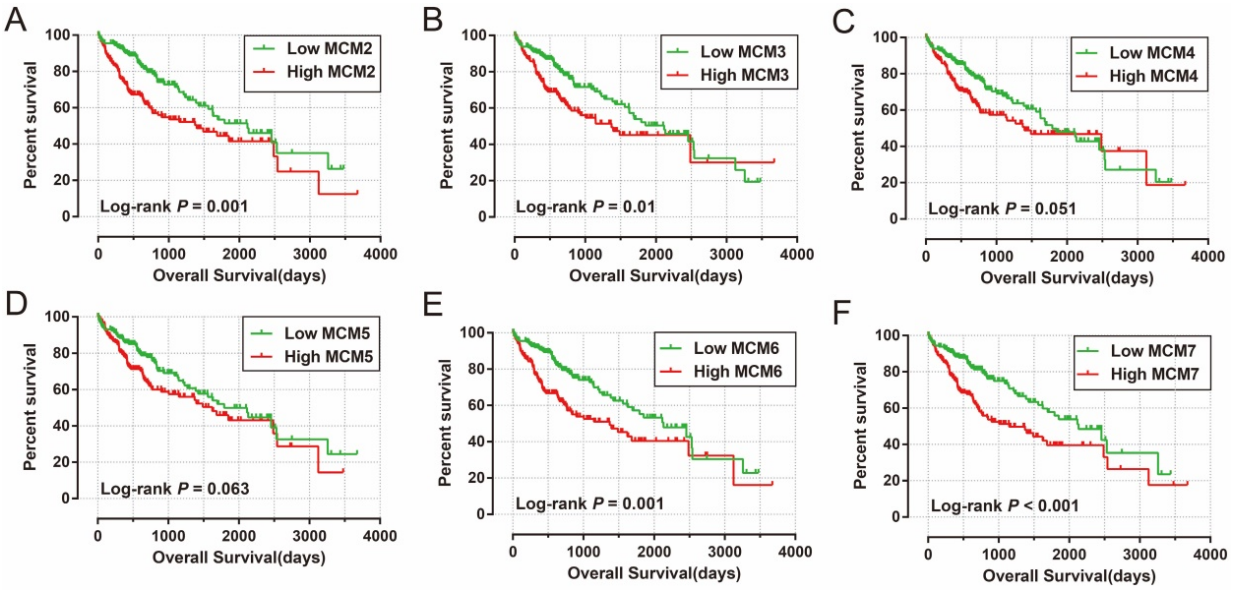


**Figure 7.** Kaplan–Meier survival curves for MCM gens in HCC of TCGA cohort. OS stratified by *MCM2* (A), *MCM3* (B), *MCM4* (C), *MCM5* (D), *MCM6* (E), and *MCM7* (F).

**Table 5.** Joint effects analysis of MCM2 and MCM6 expression in HCC patients OS

| Group | MCM2 | MCM6 | Patients | NO. of event | MST | Crude HR (95% CI) | Crude P | Adjusted HR (95% CI) | Adjusted P § |
|---|---|---|---|---|---|---|---|---|---|
| **GSE14520 cohort** | | | **n=212** | | **months** | | | | |
| A | High | High | 86 | 39 | 53 | 1 | | 1 | |
| B | High | Low | 20 | 9 | NA | 0.874(0.423-1.805) | 0.716 | 0.803(0.384-1.679) | 0.56 |
| C | low | High | 20 | 8 | NA | 0.747(0.349-1.598) | 0.452 | 0.791(0.365-1.714) | 0.552 |
| D | Low | Low | 86 | 26 | NA | 0.537(0.327-0.883) | 0.014 | 0.562(0.339-0.929) | 0.025 |
| **TCGA cohort** | | | **n=370** | | **days** | | | | |
| a | High | High | 153 | 69 | 1005 | 1 | | 1 | |
| b | High | Low | 32 | 7 | 2542 | 0.393(0.181-0.857) | 0.019 | 0.444(0.202-0.977) | 0.044 |
| c | low | High | 32 | 7 | NA | 0.431(0.198-0.938) | 0.034 | 0.442(0.190-1.028) | 0.058 |
| d | Low | Low | 153 | 47 | 2116 | 0.502(0.346-0.728) | <0.001 | 0.584(0.388-0.881) | 0.01 |

Notes: § Adjusted for tumor size, cirrhosis, BCLC stage in GSE14520 cohort; and adjusted for tumor stage and radical resection in TCGA cohort. MCM, minichromosome maintenance; HCC, hepatocellular carcinoma; OS, overall survival; MST, median survival time; HR, hazard ratio; CI, confidence interval; TCGA, The Cancer Genome Atlas; NA, not available.

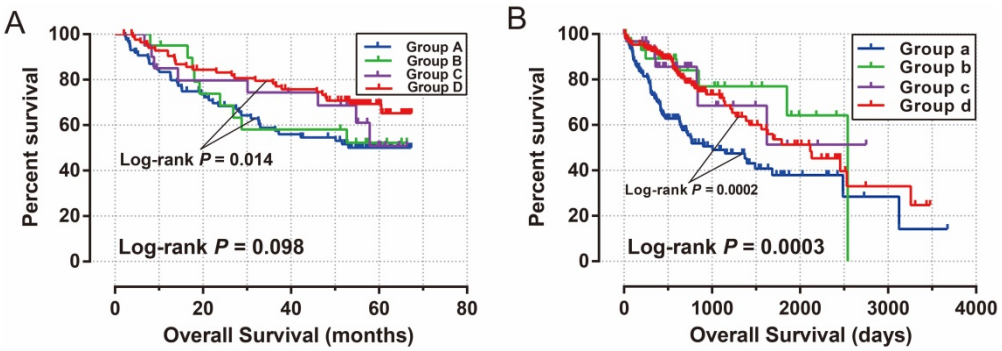


**Figure 8.** Kaplan–Meier survival curve for joint effects analysis of *MCM2* and *MCM6* genes in HCC patients. (A) Joint effects analysis of *MCM2* and *MCM6* in GSE14520 cohort; (B) Joint effects analysis of *MCM2* and *MCM6* in TCGA cohort.

## Prognostic signature construction

The *MCM2* and *MCM6* were associated with a significantly different survival in the joint effects survival analysis; however, the combination of *MCM2* and *MCM6* in prognostic prediction still needed further development. Our previous study divided the patients into high and low risk groups using a risk score model based on the expression of genes [25]; therefore, *MCM2* and *MCM6* expression were used for further prognostic signature construction. In the GSE14520 cohort, the regression coefficient (β) that was derived from the multivariate Cox proportional hazards regression model and the risk score formula was: risk score = expression of *MCM2* × 0.181 + expression of *MCM6*×1.552. Survival analysis of the prognostic signature in the GSE14520 cohort suggested that patients with a high risk score had a significantly increased risk of death in HBV-related HCC compared to the patients with a low risk score (adjusted *P*=0.026; adjusted HR=1.656; 95% CI=1.063–2.581; **Table 6; Figure 9A, B**). Time-dependent ROC analysis of the risk score indicated that the prognostic signature performed well in the HBV-related HCC OS prediction of the GSE14520 cohort, as the AUC of the ROC curve was 0.548, 0.598, 0.607, and 0.612 for 1-, 2-, 3-, and 5-year survival (**Figure 9C**), respectively.

The multivariate Cox proportional hazards regression model was used in the validation cohort of TCGA HCC patients with the following risk score formula: risk score=expression of *MCM2* × 0.0878 + expression of *MCM6*×0.3056. Patients with a high risk score had a significantly increased risk of death in HCC (adjusted *P*=0.034; adjusted HR=1.512; 95% CI=1.033–2.213; **Table 6; Figure 10A, B**), compared to the patients with a low risk score. The AUC of the time-dependent ROC curve was 0.706, 0.673, 0.662, and 0.593 for 1-, 2-, 3-, and 5-year survival (**Figure 10C**), respectively.

**Table 6.** Survival analysis of MCM gene expression prognostic signature in HCC patients

| Variables | Patients | NO. of event | MST | Crude HR (95% CI) | Crude P | Adjusted HR (95% CI) | Adjusted P § |
|---|---|---|---|---|---|---|---|
| **GSE14520** | **n=212** | | **months** | | | | |
| **Low risk** | 106 | 35 | NA | 1 | | 1 | |
| **High risk** | 106 | 47 | 57 | 1.643(1.060-2.547) | 0.026 | 1.656(1.063-2.581) | 0.026 |
| **TCGA** | **n=370** | | **days** | | | | |
| **Low risk** | 185 | 55 | 2131 | 1 | | 1 | |
| **High risk** | 185 | 75 | 1397 | 1.751(1.234-2.485) | 0.002 | 1.512(1.033-2.213) | 0.034 |

**Notes:** § Adjusted for tumor size, cirrhosis, BCLC stage in GSE14520 cohort; and adjusted for tumor stage and radical resection in TCGA cohort. MCM, minichromosome maintenance; MST, median survival time; HR, hazard ratio; CI, confidence interval; TCGA, The Cancer Genome Atlas.

## GSEA

GSEA of *MCM2* and *MCM6* were also performed in both the GSE14520 and TCGA cohorts.

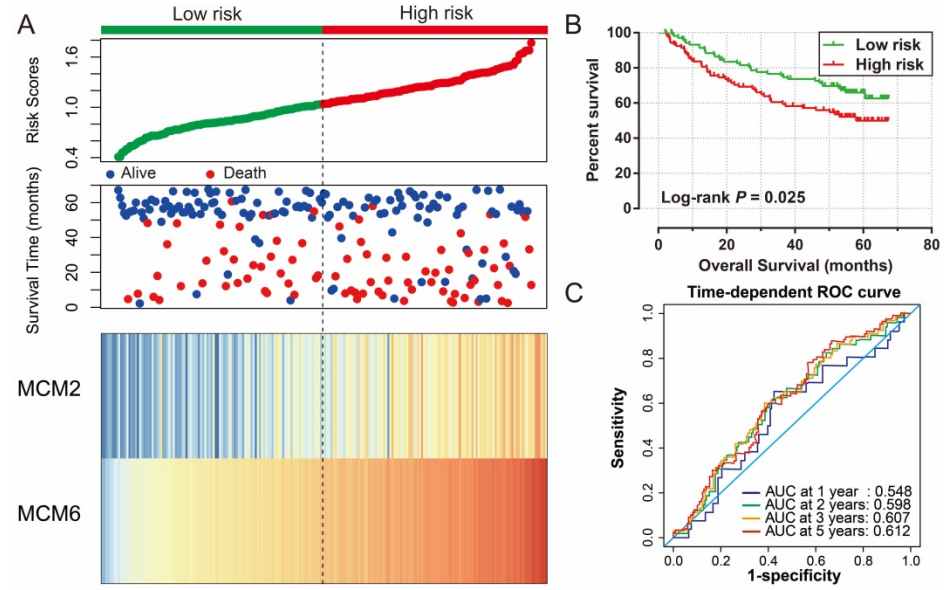


**Figure 9.** Prognostic risk score model analysis of *MCM2* and *MCM6* genes in HBV-related HCC patients of GSE14520 cohort. (A) From top to bottom are the risk score, patients' survival status distribution, and *MCM2* and *MCM6* genes heat map for low- and high-risk groups. (B) Kaplan–Meier curves for low- and high-risk groups. (C) ROC curve for predicting survival in HCC patients by the risk score.

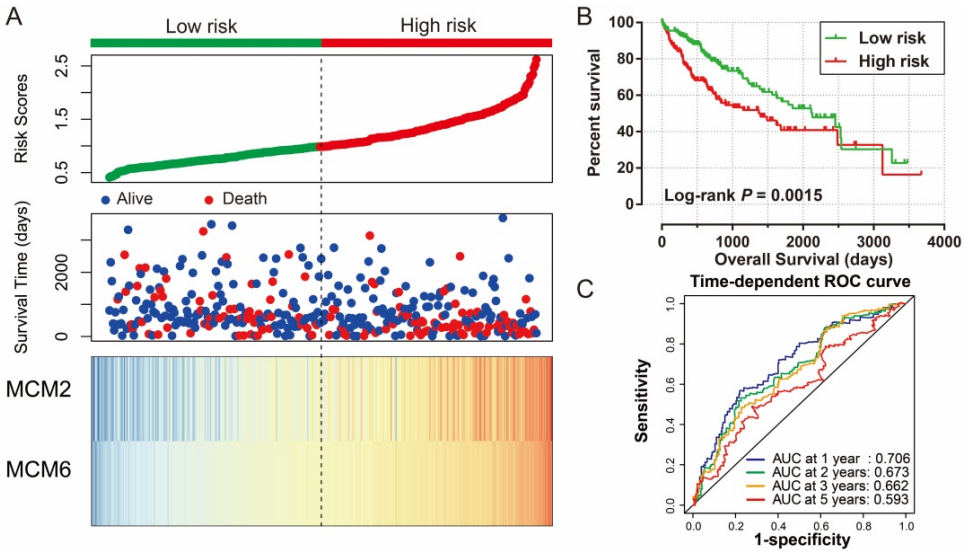


**Figure 10.** Prognostic risk score model analysis of *MCM2* and *MCM6* genes in HCC patients of TCGA cohort. (A) From top to bottom are the risk score, patients' survival status distribution, and *MCM2* and *MCM6* genes heat map for low- and high-risk groups. (B) Kaplan–Meier curves for low- and high-risk groups. (C) ROC curve for predicting survival in HCC patients by the risk score.

The genome-wide expression profile dataset of the GSE14520 and TCGA cohorts were divided into two groups according to the median values of the *MCM2* and *MCM6* genes, respectively. GSEA results of the GSE14520 cohort are shown in **Figure 11A–L** and **Table S1–4**, which suggested that both the high expression of *MCM2* and *MCM6* were significantly correlated with cell cycle process, P53 regulation pathway, liver cancer survival, liver cancer progression G1 and G2, and DNA repair. The *MCM2* and *MCM6* GSEA results in the GSE14520 cohort could also be validated in the TCGA HCC cohort, and high expressions of *MCM2* and *MCM6* were also significantly correlated with cell cycle process, P53 regulation pathway, liver cancer survival, liver cancer progression G1 and G2, and DNA repair (**Figure 12A–L** and **Table S5–8**).

## Discussion

The MCM genes play a critical role in DNA replication [8, 36, 37]. The MCM2–7 gene family is comprised of six structurally related proteins, which can form a hexameric complex, and this complex is an essential component in early G1 phase [8, 36, 37]. Our gene function enrichment analysis also suggested that MCM2–7 genes were significantly enriched in DNA replication and cell cycle biological processes and pathways. Co-expression analysis demonstrated that

MCM2–7 genes were strongly co-expressed with each other at both the gene and protein levels, as well as in HCC tumor tissues.

Extensive studies have reported that MCM2–7 genes are potential diagnostic markers in multiple cancers. Previous studies indicated that MCM2 is upregulated in colorectal cancer tumor tissue, and could be used as a diagnostic marker using immunocytochemical analysis from patients' tissues or colonocytes retrieved from the fecal surface [38, 39]. Similar immunocytochemical detection of MCM2 in cells retrieved from urine also showed a diagnosis value in bladder cancer [40] and cervical cancer screening [41]. The immunocytological evaluation of MCM3 can be used for early detection of oral squamous cell carcinoma [42]. The potential

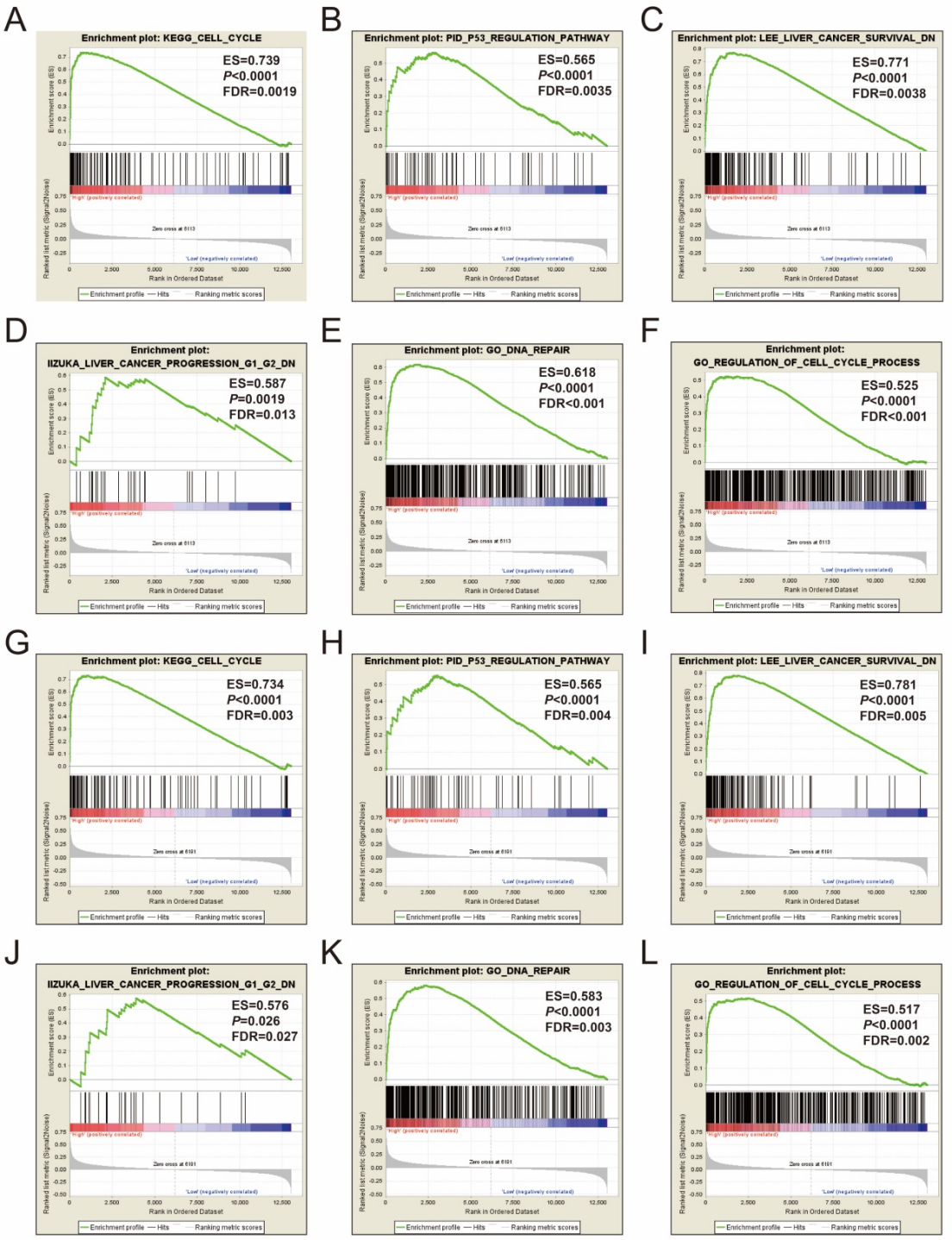


**Figure 11.** GSEA results of MCM2 and MCM6 in GSE14520 HBV-related HCC patients. (A-D) GSEA results of c2 reference gene sets for high MCM2 expression groups; (E–F) GSEA results of c5 reference gene sets for high MCM2 expression groups. (G-J) GSEA results of c2 reference gene sets for high MCM6 expression groups; (K–L) GSEA results of c5 reference gene sets for high MCM6 expression groups.

diagnostic value of MCM5 has also been investigated in genito-urinary tract cancer [11, 43], oesophageal cancer [12], pancreaticobiliary malignancy [44, 45], and cervical cancer screening [41]. In addition, MCM7 can be used for the early diagnosis of gastric cancer [46], and differential diagnosis between reactive mesothelial cells and malignant mesothelioma cells [47, 48]. A study by Saydam et al. reported that MCM2–7 genes were upregulated in meningiomas tumor tissues and could serve as potential diagnostic markers [49]. Consistent with the study by Saydam and his co-workers, our current study also observed that MCM2–7 genes were upregulated in HCC tumor tissues, and ROC analysis suggested that MCM2–7 genes may be potential diagnostic markers in HCC.

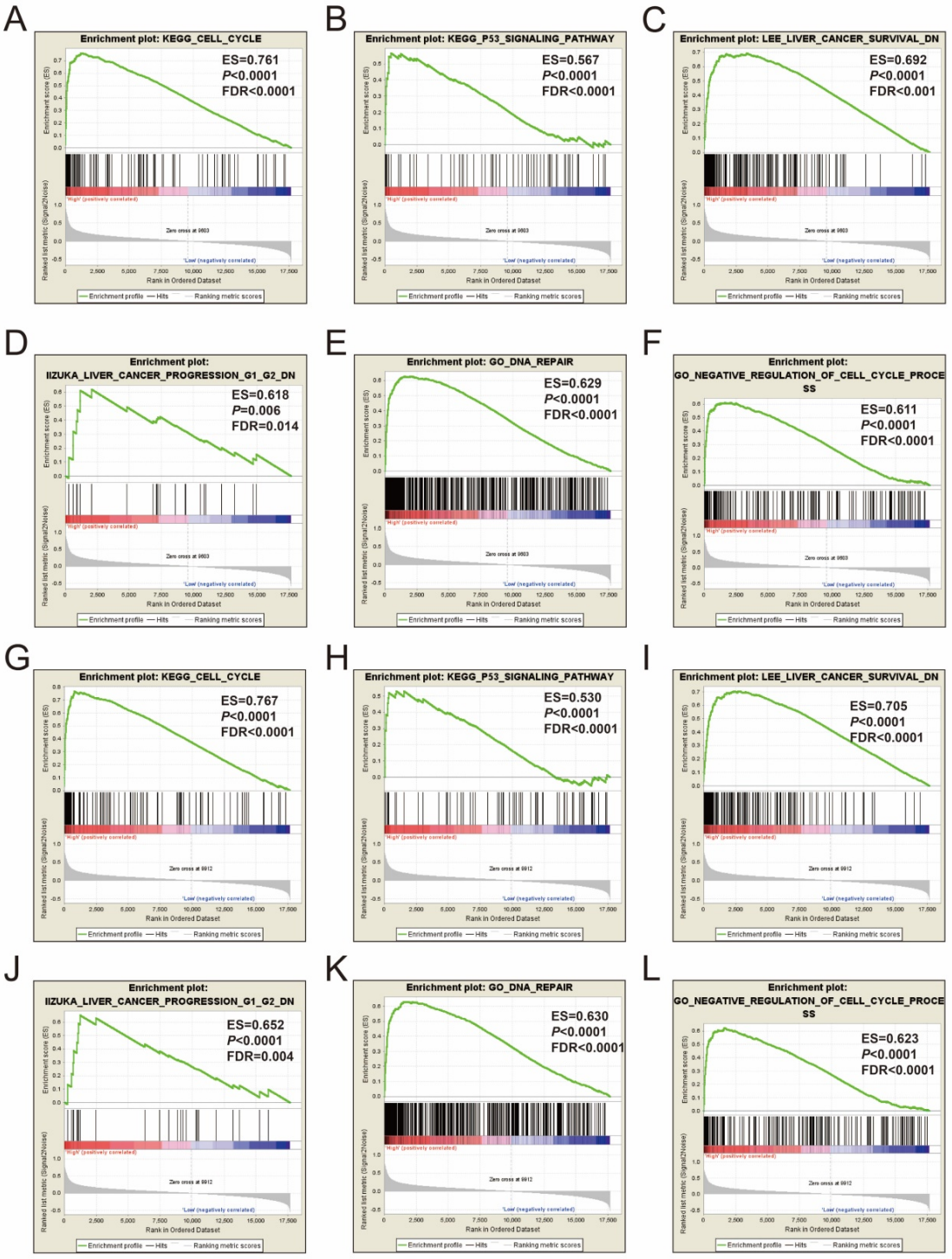


**Figure 12.** GSEA results of MCM2 and MCM6 in TCGA HCC patients. (A-D) GSEA results of c2 reference gene sets for high MCM2 expression groups; (E–F) GSEA results of c5 reference gene sets for high MCM2 expression groups. (G-J) GSEA results of c2 reference gene sets for high MCM6 expression groups; (K–L) GSEA results of c5 reference gene sets for high MCM6 expression groups

In the present study, we observed that the expression of *MCM2, MCM4, MCM5,* and *MCM6* were significantly associated with HBV-related HCC OS in the GSE14520 cohort, whereas expression of *MCM2, MCM6,* and *MCM7* were correlated with HCC OS in the TCGA cohort. Joint effects survival analysis suggested that patients with low expression of both *MCM2* and *MCM6* had a significantly decreased risk of death in HBV-related HCC compared to the patients with high expression of both *MCM2* and *MCM6*. In addition, the risk score model, which constructed based on the expression of *MCM2* and *MCM6* in the GSE14520 and TCGA cohorts, also could divided the patients into high- and low-risk groups, and patients with high risk scores were significant associated with a poor OS. However, the prognostic values of MCM2–7 genes in multiple cancers also have been reported in previous studies. Numerous studies have demonstrated that the high expression of MCM2 predicts a poor prognosis in patients with gastric cancer [50-52], lung cancer [10, 53], ovarian adenocarcinomas [54], and muscle-invasive urothelial bladder carcinomas [55]. Additionally, the expression of MCM2 is also an independent predictor of recurrence in stage Ta/T1 bladder cancer [56]. High expression of MCM3 and MCM4, identified by immunohistochemistry, were significantly associated with OS in patients with astrocytoma [57] and esophageal adenocarcinoma [58], respectively. Expression of MCM5 also increased markedly in lung cancer and cervical cancer, and patients with a high expression of MCM5 had a significantly increased risk of death [59, 60]. Immunohistochemical staining of MCM6 showed a strong correlation between MCM6 expression and OS in patients with non-small cell lung carcinoma [61], low-grade chondrosarcoma [62], mantle cell lymphoma [63], and endometrioid endometrial adenocarcinoma [64], and these patients were significantly correlated with a poor OS. Furthermore, high MCM6 immunohistochemical staining significantly increased the risk of recurrence in patients with meningiomas, as well as correlated with the histological grade [65].

Similar results of MCM7 expression in cancer prognosis, identified by immunohistochemical staining, was found in non-small cell lung cancer [66, 67], colorectal cancer [68, 69], oral squamous cell carcinoma [70], HCC [71-73], and oesophageal squamous cell carcinoma [74]. These studies suggested that the MCM7 gene may serve as a prognostic biomarker, and high MCM7 expression in these cancers were significantly associated with a poor OS. Consistent with the results of the MCM7 gene in cancer OS, high expression of MCM7 also significantly correlated with a poor RFS of colorectal cancer [68], gastric adenocarcinoma [75], pituitary adenoma [76] and meningiomas [77], and lymph node metastasis of oral squamous cell carcinoma [78]. By reviewing these studies, a potential prognostic role for MCM genes in HCC was identified in the current study and was consistent with previous studies, which indicated that these MCM genes may serve as oncogenes in cancer. However, our findings still need further validation.

Due to the function of the MCM genes, they have been reported to play an important multi-aspect role in HCC, such as in diagnosis, progression, and prognosis. Previous studies substantiated that MCM2 was a novel marker to assess the progression from liver cirrhosis to HCC [79], and proliferation and metastasis of HCC cells could be inhibited by long noncoding RNA FTX through binding MCM2 and miR-374a [80]. In addition, MCM2 could serve as a prognostic biomarker and therapeutic target for HCC [81, 82], and MCM7 also can act as a prognostic biomarker for HCC [71-73]. Polymorphisms of *MCM4* rs2305952 may be associated with susceptibility of HCC [83], and plasma MCM6 serves as a diagnostic biomarker for HCC patients, especially in patients with AFP-negative and small HCC [84].

GSEA in the current study indicated that *MCM2* and *MCM6* were significantly associated with liver cancer survival and progression, and the potential mechanism of *MCM2* and *MCM6* in HCC prognosis may involve signal pathway and biological processes of the cell cycle, DNA repair, and p53, which were correlated with their biological functions. As is well-known, MCM genes play a critical role in DNA replication and participate in the cell cycle process [8]. Previous studies also demonstrated that the function of the MCM2 gene was to participate in the p53 pathway in non-small cell lung carcinomas [85] and in a mouse fibroblast 3T3 cell line[86], followed by cellular apoptosis. Furthermore, immuno-cytochemistry of the MCM2 and p53 combination can be used for distinguishing benign cells from malignant cells in squamous cell carcinoma [87] and pancreaticobiliary adenocarcinoma [88]. However, the functional correlation between p53 and MCM6 has not been reported in previous studies. Due to the co-expression and GSEA of *MCM6* and *MCM2*, we concluded that *MCM6* may participate in the p53 pathway by affecting *MCM2* expression. However, this hypothesis still needs further experimental confirmation.

There are some limitations in the current study that need clarification. All data in the current study were obtained from public databases and the clinical parameters were incomplete; therefore, we could not perform a comprehensive survival analysis of MCM

genes that considered all the potential prognostic factors of HCC in multivariate Cox proportional hazards regression model analysis. Second, due to the different sources of HCC patients and multiple factors that influence the HCC prognosis, we could not construct a unified risk score model that was based on *MCM2* and *MCM6* expression levels for prognosis prediction in patients with HCC. Third, by comparison with the previous study, the limitation of our current study was that it only investigated the association between the mRNA expression of the MCM genes and HCC prognosis; however, the relationship between the MCM protein level and HCC prognosis prediction still needs further exploration.

Despite these limitations, in the present study, we have identified and validated the diagnostic and prognostic values of the expression of the MCM genes in patients with HCC, and also investigated the potential mechanism of *MCM2* and *MCM6* in HCC prognosis through GSEA. Once these results are verified the diagnostic and prognostic values of MCM genes at the protein level, these genes may have a potential clinical application value in HCC diagnosis, cancer management and targeted therapy. However, prospective validation with a larger sample size is necessary before the MCM genes can be included in diagnosis and prognostic monitoring for patients with HCC.

## Conclusions

In the present study, we found that all MCM genes were significantly upregulated in tumor tissue, and had a potential diagnostic value in patients with HCC. Survival analysis in the GSE14520 and TCGA cohorts suggested that *MCM2* and *MCM6* may serve as potential prognostic biomarkers in patients with HCC. Survival analysis of the risk score model and joint effects analysis indicated that the combination of *MCM2* and *MCM6* could also serve as an indicator for HCC prognosis prediction. However, our findings still need further validation, and the prognostic values of other MCM genes still need prospective validation in a larger number of patients.

## Abbreviations

MCM, minichromosome maintenance; MST, median survival time; RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; AFP, α-fetoprotein; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ROC, receiver operating characteristic; ES, enrichment score; FDR, false discovery rate; GSEA, gene set enrichment analysis.

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### Authors' Contributions

Xiwen Liao, Xiaoguang Liu and Tao Peng designed this manuscript; Xiwen Liao, Xiaoguang Liu, Chengkun Yang, Xiangkun Wang, Tingdong Yu, Chuangye Han, Ketuan Huang, Guangzhi Zhu, Hao Su,Wei Qin, Rui Huang, Long Yu, Jianlong Deng, Xianmin Zeng, Xinping Ye, and Tao Peng conducted the study, collected and analyzed the data. Xiwen Liao wrote and revised the manuscript, Xiaoguang Liu contributed to the data interpretation, and Tao Peng guided the writing.

## Supplementary Material

Supplementary figures.
http://www.jcancer.org/v09p2357s1.pdf
Supplementary tables.
http://www.jcancer.org/v09p2357s2.xlsx

## Competing Interests

The authors have declared that no competing interest exists.

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