

Diagnostic and prognostic value of MCM3 and its interacting proteins in hepatocellular carcinoma

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Abstract. Aberrant DNA replication is one of the driving forces behind oncogenesis. Furthermore, minichromosome maintenance complex component 3 (MCM3) serves an essential role in DNA replication. Therefore, in the present study, the diagnostic and prognostic value of MCM3 and its interacting proteins in hepatocellular carcinoma (HCC) were investigated. By utilizing The Cancer Genome Atlas (TCGA) database, global MCM3 mRNA levels were assessed in HCC and normal liver tissues. Its effects were further analyzed by reverse transcription-quantitative PCR (RT-qPCR), western

blotting and immunohistochemistry in 78 paired HCC and adjacent tissues. Functional and pathway enrichment analyses were performed using the Search Tool for the Retrieval of Interacting Genes database. The expression levels of proteins that interact with MCM3 were also analyzed using the TCGA database and RT-qPCR. Finally, algorithms combining receiver operating characteristic (ROC) curves were constructed using binary logistic regression using the TCGA results. Increased *MCM3* mRNA expression with high α -fetoprotein levels and advanced Edmondson-Steiner grade were found to be characteristic of HCC. Survival analysis revealed that high MCM3 expression was associated with poor outcomes in patients with HCC. In addition, MCM3 protein expression was associated with increased tumor invasion in HCC tissues. MCM3 and its interacting proteins were found to be primarily involved in DNA replication, cell cycle and a number of binding processes. Algorithms combining ROCs of MCM3 and its interacting proteins were found to have improved HCC diagnosis ability compared with MCM3 and other individual diagnostic markers. In conclusion, MCM3 appears to be a promising diagnostic biomarker for HCC. Additionally, the present study provides a basis for the multi-gene diagnosis of HCC using MCM3.

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Abbreviations: MCM3, minichromosome maintenance complex component 3; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; RT-qPCR, reverse transcription-quantitative PCR; ROC, receiver operating characteristic; AFP, α -fetoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; PPI, protein-protein interaction; STRING, Search Tool for the Retrieval of Interacting Genes; MCODE, Molecular Complex Detection; OS, overall survival; RFS, recurrence-free survival; MF, molecular functions; CHK1, checkpoint kinase 1; DHX9, DEAH (Asp-Glu-Ala-His) box helicase 9; DDR, DNA damage response; AUC, area under the curve; RSEM, RNA-Seq by Expectation-Maximization; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; E-S grade, pathological Edmondson-Steiner grade

Key words: hepatocellular carcinoma, minichromosome maintenance complex component 3, diagnostic ability, multi-gene diagnosis, biomarker

Introduction

Minichromosome maintenance complex component (MCM)3, one of the highly conserved MCMs, is involved in prokaryotic and eukaryotic genome replication (1). MCM forms a hexameric protein complex, which is a key component of the pre-replication complex. This complex is involved in the formation of the replication fork and in the recruitment of other DNA replication proteins (2). MCM3 is a subunit/component of the hexameric protein complex that consists of MCM2-7 and directly interacts with MCM5 (3). MCM3 is essential for the initiation of DNA replication and is involved in ensuring that DNA replication is initiated precisely once per cell cycle (4). The initiation of DNA replication and cell cycle

progression is triggered by acetylation of MCM3 (4), and cells lacking MCM3 fail to proceed into S phase and proliferate (5). Furthermore, MCM3 is a point of direct contact between Kelch-like ECH-associated protein 1 (KEAP1; a substrate adaptor protein for a cullin-3-based E3 ubiquitin ligase) and the MCM2-7 hexamer. KEAP1-mediated MCM3 ubiquitylation may also impact helicase activation (6).

Hepatocellular carcinoma (HCC), a common malignant tumor, accounts for 90% of primary liver malignancies (7). HCC is the second most common cause of cancer-associated mortality worldwide and this proportion continues to increase (8). In the United States alone, annual HCC-related deaths have doubled from 5,112 in 1999 to 11,073 in 2016 (9). A high incidence of HCC has also been noted in China (10). Hepatitis virus infection (HBV or HCV), cirrhosis, HIV co-infection, alcoholism, obesity, aflatoxin B1 intake and inherited factors are associated with increased incidence of HCC (7-8,10). Current treatments for HCC, including surgical resection, liver transplantation and occasionally ablative therapies, only provide limited benefits. Survival of patients with HCC is limited even following liver excision or liver transplantation (8). The disease is refractory to classic chemotherapy and is unsuitable for radiation treatment due to the chance of developing liver toxicity (7-8,10). Due to the difficulties associated with early diagnosis and the high incidence of tumor recurrence, identification of novel HCC treatment methods is required to improve patient outcomes.

MCM proteins, which are overexpressed in various neoplasms, can be used as proliferation markers to analyze the behavior of diverse neoplasms (11,12). A previous study has demonstrated that overexpression of MCM3 enhances anchorage-independent cell growth, cell migration and invasion abilities of medulloblastoma cells (13). These results suggest that MCM3 is likely to be associated with tumor migration and invasion. Increased MCM3 expression has also been observed in melanoma, prostate cancer, cervical squamous cell carcinoma and salivary gland tumors (1,5,14). These studies indicate that MCM3 may be a potential proliferation marker in the diagnosis of certain tumors (1,5,13,14). In certain tumors, MCM3 may also be detected in non-proliferating cells, which indicates their readiness to enter the cell cycle (15).

There is limited research regarding MCM3 in HCC. Therefore, the association between MCM3 and clinical features of HCC remains unclear. The present study assessed the effect of MCM3 in HCC. MCM3 expression was analyzed using The Cancer Genome Atlas (TCGA) database. mRNA and protein expression levels were detected in HCC and paired adjacent tissues. Additionally, the association between MCM3 expression and the clinicopathological features of patients with HCC were evaluated, including common diagnostic marker AFP and noninvasive liver function parameters ALT and AST, which are commonly tested in clinical practice to indicate prognosis (16,17). Subsequently, functional and pathway enrichment analyses were carried out and a protein-protein interaction (PPI) network of MCM3 was established. The present study bridges the gap in understanding the underlying molecular mechanisms of MCM3 and its interacting proteins in HCC. Finally, the expression of the proteins that interact with MCM3, including MCM family proteins and non-MCM family proteins, were analyzed in the context of HCC.

Algorithms combining the receiver operating characteristic (ROC) curves of MCM3 and its interaction partners were constructed to improve the diagnosis of HCC when compared with MCM3 alone.

Materials and methods

Patients and HCC specimen. A total of 78 patients (age range, 28-68 years; median age, 45 years) with HCC who were diagnosed according to the Chinese primary liver cancer guidelines (18) and treated at the Department of Hepatobiliary Pancreatic Surgery of the Affiliated Hospital of Guilin Medical University (Guilin, P.R. China) between January 2017 and August 2018 were prospectively recruited in the present study. All patients had not received any antitumor therapy prior to surgery. Surgical indications of these patients met the following criteria based on the Chinese primary liver cancer guidelines (18): i) Patients with good liver function reserve and HCC staging Ia, Ib and IIa; ii) patients with HCC staging IIb and IIIa who have <3 tumor nodules; and iii) patients with HCC staging IIb and IIIa that meet particular conditions, including: a) Patients have >3 tumor nodules that are localized in the same segment or lobe, or if radiofrequency ablation can be performed for some lesions during surgery; b) the tumor is localized in one lobe and the tumor emboli are expected to be completely resected during surgery; c) patients that have obstructive jaundice because of tumor emboli in the bile duct and the intrahepatic tumors are resectable; and d) patients with portal lymph node metastases, intraoperative lymph node dissection or postoperative external radiation therapy. HCC samples, including tumor tissue and adjacent tissues (≥ 2 cm distance from the tumor margin) were then collected during radical resection or partial resection. The demographic and clinicopathological characteristics, including age, sex, smoking, drinking, Hepatitis B virus infection, α -fetoprotein (AFP), alanine aminotransferase (ALT), aspartate transaminase (AST), tumor size and pathological Edmondson-Steiner (E-S) grade (19), were also obtained. The present study was approved by the Ethics Committee of The Affiliated Hospital of Guilin Medical University and written informed consent was obtained from all patients.

Data collection and analysis from TCGA. RNA-seq data quantified using RNA-Seq by Expectation-Maximization (RSEM) were downloaded from TCGA (<https://tcga.xenahubs.net>). In particular, the level-3 data were downloaded from TCGA data coordination center. In order to acquire TCGA data comparable to the subject of the present research, only patients with HCC were included in the analysis (n=363), and were paired with non-HCC (normal liver) samples for statistical analysis (n=50). In addition, data regarding clinical features of patients for all downloaded datasets (LIHC_clinicalMatrix) were collected.

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was used to determine mRNA expression levels of different genes in 78 HCC tissue samples. Total RNA from HCC specimens was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA (1 μ g) was reverse transcribed to cDNA using a two-step RT Kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. Complementary

Table I. Primers utilized for reverse transcription-quantitative PCR amplification.

Gene	Upstream primer sequence (5'-3')	Downstream primer sequence (5'-3')
MCM2	ATCTACGCCAAGGAGAGGGT	GTAATGGGGATGCTGCCTGT
MCM3	TGGCCTCCATTGATGCTACC	GGACGACTTTGGGACGAACT
MCM5	TCATCTCCAAGAGCATCGCC	CCTCGGCGAGTAAGTCCATC
CHK1	AATGCTCGCTGGAGAATTGC	CACCACCTGAAGTGACTCGG
DHX9	GCCAATTTCTGGCCAAAGCA	CGAGGCTCAATGGGGAGTTT
β -actin	CAGGCACCAGGGCGTGAT	TAGCAACGTACATGGCTGGG

MCM, minichromosome maintenance complex component; CHK1, checkpoint kinase 1; DHX9, DEAH (Asp-Glu-Ala-His) box helicase 9.

DNA was amplified using the StepOnePlus system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR Green master mix [Roche Diagnostics (Shanghai) Co., Ltd.]. The RT-qPCR conditions used were as follows: Initiation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, final extension at 60°C for 10 min. mRNA expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method (20). β -actin was used as an internal control. The primers used for RT-qPCR amplification are shown in Table I.

Western blotting. Total protein was extracted from tissue (5 mg) using 300 μ l ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology) with proteinase inhibitor and homogenized using an electric homogenizer. Samples were centrifuged at 12,000 rpm for 20 min at 4°C and the supernatant was collected. Protein concentration was determined using a BCA assay (Beijing Leagene Biotech, Co., Ltd.). Total protein was mixed with 5X loading buffer mix and boiled at 100°C for 5 min. Equal amounts of total protein (20 μ g/lane) were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane using a Bio-Rad SemiDry apparatus. The membranes were blocked in TBS + Tween 20 (TBST; 0.1% Tween 20) containing 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 1 h. Anti-MCM3 primary antibody (diluted in TBST; 1:1,000; cat. no. ab128923; Abcam) and the loading control anti- β -actin antibody (diluted in TBST; 1:4,000, cat. no. HRP-60008; ProteinTech Group, Inc.) were added to the membranes at 4°C overnight. The membranes were rinsed three times for 5 min with TBST and then incubated with HRP-conjugated secondary antibody (diluted in TBST; 1:3,000; cat. no. SA00001-2; ProteinTech Group, Inc.) for 1 h at room temperature. The membranes were rinsed three times for 5 min with TBST and protein bands were visualized using the chemiluminescent substrate BeyoECL Plus kit (Beyotime Institute of Biotechnology). The FluorChem HD2 chemiluminescence system (ProteinSimple) was used for protein visualization. Protein expression was semi-quantified using ImageJ software (version 1.52a; National Institutes of Health) with β -actin as the loading control.

Immunohistochemistry (IHC). All tissues were fixed in 4% paraformaldehyde for 24 h at room temperature, embedded in paraffin, and sliced into 4- μ m sections. Embedded sections were deparaffinized with xylene and rehydrated with a descending ethanol series (100, 95, 90 and 85%), followed by antigen retrieval in citrate acid buffer for 15 min using

an autoclave at 121°C. Subsequently, protein expression was analyzed using the SP-9000 two-step immunohistochemical staining kit (OriGene Technologies, Inc.) according to the manufacturer's protocol. Endogenous peroxidase activity was blocked with hydrogen peroxide at room temperature for 15 min. After a 10-min incubation with goat serum at room temperature, sections were incubated with anti-MCM3 primary antibody (dilution in PBS; 1:1,000; cat. no. ab128923; Abcam) overnight at 4°C in a humidified chamber. Negative controls were incubated with PBS instead of the primary antibody. After washing with PBS, samples were incubated with the biotinylated secondary antibody at room temperature for 15 min. Sections were incubated with horseradish peroxidase at room temperature for 15 min and developed in 3,3'-diaminobenzidine peroxidase substrate solution. Finally, sections were counterstained with hematoxylin at room temperature for 5 min, dehydrated and mounted in resin blocks.

Two independent pathologists evaluated the degree of IHC staining under a light microscope. In each sample, $\geq 1,000$ cells were counted and 10 high-power fields (magnification, x400) were analyzed. The average number was considered for the whole sample. The average labelling index of MCM3 from 10 randomly selected high-power fields was recorded as follows: i) 0, no staining, or faint staining intensity in <10% of cells; ii) 1+, faint nuclear/cytoplasmic staining in $\geq 10\%$ of cells; iii) 2+, moderate and incomplete nuclear staining in $\geq 10\%$ of cells; and iv) 3+, strong nuclear staining in $\geq 10\%$ of cells (21). Specimens exhibiting immunostaining of 3+ were defined as high expression of MCM3; expression levels of 1+ or 2+ were defined as low expression, and 0 was defined as negative expression.

PPI network construction and module analysis. The functional interactions between proteins can provide context in molecular mechanisms of MCM3 and its interacting proteins in the development of HCC. In the present study, a PPI network consisting of MCM3 and its interacting proteins was constructed using the Search Tool for the Retrieval of Interacting Genes (STRING) software tool (version 11.0; <http://string-db.org>). Cytoscape v17 software (www.cytoscape.org) with Molecular Complex Detection (MCODE; version 1.6.1) (22) was used to visualize and screen modules of the PPI network as previously described (23).

Functional and pathway enrichment analysis. MCM3 and its interacting proteins were used to perform Gene Ontology

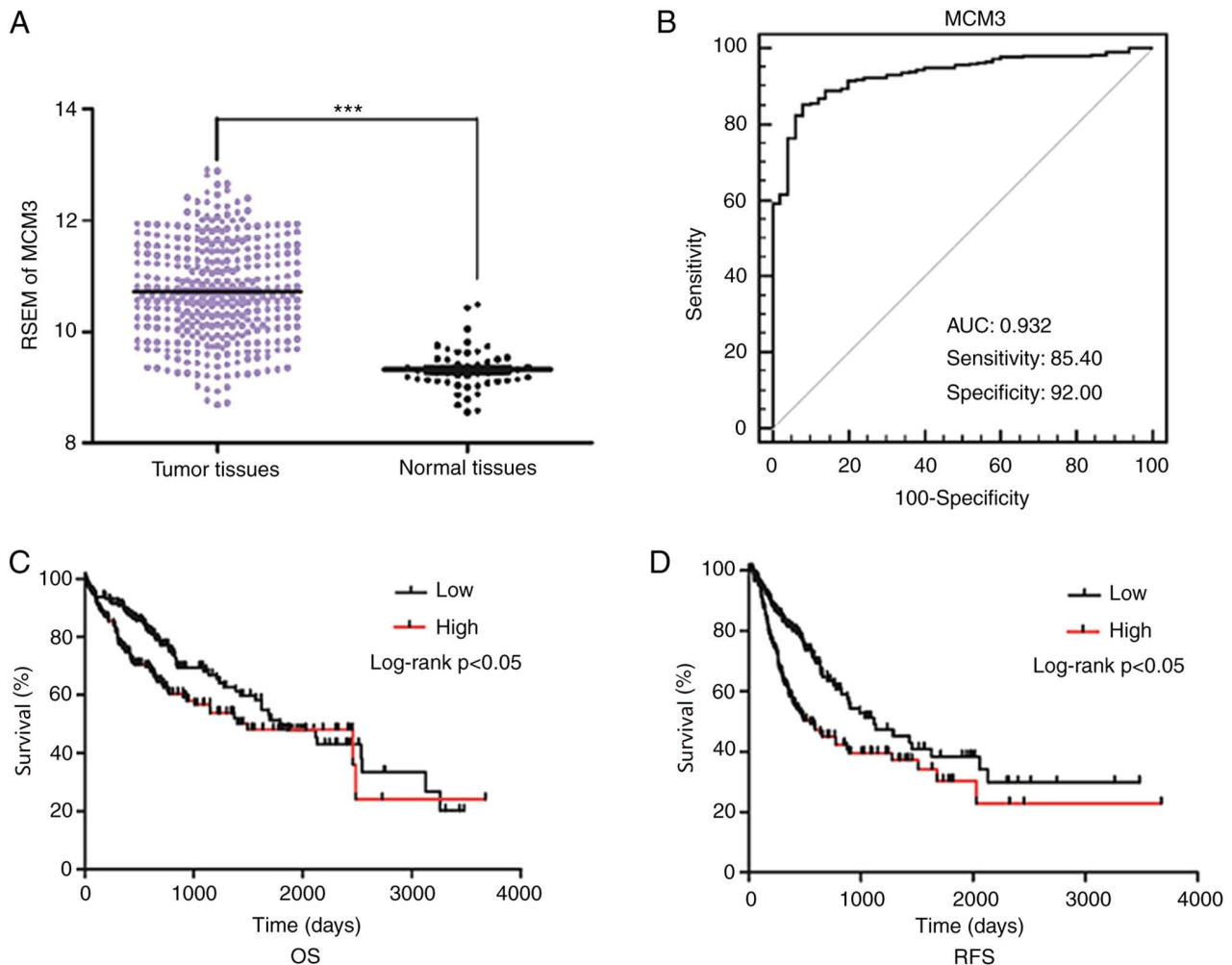


Figure 1. Data mining of MCM3 expression from The Cancer Genome Atlas database. (A) MCM3 mRNA expression in HCC tissues and normal liver tissues. mRNA was normalized by RSEM. Higher MCM3 mRNA expression was found in HCC tissues compared with in normal liver tissues ($P < 0.05$). (B) Receiver operating characteristic curve of MCM3 for diagnosis of HCC. (C) OS in patients with high expression levels of MCM3 compared with in patients with low expression levels of MCM3. (D) RFS in patients with high expression levels of MCM3 compared with in patients with low expression levels of MCM3. *** $P < 0.05$. MCM3, minichromosome maintenance complex component 3; HCC, hepatocellular carcinoma; RSEM, RNA-Seq by Expectation-Maximization; AUC, area under the curve; OS, Overall survival; RFS, recurrence-free survival.

(GO; <http://www.geneontology.org>) and Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg>) pathway enrichment analysis in STRING. $P < 0.05$ was set as the cut-off criterion.

Statistical analysis. Statistical analysis was performed using the SPSS software (version 22.0; IBM Corp.). Data are presented as the mean \pm standard deviation. Unpaired comparisons between 363 HCC tissues and 50 normal tissues were analyzed using Student's unpaired t-test and Welch t-test. Comparisons between two paired groups (78 HCC tissues vs. paired adjacent tissues) were conducted using Wilcoxon signed-rank test for IHC data and Student's paired t-test for RT-qPCR results. The association between MCM3 expression and clinicopathological factors in patients with HCC was determined using χ^2 test with Fisher's exact test. The ROC curves and the area under the curve (AUC) were used to evaluate the diagnostic value of different genes in HCC. Pairwise comparisons of ROC curves were conducted using MedCalc (version 9.2.0.1; MedCalc Software Ltd.) and the best cut-off value for each gene was defined as the point

with maximum Youden index (sensitivity + specificity - 1) on the ROC curve. Based on the mRNA data (RSEM) extracted from TCGA liver hepatocellular carcinoma dataset, binary logistic regression analysis was used to generate algorithms combining ROCs of MCM3 and its interacting proteins. Enrichment analysis was performed in the STRING database. False discovery rate < 0.05 was considered statistically significant. Overall survival (OS) and recurrence-free survival (RFS) time were stratified according to the median expression levels of RSEM of different genes (high and low). Survival analysis was conducted using Kaplan-Meier analysis with a log-rank test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Analyses of MCM3 using the TCGA database. The MCM3 mRNA expression levels in 413 selected tissues (363 tumor tissues and 50 normal tissues) were obtained from TCGA. Increased MCM3 expression was observed in the tumor tissues (Fig. 1A). When the associations between MCM3 and clinical

Table II. Association between MCM3 expression levels and clinicopathologic characteristics in hepatocellular carcinoma in The Cancer Genome Atlas dataset (n=363).

Clinical features	Cases, n	MCM3 levels (RSEM; mean \pm SD)	P-value
Sample			
LIHC	363	10.7220 \pm 0.8776	<0.001
Normal	50	9.3221 \pm 0.3847	
Age at diagnosis, years			
\leq 45	48	10.9396 \pm 0.9240	0.065
>45	314	10.6881 \pm 0.8683	
Unknown	1		
Sex			
Female	117	10.8264 \pm 0.8170	0.118
Male	246	10.6724 \pm 0.9024	
AFP in serum, ng/ml			
\leq 20	143	10.4980 \pm 0.8607	<0.001
>20	129	10.9507 \pm 0.8032	
Unknown	91		
E-S grade			
I-II	225	10.5271 \pm 0.8640	<0.001
III-IV	133	11.0550 \pm 0.8095	
Unknown	5		
Child-Pugh classification			
A	213	10.6595 \pm 0.8948	0.806
B	21	10.6102 \pm 0.6849	
C	1		
Unknown	128		
Lymph node			
N0	246	10.7852 \pm 0.8700	0.441
N1	3	11.1746 \pm 0.4966	
Unknown	114		
Metastasis			
M0	260	10.7999 \pm 0.8767	0.432
M1	4	10.4529 \pm 0.7069	
Unknown	99		
HBsAg infection			
Yes	137	10.8366 \pm 0.9352	
No	0		
Unknown	226		

LIHC, liver hepatocellular carcinoma; RSEM, RNA-Seq by Expectation-Maximization; MCM3, minichromosome maintenance complex component 3; AFP, α -fetoprotein; E-S, pathological Edmondson-Steiner; HBsAg, hepatitis B surface antigen.

features, including age, sex, AFP, E-S grade, Child-Pugh classification (24), hepatitis B surface antigen infection and lymph node metastasis, were evaluated in HCC, elevated MCM3 expression was identified to be associated with high AFP levels, as well as advanced E-S grade (Table II). However,

no association was observed between MCM3 expression and other clinical factors. In addition, Kaplan-Meier analysis indicated that patients with high MCM3 expression had poorer OS and RFS rates than patients with low MCM3 expression (Fig. 1C and D).

To further assess the diagnostic utility of MCM3 in HCC, a ROC curve with an AUC of 0.932 was constructed by plotting sensitivity vs. specificity. At a cut-off point of 9.757, which was determined using the Youden's index, the sensitivity and specificity were 85.40 and 92.00%, respectively (Fig. 1B).

MCM3 expression in HCC and adjacent tissues. High MCM3 expression at the mRNA and protein levels in HCC tissues was observed compared with in paired adjacent tissues (Fig. 2). The RT-qPCR results indicated that MCM3 mRNA expression was higher in the HCC tissues than in the adjacent tissues (Fig. 2A). Immunoblotting and IHC demonstrated increased MCM3 expression in HCC at the protein level (Fig. 2B and C). Interestingly, MCM3 proteins were primarily observed in the nuclei of tumor cells (Fig. 2C). The statistical analysis of IHC data revealed that there was a significant difference between HCC and adjacent normal tissues, with MCM3 being highly expressed in 37 (47.4%) of the 78 HCC tissues, whereas there were only 13 (16.7%) tissues with high MCM3 expression among the adjacent normal tissues (Table III).

Association between MCM3 expression and clinical features of patients with HCC. The analysis of the association between MCM3 expression and the clinical features of the respective patients revealed that enhanced MCM3 expression was associated with invasiveness of the tumor cells (Table IV). However, there was no association identified between MCM3 expression and other clinical features, such as age, sex, smoking, drinking, HBsAg infection, AFP, E-S grade, ALT, AST and metastasis, in patients with HCC (Table IV).

Construction of a PPI network and module analysis. A PPI network of MCM3 and the proteins that interact with it, including MCM2-7 and other genes, was generated with STRING and visualized using Cytoscape (Fig. 3A). MCM4/6/7 formed a core subcomplex, while MCM3 was associated with both MCM2 and MCM5 and served a regulatory role in the MCM complex (25). A significant module was obtained from the PPI network by applying MCODE in Cytoscape (Fig. 3B). The module containing MCM3 was associated with 'DNA replication', 'cell cycle', 'nucleoplasm' and 'binding' (P<0.05, Fig. 3C).

GO functional enrichment analysis and KEGG pathway enrichment analysis. The significant terms of GO and KEGG enrichment analysis obtained using STRING are shown in Table V. MCM3 and its interacting proteins were mainly involved in biological processes, such as 'DNA replication', 'cell cycle', 'cell cycle processes', and 'chromosome organization'. The molecular functions (MFs) mainly included 'helicase activity' and numerous binding processes, including 'ATP binding', 'DNA binding', 'heterocyclic compound binding', 'organic cyclic compound binding' and 'chromatin binding'. Cell component annotations suggested that the

Table III. Expression levels of MCM3 in HCC and adjacent tissues.

MCM3 levels	Labelling index	HCC tissues, n (%)	Adjacent tissues, n (%)	P-value
Negative expression	0	8 (10.3)	16 (20.5)	<0.001
Low expression	1+	11 (14.1)	25 (32.0)	
	2+	22 (28.2)	24 (30.8)	
High expression	3+	37 (47.4)	13 (16.7)	

0, no or faint staining intensity in <10% of cells; 1+, faint nuclear/cytoplasmic staining in ≥10% of cells; 2+, moderate and incomplete nuclear staining in ≥10% of cells; 3+, strong nuclear staining in ≥10% of cells; MCM3, minichromosome maintenance complex component 3; HCC, hepatocellular carcinoma.

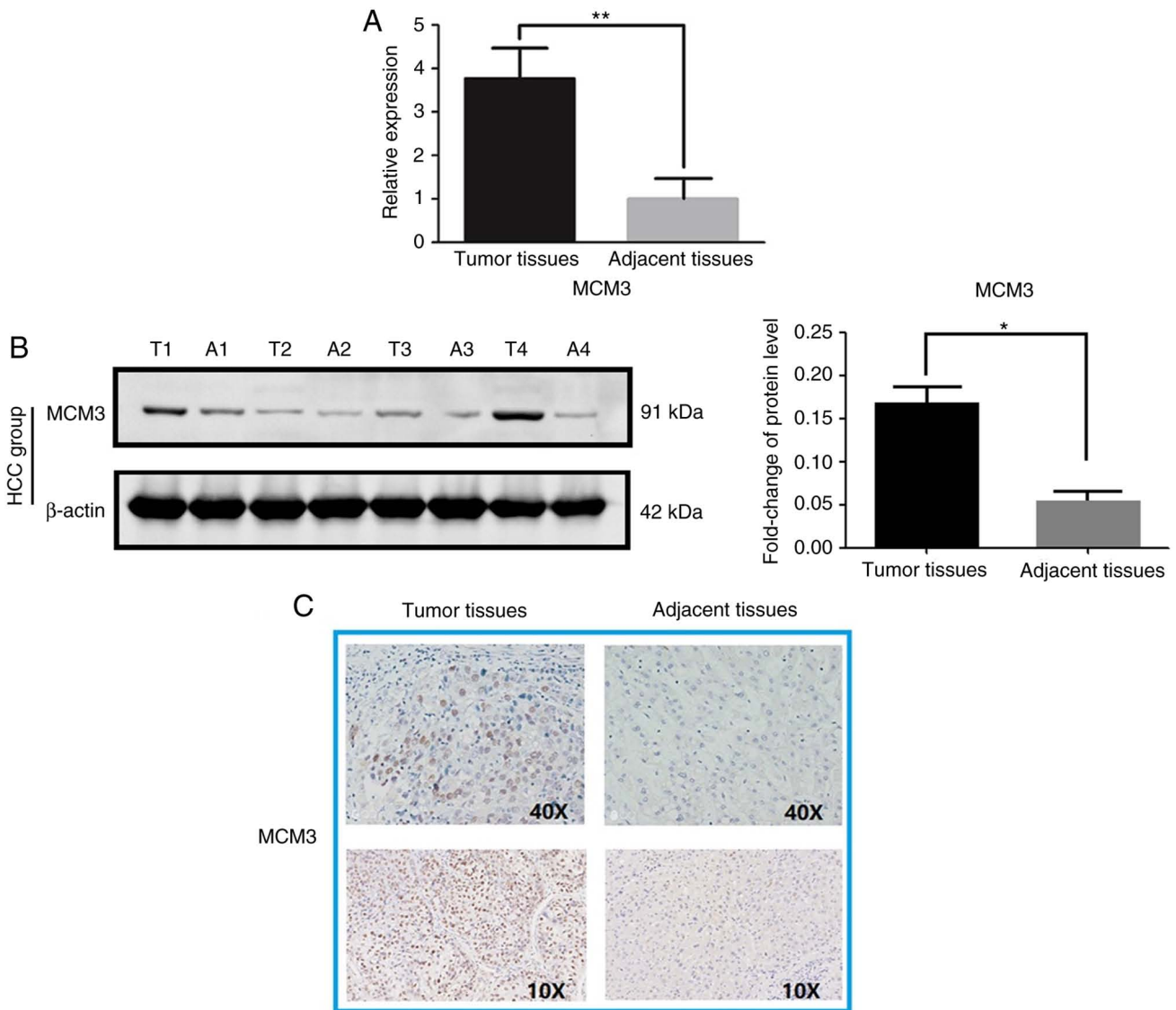


Figure 2. MCM3 expression in HCC tissues and the adjacent tissues. (A) Relative mRNA levels of MCM3 in HCC tissues and the corresponding adjacent tissues. (B) Protein levels of MCM3 in HCC tissues and the adjacent tissues detected by western blotting. (C) Immunohistochemical staining of MCM3 in HCC tissues and the adjacent tissues. T, tumor tissue; A, adjacent tissue; MCM3, minichromosome maintenance complex component 3; HCC, hepatocellular carcinoma. *P<0.05, **P<0.01.

proteins were enriched predominantly in the ‘nucleoplasm’ and ‘protein-containing complex’. KEGG enrichment analysis suggested that MCM3 and its interacting proteins were associated with the ‘cell cycle’ and ‘DNA replication’.

Expression levels of the MCM3 interactome [MCM2, MCM5, checkpoint kinase 1 (CHK1), and DEAH (Asp-Glu-Ala-His) box helicase 9 (DHX9)] in HCC. TCGA and RT-qPCR analysis revealed significantly higher expression levels of MCM2,

Table IV. Association between MCM3 expression levels and clinicopathological characteristics in hepatocellular carcinoma (n=78).

Clinical features	Cases, n	MCM3 levels		P-value
		Low/Negative, n (n=41)	High, n (n=37)	
Age, years				
≤45	31	17	14	0.819
>45	47	24	23	
Sex				
Male	55	31	24	0.330
Female	23	10	13	
Smoking				
Yes	32	19	13	0.362
No	46	22	24	
Drinking				
Yes	36	19	17	1.000
No	42	22	20	
HBsAg infection				
Yes	54	28	26	1.000
No	24	13	11	
AFP, ng/ml				
≤20	26	14	12	1.000
>20	52	27	25	
ALT, U/l				
≤40	41	23	18	0.650
>40	37	18	19	
AST, U/l				
≤40	37	20	17	0.824
>40	41	21	20	
E-S grade				
I-II	33	17	16	1.000
III-IV	45	24	21	
Tumor size (cm)				
≤5	32	18	14	0.649
>5	46	23	23	
Metastasis				
Yes	25	14	11	0.809
No	53	27	26	
Invasion				
Yes	51	22	29	0.032
No	27	19	8	

MCM3, minichromosome maintenance complex component 3; HBsAg, hepatitis B surface antigen; AFP, α -fetoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; E-S, pathological Edmondson-Steiner.

MCM5, CHK1 and DHX9 in HCC tissues compared with in normal or adjacent tissues (Fig. 4A and B).

Impact of MCM3 interactome (MCM2, MCM5, CHK1 and DHX9) in patients with HCC. Based on the data from TCGA,

the area under the curve (AUC) of HCC samples expressing MCM2, MCM5, CHK1 and DHX9 was 0.930, 0.895, 0.883 and 0.727, respectively, according to RSEM analysis (Table VI). The sensibility and specificity of these genes in indicating HCC diagnosis are shown in Table VI. Furthermore, high expression levels of MCM2 and CHK1 were associated with poor OS and RFS (Fig. 4C and D).

In order to improve the HCC diagnosis efficiency, three combined models were generated. The ROC curve of model 1, which had the highest specificity of any of the models, combined the utility of CHK1 and MCM3 (Fig. 5A). The sensitivity and specificity of model 1 were 88.15 and 100%, respectively (Table VI). Model 2, combining the utility of DHX9 and MCM3, had a sensitivity and specificity of 99.17 and 94.00%, respectively, and its sensitivity was the highest of the three models (Table VI; Fig. 5B). Model 3 combined the utility of MCM2, MCM3 and MCM5, and had a sensitivity and specificity of 98.35 and 94.00%, respectively (Table VI; Fig. 5C). The AUCs of models 1, 2 and 3 were 0.975, 0.967 and 0.989, respectively, which were higher than those of MCM3 or any of its interacting proteins alone (Table VI; Fig. 5). The specific algorithm of each model is indicated in Table VI.

Discussion

Studies have indicated that MCM3 is overexpressed in certain carcinomas, such as melanoma, prostate cancer, cervical squamous cell carcinoma and salivary gland tumors (1,5,12). However, little research has been conducted on MCM3 in HCC. Recent studies have only focused on the prognostic or diagnostic value of MCM3 in HCC (26,27), whereas the effect of MCM3 in Chinese patients with HCC remains unclear. Furthermore, the aforementioned studies did not reveal an association between MCM3 and HCC clinical features or the diagnostic ability of MCM3 in combination with its interacting proteins. In the present study, MCM3 mRNA and protein expression was found to be higher in HCC tissues than in adjacent tissues. Analysis of MCM3 using the TCGA database revealed that high MCM3 expression was associated with high AFP levels and an advanced E-S grade. Furthermore, high MCM3 expression was associated with poor OS and RFS, suggesting that high MCM3 expression levels may be predictive of a poor OS and RFS in HCC. Furthermore, in HCC tissues collected from a cohort of Chinese patients, high mRNA expression levels of MCM3 were associated with HCC invasion. Previous studies have suggested that tumor invasion is associated with poor outcomes in numerous types of cancer, including HCC (28-32). The present study suggested that MCM3 may be a novel prognostic marker of HCC and that high MCM3 expression may be associated with poor disease outcomes. AFP is a common diagnostic marker for patients with HCC, which is also used to evaluate tumor recurrence (17). A previous study demonstrated that high AFP expression was related to the poor prognosis of patients, and the higher the expression of serum AFP, the worse the prognosis of patients (17). However, there was no association identified between MCM3 expression levels and AFP levels (or E-S grade) in Chinese patients with HCC. On the one hand, TCGA mainly consists of data from Caucasian populations, whereas the current study included HCC tissues from a Chinese

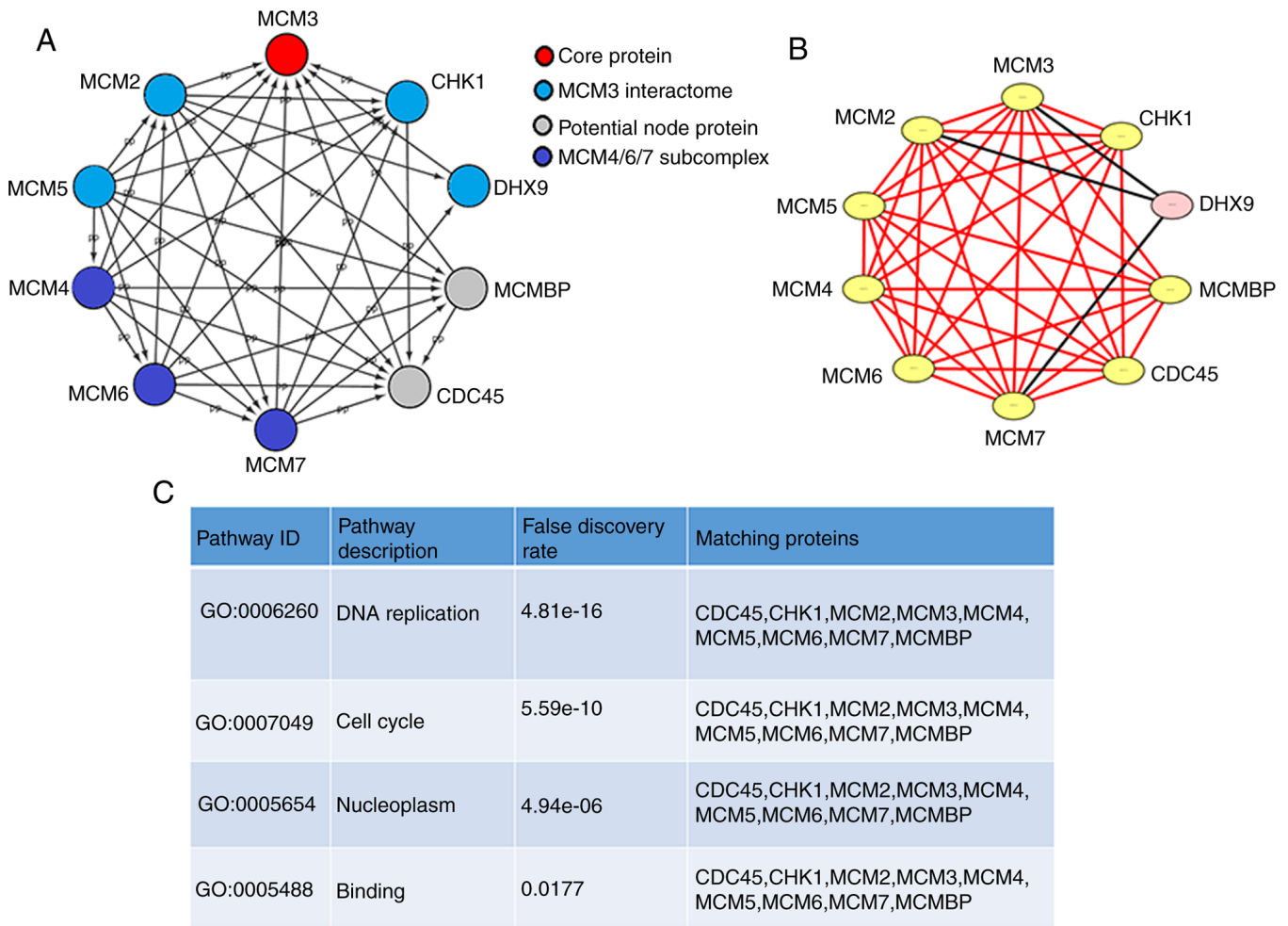


Figure 3. PPI network and core module of MCM3. (A) PPI network of MCM3 (B) Core module for MCM3 and its interaction partners. Yellow nodes represent proteins from the same module. Pink nodes represent proteins not belonging to a specific module. (C) Enrichment and pathway analysis of the module. PPI, protein-protein interaction; MCM, minichromosome maintenance complex component; CHK1, checkpoint kinase 1; DHX9, DEAH (Asp-Glu-Ala-His) box helicase 9; CDC45, cell division control protein 45 homolog; MCMBP, minichromosome maintenance complex binding protein; GO, Gene Ontology.

population. Hence, ethnic diversity may be responsible for the observed difference. However, there was a considerable difference in the sample size between the collected HCC samples and samples from TCGA, which may also be a possible reason why there was no association between MCM3 expression and AFP levels in the current cohort. Therefore, further analysis including more HCC samples from Chinese patients and additional ethnicities would help to elucidate the discrepancy in the outcome observed between TCGA and the analyzed cohort of Chinese patients. Furthermore, it would be helpful to understand the association between the expression levels of MCM3 and AFP levels.

The GO and KEGG enrichment analysis revealed that MCM3 and its interacting proteins, including DHX9, CHK1 and MCM components (MCM2/5), were associated with 'DNA replication' and 'cell cycle'. Interestingly, the MFs showed that they participated in numerous binding processes, indicating that abnormal expression of MCM3 and its interacting proteins may have adverse effects in normal cells, which could be a vital trigger leading to HCC.

The regulation of cell proliferation is fundamental for cell growth and the prevention of cancer. An important step in the regulation of cell proliferation lies in regulating the initiation

of DNA synthesis (33). MCM proteins are essential DNA replication factors serving a role in initiating DNA synthesis once per cell cycle (34). Altered levels of MCM proteins have been detected in malignant human cancer cells, such as melanoma, prostate cancer, cervical squamous cell carcinoma, salivary gland tumors, and pre-cancerous cells undergoing malignant transformation (34). DNA replication triggered by the activity of the MCM2-7 complex consists of the MCM4/6/7 core complex and the loosely associated MCM2 and MCM3/5 (25). Previous studies have indicated that both the MCM2 protein and MCM3/5 complex inhibit MCM4/6/7 helicase activity by disassembling the MCM4/6/7 hexamer into the MCM2/4/6/7 and MCM3/4/5/6/7 complexes, respectively (25,35). The inhibitory role of MCM3 may be based on the simple mechanism that free MCM3 and/or MCM3/5 decrease the amount of their adjacent MCM partners, which is associated with the presence of 2-3 times higher MCM3 levels than those of any of the other five MCM proteins (35). Furthermore, MCM3/5 has a direct role in transcription, and increased levels of MCM5 are associated with the activation of transcription (36).

In the present study, MCM2, MCM3 and MCM5 levels were identified to be elevated in HCC. Juríková *et al* (36)

Table V. GO and KEGG pathway enrichment analyses of MCM3 and its node genes.

Category	Pathway ID	Pathway description	Gene count	Matching proteins
Biological process	GO:0006260	DNA replication	10	CDC45, CHK1, DHX9, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7, MCMBP
Biological process	GO:0006270	DNA replication initiation	7	CDC45, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7
Biological process	GO:0006261	DNA-dependent DNA replication	8	CDC45, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7, MCMBP
Biological process	GO:0000082	G1/S transition of mitotic cell cycle	7	CDC45, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7
Biological process	GO:0032508	DNA duplex unwinding	6	CDC45, DHX9, MCM2, MCM4, MCM6, MCM7
Biological process	GO:0006268	DNA unwinding involved in DNA replication	4	MCM2, MCM4, MCM6, MCM7
Biological process	GO:0007049	Cell cycle	9	CDC45, CHK1, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7, MCMBP
Biological process	GO:0022402	Cell cycle process	8	CDC45, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7, MCMBP
Biological process	GO:0051276	Chromosome organization	8	CDC45, CHK1, DHX9, MCM2, MCM4, MCM6, MCM7, MCMBP
Biological process	GO:0033044	Regulation of chromosome organization	3	CDC45, CHK1, MCM2
Biological process	GO:0006282	Regulation of DNA repair	2	CHK1, DHX9
Biological process	GO:0060968	Regulation of gene silencing	2	CDC45, DHX9
Biological process	GO:0031570	DNA integrity checkpoint	2	CDC45, CHK1
Biological process	GO:1902275	Regulation of chromatin organization	2	CDC45, CHK1
Cell component	GO:0042555	MCM complex	6	MCM2, MCM3, MCM4, MCM5, MCM6, MCM7
Cell component	GO:0005654	Nucleoplasm	10	CDC45, CHK1, DHX9, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7, MCMBP
Cell component	GO:0044427	Chromosomal part	7	CDC45, CHK1, DHX9, MCM2, MCM3, MCM7, MCMBP
Cell component	GO:0000228	Nuclear chromosome	6	CDC45, CHK1, DHX9, MCM2, MCM3, MCMBP
Cell component	GO:0000785	Chromatin	5	CHK1, DHX9, MCM2, MCM7, MCMBP
Cell component	GO:0044454	Nuclear chromosome part	5	CDC45, DHX9, MCM2, MCM3, MCMBP
Cell component	GO:0032991	Protein-containing complex	9	CDC45, CHK1, DHX9, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7
Cell component	GO:0005813	Centrosome	4	CDC45, CHK1, DHX9, MCM3
Cell component	GO:0043596	Nuclear replication fork	2	CDC45, MCM3
Cell component	GO:0015630	Microtubule cytoskeleton	5	CDC45, CHK1, DHX9, MCM2, MCM3
Cell component	GO:0032993	Protein-DNA complex	2	CDC45, MCM3
Cell component	GO:0000790	Nuclear chromatin	2	DHX9, MCMBP
Molecular function	GO:0004386	Helicase activity	8	CDC45, DHX9, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7
Molecular function	GO:0003678	DNA helicase activity	5	CDC45, DHX9, MCM4, MCM6, MCM7
Molecular function	GO:0003688	DNA replication origin binding	4	CDC45, DHX9, MCM2, MCM5
Molecular function	GO:0003697	Single-stranded DNA binding	5	CDC45, DHX9, MCM4, MCM6, MCM7
Molecular function	GO:0005524	ATP binding	8	CHK1, DHX9, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7
Molecular function	GO:0003677	DNA binding	8	CDC45, DHX9, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7

Table V. Continued.

Category	Pathway ID	Pathway description	Gene count	Matching proteins
Molecular function	GO:0043138	3'→5' DNA helicase activity	2	CDC45, DHX9
Molecular function	GO:1901363	Heterocyclic compound binding	9	CDC45, CHK1, DHX9, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7
Molecular function	GO:0097159	Organic cyclic compound binding	9	CDC45, CHK1, DHX9, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7
Molecular function	GO:0003824	Catalytic activity	9	CDC45, CHK1, DHX9, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7
Molecular function	GO:0003682	Chromatin binding	3	CDC45, DHX9, MCMBP
Molecular function	GO:0005488	Binding	10	CDC45, CHK1, DHX9, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7, MCMBP
KEGG	hsa04110	Cell cycle	8	CDC45, CHK1, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7
KEGG	hsa03030	DNA replication	6	MCM2, MCM3, MCM4, MCM5, MCM6, MCM7

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MCM, minichromosome maintenance complex component; CDC45, cell division control protein 45 homolog; CHK1, checkpoint kinase 1; DHX9, DEAH (Asp-Glu-Ala-His) box helicase 9; MCMBP, minichromosome maintenance complex binding protein.

Table VI. Sensitivity and specificity of hepatocellular carcinoma diagnosis by analyzing the expression of MCM3 and its interacting proteins.

Diagnostic marker	AUC	Sensitivity, %	Specificity, %
MCM2	0.930	85.95	88
MCM3	0.932	85.40	92
MCM5	0.895	74.93	92
CHK1	0.883	75.76	90
DHX9	0.727	52.34	96
Model 1 (CHK1 and MCM3) ^a	0.975	88.15	100
Model 2 (DHX9 and MCM3) ^b	0.967	99.17	94
Model 3 (MCM2, MCM3 and MCM5) ^c	0.989	98.35	94

^aAlgorithm, $Y=1.996x \text{ CHK1} + 3.174x \text{ MCM3} - 41.662$; ^bAlgorithm, $Y=-11.523x \text{ DHX9} + 10.173x \text{ MCM3} + 22.341$; ^cAlgorithm, $Y=-21.378x \text{ MCM5} + 3.841x \text{ MCM3} + 14.988x \text{ MCM2} + 49.305$. MCM, minichromosome maintenance complex component; CHK1, checkpoint kinase 1; DHX9, DEAH (Asp-Glu-Ala-His) box helicase 9; AUC, area under the curve.

suggested that the expression levels of all MCM proteins are associated with each other in disease prognosis and that co-overexpression of the six MCM proteins is higher in breast cancer compared with control samples. Depletion or mutation of one MCM protein has been revealed to decrease the levels of all other MCM proteins (35). The present results indicated that MCM2, MCM3 and MCM5 expression was increased in HCC. Furthermore, high levels of MCM2 and MCM3 were associated with poor OS and RFS. Hua *et al* (37) reported that elevated MCM2 and MCM3 mRNA levels are associated with poor outcomes in patients with glioma and these proteins could be clinically useful molecular prognostic markers for glioma. Similarly, research has indicated that MCM2 and MCM3 act as oncogenes in osteosarcoma by conducting

in vitro experiments (38). However, in HCC, the role and the underlying mechanism of MCM2, MCM3 and MCM5 remain unclear.

DHX9 is an enzyme that catalyzes the ATP-dependent unwinding of double-stranded RNA and DNA-RNA complexes. DHX9 mainly binds to inverted-repeat Alu elements and inhibits the production of circular RNAs (39). Abnormal DHX9 expression has been detected in different tumors, including HCC, colorectal cancer, lung cancer, osteosarcoma, breast cancer and prostate cancer (38-41). A previous study indicated that DHX9 interacts with MCM2 and MCM3 in osteosarcoma cells (38). In addition, the decreased growth of osteosarcoma cells due to MCM2 or MCM3 knockdown is reversed by DHX9 overexpression, indicating that MCM2

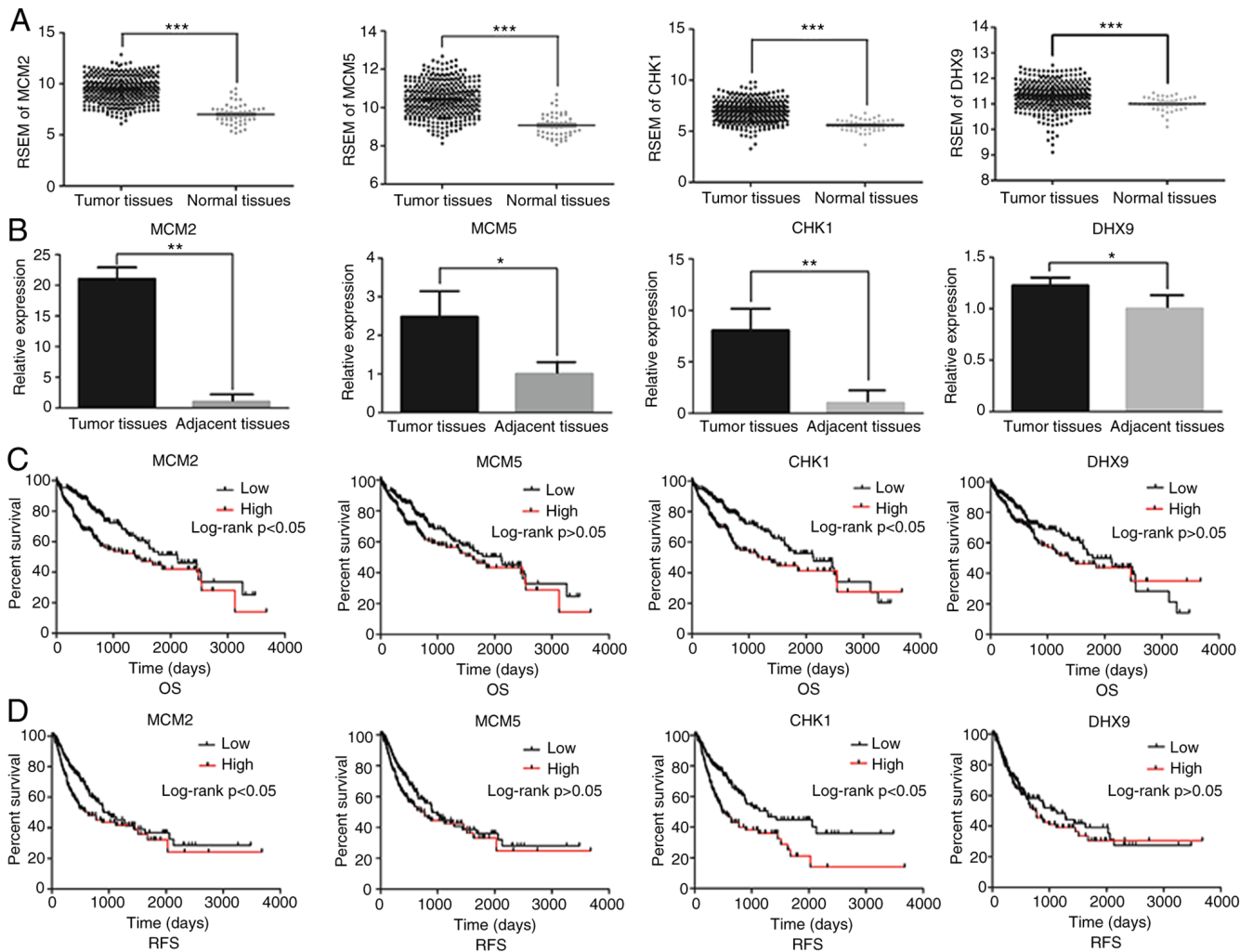


Figure 4. Impact of MCM3 interacting proteins in HCC. (A) mRNA expression of MCM2, MCM5, CHK1 and DHX9 in HCC tissues and normal liver tissues from The Cancer Genome Atlas database. mRNA expression was normalized by RSEM. (B) Relative mRNA expression levels of MCM2, MCM5, CHK1 and DHX9 in HCC and adjacent tissues from the patients recruited in the present study. (C) OS curves and (D) RFS curves for HCC samples expressing high and low levels of the indicated MCM3 interacting proteins. MCM, minichromosome maintenance complex component; CHK1, checkpoint kinase 1; DHX9, DEAH (Asp-Glu-Ala-His) box helicase 9; HCC, hepatocellular carcinoma; RSEM, RNA-Seq by Expectation-Maximization; OS, overall survival; RFS, recurrence-free survival. *P<0.05, **P<0.01, ***P<0.001.

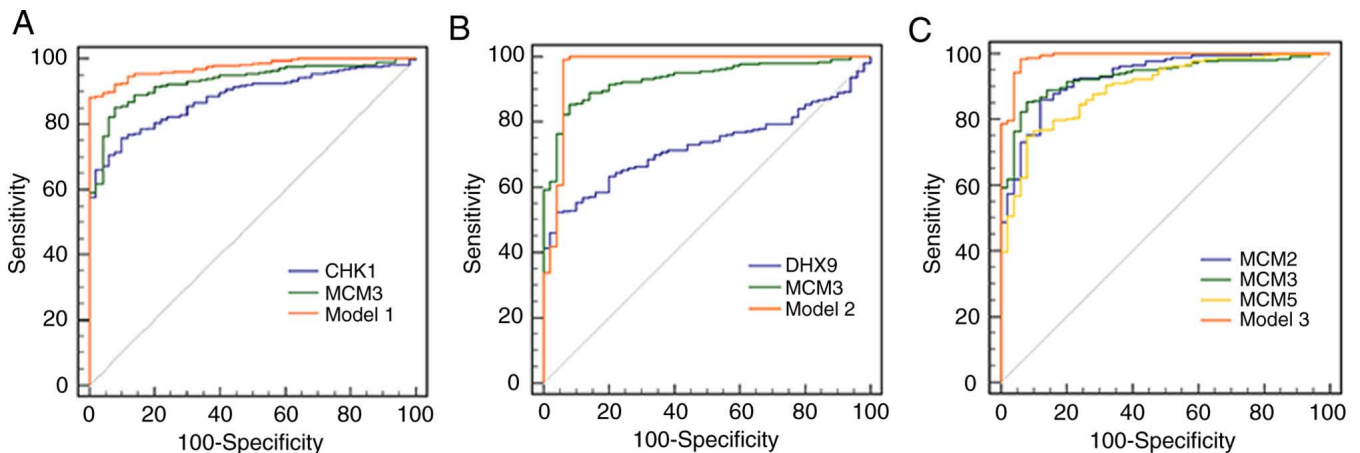


Figure 5. ROC curves of different genes and combined models for diagnosis of hepatocellular carcinoma. The orange solid ROC curve represents models with combined proteins. (A) ROC curves of CHK1, MCM3 and model 1. Model 1 was the combination of CHK1 and MCM3. P<0.05 for AUC of Model 1 vs. CHK1 or MCM3. (B) ROC curves of DHX9, MCM3 and model 2. Model 2 was the combination of DHX9 and MCM3. P<0.05 for AUC of Model 2 vs. DHX9 or MCM3. (C) ROC curves of MCM2, MCM3, MCM5 and model 3. Model 3 was the combination of MCM2, MCM3 and MCM5. P<0.05 for AUC of Model 3 vs. MCM2, MCM3 or MCM5. ROC, receiver operating characteristic; CHK1, checkpoint kinase 1; MCM, minichromosome maintenance complex component; AUC, area under the curve; DHX9, DEAH (Asp-Glu-Ala-His) box helicase 9.

and MCM3 activity is DHX9-dependent (38). In the present study, DHX9 mRNA levels were found to be increased in HCC tissues compared with in adjacent tissues. It has been reported that MCM2 and MCM3 function through DHX9 and that the MCM2/3-DHX9 axis may mediate transcription, translation and DNA replication in osteosarcoma (42,43). The findings of the present study revealed that DHX9 may coordinate with MCM2/MCM3 in HCC, suggesting that targeting the MCM2/3-DHX9 axis may be a feasible and effective strategy for HCC treatment (38). However, the exact role of the MCM2/3-DHX9 axis in proliferation of HCC requires further study.

CHK1 is a central protein involved in the DNA damage response (DDR), particularly responding to damage caused by cytotoxic agents. Increased CHK1 expression has been identified in a variety of human tumors, including breast, colon, gastric and nasopharyngeal cancer (44). In the present study, CHK1 was found to be overexpressed in HCC, which is consistent with previous studies (45,46). Han *et al* (47), suggested that CHK1 serves a fundamental role in the phosphorylation of MCM3 at Ser-205 under normal cell growth conditions. MCM3 phosphorylation by CHK1 negatively inhibits normal DNA replication, whereas CHK1 inhibition leads to increased DNA replication (47). The present study demonstrated that CHK1 and MCM3 were upregulated in HCC, and high CHK1 expression was associated with poor OS and RFS. As higher CHK1 expression is associated with an enhanced ability to respond to DNA damage, thereby promoting DNA replication (44), HCC cells may display increased proliferation and improved resistance to DNA damage compared with normal cells. A previous study has also suggested that malignant tumor cells can survive severe DNA replication stress, which results in the DDR (48). CHK1 could phosphorylate multiple targets in the DDR, such as MCM3 and p53 (47,48).

ROC curves were constructed based on the TCGA data. The AUC for MCM3 was larger than that for MCM2, MCM3, CHK1 and DHX9, suggesting that MCM3 may be a valuable marker in the diagnosis of HCC. The three combined models displayed enhanced HCC diagnostic ability compared with MCM3 alone. At optimal cut-offs with the maximal Youden index, model 1 had the highest specificity, model 2 had the highest sensitivity and the AUC of model 3 was the largest. The results of the present study suggest that the properties of multi-gene HCC diagnosis are affected by the genes included in the combined model. MCM3 and its combined models had higher sensitivities and specificities than existing HCC diagnostic markers, such as glypican-3 and AFP, which had pooled sensitivities and specificities of 0.55 (95% CI, 0.52-0.58) and 0.58 (95% CI, 0.54-0.61), and 0.54 (95% CI, 0.51-0.57) and 0.83 (95% CI, 0.80-0.85), respectively (49).

In conclusion, higher MCM3 expression levels were identified in HCC than in normal liver tissues, and high MCM3 expression was associated with high AFP levels and advanced E-S grade. Additionally, high MCM3 expression was associated with poor OS and RFS. Furthermore, in HCC tissues collected from Chinese patients, high MCM3 expression was associated with HCC invasion. Functional and pathway enrichment analysis revealed that MCM3 and its interacting proteins were primarily involved in 'DNA replication', 'cell

cycle' and in the binding process with numerous substrates (DNA/protein/organic compounds). Similarly, TCGA and RT-qPCR analysis demonstrated that the expression levels of MCM3 interactome proteins (MCM2/5, CHK1 and DHX9) were increased in HCC. An algorithm combining the ROCs of MCM3 and the other interaction partners was more specific and sensitive for the diagnosis HCC than other conventional HCC diagnostic markers. The present study revealed that MCM3 and its interacting proteins may be potential novel diagnostic markers for HCC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HTL, XW, BW, ZQL and XLY contributed to the conception and design of the study, analysis of the data, interpretation of the results and the writing of the manuscript. WXJ, XW, HTL, YZX, JYL, MNS, SXC, NFM and DZ conducted the experiments and contributed to the acquisition of data. XW, WPZ, JQ, PL and QLZ analyzed the data. XLY reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This research project was approved by the Ethics and Human Subjects Committee (EHSC) of Guilin Medical University and conformed to the provisions of the Declaration of Helsinki. Informed consent was obtained from each patient included in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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