Research Article

MCM6 Promotes Hepatocellular Carcinoma Progression via the Notch Pathway: Clinical, Functional, and Genomic Insights

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Objective. To evaluate the expression profile of MCM6 in HCC and the relationship between MCM6 level and clinicopathological parameters through bioinformatics analysis of several databases.

Methods. MCM expression level, clinical parameters, survival data, and gene set enrichment analysis were analyzed by bioinformatics database, including Oncomine™, UALCAN, HCCDB, TCGA, cBioPortal, and LinkedOmics. Real-time PCR, western blotting, and IHC staining were conducted to identify the expression of MCM6 in HCC compared to normal liver tissues.

Results. Bioinformatics analysis indicated that the mRNA of MCM6 was obviously increased in multiple cancer types, especially in HCC. MCM6 level was positively associated with multiple clinical parameters (stage 3 and grades 3 and 4) and negatively associated with patient outcomes (overall survival). Moreover, enrichment of functions and signaling pathways analysis of MCM6 suggested that MCM6 might mediate DNA replication and cellular metabolism to promote the development and progression of HCC. Furthermore, IHC staining and western blotting indicated that the MCM6 was enhanced in HCC tissue, and MCM6 could promote HCC proliferation in activating Notch pathway via WB and bioinformatic analysis.

Conclusion. This study actually revealed the expression and related functions of MCM6 in HCC. Furthermore, MCM6 is a carcinogenic role in activating Notch pathway to promote HCC cell proliferation, which may be a new prognostic biomarker and therapeutic target for HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most lethal human malignancy globally, with 841,080 new and 781,631 deaths cases estimated for 2018 [1]. The World Health Organization has predicted that more than 1 million people will die from HCC in 2030 [2]. Moreover, the incidence and death rates of HCC are increasing in developing countries [3]. Despite the improvements in surgery combined with radiotherapy and chemotherapy, the high mortality and low 5-year survival rate of HCC are primarily due to the complex pathophysiological processes, including hepatitis, liver fibrosis, and abnormal regeneration of liver cells. Identifying key multigene networks in multiple steps is necessary to enhance the early metaphase diagnosis rate [2]. Early diagnosis of HCC may be able to prolong the lifespan of patients with HCC as much as possible [4]. Therefore, it is of great clinical significance to explore accurate biomarkers for early diagnosis and potential treatment targets in HCC.

Minichromosome maintenance (MCM) proteins family, a classical family on the eukaryotic genome replication, in Saccharomyces cerevisiae, was found first, which were necessary for the maintenance of extrachromosomal DNA [5]. During the DNA replication licensing process, MCM2-7 forms a hexameric ring-shaped complex to promote DNA replication of chromatin by binding to a DNA replication
Comparison of MCM6 across 3 analysis

Over-expression

| Median rank | \( p \)-value | Gene |
|-------------|---------------|------|
| 63.0        | 6.47E-11      | MCM6 |

Legend
1. Hepatocellular carcinoma vs. normal
   Chen liver, Mol Biol cell, 2002 Roessler liver2, cancer Res, 2010
2. Hepatocellular carcinoma vs. normal
   Roessler liver, cancer Res, 2010

The rank for a gene is the median rank for that gene across each of the analysis. The \( p \)-value for a gene is its \( p \)-value for the median-ranked analysis.

Figure 1: Continued.
starting point in the middle to late cell cycle to the early G1 phase [6]. A biochemical and structural study indicated that the hexameric ring-shaped complex was a hetero-hexameric AAA+ ATPase, which has helicase activity, while the N-terminal region plays the role of organizing center in the replication in eukaryotic cells [7].

MCM6, as an important member of the MCM family, could interact with Cdt1 at NTD to stabilize the coil structure [8], be phosphorylated by DDK at NTD terminus to activate pre-RC [9], and be involved in translocation along single-stranded DNA in the MCM2-7 complex [8]. It is reported that MCM6, as a transcriptional regulation factor, could activate MEK/ERK signaling pathway to mediate EMT progression, resulting in metastasis of HCC [10]. Chen et al. [11] found that the direct interaction between MCM6 and p53-binding protein 1 (53BP1) was involved in 53BP1 chromatin fraction and foci formation in HepG2 cells. However, little is known about the role and mechanism of MCM6 in hepatocarcinogenesis. Until now, the previous studies indicated that the expression of MCM6 was observed in 70 patients with HCC, and it may relate to poor prognosis [10].

This study evaluated the expression profile of MCM6 in HCC and the relationship between MCM6 level and clinicopathological parameters through bioinformatics analysis of several databases. In addition, the biological functions and genomic changes correlated with biological functions in HCC were examined. The present study may provide a novel and potential molecule target for the early diagnosis and targeted therapy of HCC.

2. Materials and Methods

2.1. Bioinformatic Analysis. Oncomine database (http://www.oncomine.org) is a web tool to extract data for the mRNA level and gene expression of MCM6, currently containing 715 datasets and 86,733 samples. A series of HCC datasets were utilized for this study, including Chen Liver, Roessler Liver, and Roessler Liver 2. HCCDB database (http://lifeome.net/database/hccdb/search.html) is an integrative molecular database of HCC based on 4000 HCC samples. We utilized the HCCDB database to confirm the level of MCM6. UALCAN database (http://ualcan.path.uab.edu) is an interactive web portal to analyze the expression of MCM6 in HCC, which is based on gene expression analysis of TCGA (The Cancer Genome Atlas). The cBioPortal database (http://cbioportal.org) is an interactive web tool for elucidating the altered expression of MCM6 in HCC, which is used to explore the multidimensional genomics datasets for multiple cancer types. LinkedOmics database (http://www.linkedomics.org/login.php), as an online tumor database, was utilized for elucidating multomics data. Users can extract multiple cancer-type protein expression profiles, which are differential expression in specific cancer. We used it to analyze the GO and KEGG pathway of MCM6 coexpression genes in HCC.

2.2. Cell Culture and Tissue Collection. Two HCC cell lines (HepG2 and SNU-368) were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin and streptomycin, which were maintained at 37°C in a humidified atmosphere of containing 5% CO2. Furthermore, a total of 30-paired HCC and paracancerous tissues were surgically obtained from the Three Gorges Hospital of Chongqing University in Wanzhou, China. This study was approved by the ethics committee of Three Gorges Hospital of Chongqing University with the written consent of all patients.

2.3. Cell Transfection. The shRNA against MCM6 or shRNA negative control were constructed by RiboBio Co. Ltd.

Figure 1: MCM6 transcription in HCC. (a) MCM6 level in cancer tissues vs. corresponding normal tissues by the Oncomine. (b) The transcriptional level of MCM6 was analyzed in HCC among three studies by Oncomine. (c) MCM6 level in the HCCDB database.
Expression of MCM6 in LIHC based on tumor grade

Expression of MCM6 in LIHC based on individual cancer stages

Figure 2: Continued.
Expression of MCM6 in LIHC based on patient’s age

TCGA samples

(d)

Expression of MCM6 in LIHC based on patient’s gender

TCGA samples

(e)

Figure 2: Continued.
siRNA was transfected into cells using riboFECT transfection reagent (Changzhou Bio-Generating Biotechnologies Co., Ltd.) according to the manufacturer’s protocol. The sequences of the siRNA were as follows: si-MCM6, GGCG CATAGTAGATTTGCA. The shRNAs were transfected by lip3000 for 6 hours. The effect of MCM6 knockdown was confirmed by western blot.

2.4. Western Blot. Western blotting was conducted according to our previous report [12]. The experiment was conducted with the approval of a formal Ethics committee and the informed consent of the patients. The primary antibodies were used in this study against MCM6 (ab201683, dissolution 1/1000), NICD (Santa Cruz, sc6014, dissolution 1/500), Notch1 (ab52627, dissolution 1/1000), Hey1 (ab154077, dissolution 1/1000), Hes1 (ab119776, dissolution 1/1000), and GAPDH (ab181603, dissolution 1/10000).

2.5. Immunohistochemistry Analysis. The slices are dewaxed and then hydrated. After blocking endogenous peroxidase, the slices were treated with a hot citric acid buffer to reveal the epitope. The sections were then incubated with a primary antibody using an immunohistochemistry kit (MXB, Fuzhou, China) according to the manufacturer’s instructions.

Immunohistochemical staining scores were assessed by staining percentage of tumor cells and staining intensity. The percentage of stained cancer cells was graded from 0 to 4: 0 (0% stained), 1 (0%-25% stained), 2 (26%-50% stained), 3 (51%-75% stained), and 4 (76%-100% stained).

Figure 2: The transcription level of MCM6 in HCC based on TCGA database. (a) MCM6 gene level in multiple cancer types vs. corresponding normal tissues (TCGA). (b) Boxplot revealing MCM6 level in normal people or HCC patients with cancer grade, (c) stage, (d) different ages, (e) gender, (f) ethnicity, and (g) weight.
Mutation data
CNA data
HCC (Inserm, 2015)
Liver (TCGA)
Liver (AMC)
–++
+++
 Alteration frequency
0.5%
1%
1.5%
Deep deletion
Mutation
Mutation spectrum
MCM6 1%
Genetic alteration
Missense mutation (Unknown significance)
Translating mutation (Unknown significance)
Deep deletion
No alterations
Not profiled
Mutation spectrum
C > A
C > G
C > T
T > A
T > C
T > G
No data
Figure 3: Continued.
Figure 3: Continued.
Similarity, stained tumor cell intensity was divided into 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The final semi-quantitative immunohistochemical fraction was obtained by multiplying the percentage of stained cancer cells by the intensity grade.

2.6. Proliferation Analysis. For the MTT analysis, two HCC cancer cell lines were seeded in 96-well (5 × 10^3) and cultured for one, two, and three days. 20μl MTT solution (Sigma-Aldrich; Merck KGaA) was used to incubate for 2 hours. Next, 150μl DMSO was added in each well. Last, the OD value was confirmed at 490 nm by a microplate reader. For the clone formation assay, cells were seeded into 24-well plates with 500 cells in each well and cultured for 14 days. When cell colonies were visible to the naked eyes, the culture was terminated—washed cells with PBS for 3 times, 5 minutes each time. Then fix with 4% paraformaldehyde for 20 minutes. The cells were washed with PBS for 3 times, 5 minutes each time, incubated with methanol for 20 minutes, and washed with PBS for 3 times, 5 minutes each time. Shake dry and stain with crystal violet for 30 minutes. Take photos and analyze the results.

2.7. Statistical Analysis. Statistical analyses for bioinformatic analysis were performed in the R Programming Language (version 3.6). Data are presented as the mean ± SD. All assays were tested in three independent experiments. Experimental data were analyzed using SPSS statistical software (version 12.0; SPSS Inc.). The significance of the group difference was evaluated by one-way analysis of variance followed by Tukey’s post hoc test. Multiple comparisons between the groups were performed using Student-Newman-Keuls method. P < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. The MCM6 Level Was Enhanced in HCC. We firstly extracted MCM6 transcription levels from a variety of cancers from TCGA. The Oncomine database indicated significant increases in MCM6 mRNA expression in HCC (Figures 1(a) and 1(b)). To further determine the expression of MCM6 in many cancers, we extracted the expression profile of MCM6 based on TCGA database. As shown in Figure 2(a), we found MCM6 transcription expression was obviously enhanced in lung cancer, head and neck squamous cell carcinomas, HCC, and so on. The 4D measurement of the HCCDB dataset showed that MCM6 was not a liver-specific gene (logFC = −1.58), and MCM6 were increased in HCC compared to normal liver tissue (logFC = 0.34) and corresponding paracancer tissue (logFC = 0.91) (Figure 1(c)). In addition, high level of MCM6 was found in a subgroup evaluation of HCC samples with various clinicopathologic features (UALCAN database). MCM6 profile was obviously increased in tumor grade,
MCM6 expression high to low

Negatively correlation genes

| Z-score | Value |
|---------|-------|
| > 3     | Red   |
| 1       | Pink  |
| 0       | Blue  |
| -1      | Blue  |
| < -3    | Blue  |

(a) MCM6 expression high to low

Figure 4: Continued.
cancer stage, patient age, gender, race, and weight (Figures 2(b)–2(g)). In summary, MCM6 can be used as a possible diagnostic marker for HCC.

3.2. MCM6 Genetic Alteration Correlated with Patient Survival in HCC Patients. In the cBioPortal dataset, we extracted the profile of MCM6 genetic alteration in HCC. Three HCC databases (AMC hepatology 2014 dataset, INSERM Nat Genet 2015 dataset, TCGA Firehose Legacy dataset) analysis results indicated that the highest alteration frequency of MCM6 was only 1% (Figures 3(a) and 3(b)). Furthermore, the patients with MCM6 alteration had significant disease/progression-free survival (Figure 3(d)), but had no significant overall survival (Figure 3(c)). UALCAN analysis suggested that these HCC patients with a high level of MCM6 had a worse prognosis (Figure 3(e)). Hence, these results indicated that MCM6 alteration might induce an unfavorable prognosis in HCC patients.

3.3. Gene Coexpression of MCM6 in HCC. The method analyzed mRNA sequencing data from 371 HCC patients by LinkedOmics analysis of genes coexpressed with MCM6. The heat map showed the top 50 significant positively and negatively coexpressed genes with MCM6, respectively (Figures 4(a) and 4(b)). The volcano blot showed all the coexpressed genes with MCM6 (Figure 4(c)). For example, NDUFAF1, LDHD, and SERPING1 were the most significantly and negatively correlated with the expression of MCM6 (Figures 5(a)–5(c)), while DTL, ZWINT, and MCM2 were the top 3 negatively correlated genes with MCM6 (Figures 5(d)–5(f)). These genes were involved in many biological progressions, such as cell proliferation, transcription, metastasis, cell cycle, and metabolism. Gene set enrichment analysis (GSEA) was used to analyze GO annotation with significant enrichment. In these differentially expressed genes (DEGs) associated with MCM6, the GO enrichment results indicated that (1) for biological process (BP), these DEGs were obviously enriched in biological regulation, metabolic process, response to stimulus, multicellular organismal process, localization, and cellular component organization (Figure 6(a)); (2) for cellular component (CC), these DEGs were significantly enriched in the membrane, nucleus, membrane-enclosed lumen, protein-containing complex, cytosol, endomembrane system, and vesicle (Figure 6(b)); (3) for molecular function (MF), these DEGs were largely enriched in protein binding, ion binding, nucleic acid binding, hydrolase activity, transferase activity, and nucleotide-binding (Figure 6(c)). The signaling pathway enrichment analysis by KEGG showed that these DEGs were primarily enriched in primary bile acid biosynthesis, valine,
Figure 5: Continued.
leucine and isoleucine degradation, arginine biosynthesis, DNA replication, fatty acid degradation, and homologous recombination (Figure 6(d)).

3.4. Identification of MCM6 Expression and Prognosis in HCC Tissues. For identifying the level of MCM6 in HCC, we detected the MCM6 expression in HCC tissues by IHC staining (Figure 7(a)). The result indicated that MCM6 were obviously enhanced in HCC tissue compared to corresponding paracancer tissue, which were consistent with the previous bioinformatics results. Moreover, Kaplan-Meier survival analysis also indicated that MCM6 was negatively correlated with the prognosis in HCC patients (Figure 7(b)), suggesting that MCM6 might be a prognostic marker for HCC patients. Therefore, these results sufficient suggested that MCM6 was significantly increased in HCC, and the high expression of MCM6 showed a worse prognosis for HCC patients, suggesting that MCM6 was a potential oncogene in HCC.

3.5. Notch Signaling Activation Is Critical for MCM6-Mediated Proliferation. Hyperproliferation is a hallmark feature of cancer. To elucidate the association between cell proliferation and MCM6 expression, we performed MTT and colony formation analysis to confirm the proliferation ability in MCM6 inhibition cells. This result showed that MCM6 inhibition could significantly repress the proliferation ability for HepG2 and SNU-368 cell lines (Figures 7(c) and 7(d)). Moreover, GSEA analysis showed that MCM6 was obviously enriched in the Notch signaling pathway (Figure 7(e)), which is closely correlated with cell proliferation in multiple cancer types. We further confirmed the expression of key factors involved in the Notch signaling pathway and EMT cascade by western blot, such as E-cadherin, N-cadherin Notch1, Hey1, and Hes1 (Figure 7(f)). MCM6 inhibition could significantly enhance the expression of E-cadherin, but repress the expression of N-cadherin, Notch1, Hey1, and Hes1, which suggested that MCM6 inhibition could downregulate the proliferation ability of HCC cells via inactivating Notch pathway and EMT cascade.

4. Discussion

MCM6, a member of minichromosome maintenance proteins, has been demonstrated that it has a high level in HCC [10]. MCM6 has multiple pathophysiological functions in carcinogenesis, especially in the hepatocarcinogenesis. Chen et al. found that MCM6 knockdown could directly inhibit p53-binding protein 1 (53BP1) foci formation and chromatin fraction in response to DNA damage, indicating that MCM6 was not just a DNA replication regulator [11]. Liu and his colleagues also revealed that MCM6 could obviously promote migration and invasion ability via activating MEK/ERK signaling pathway to induce EMT cascades [10]. However, there are few studies mentioning the ancillary effects beyond DNA replication. And the ectopic expression of MCM6 in the formation of HCC is still largely unknown. This study was based on the bioinformatics analysis of databases. After analysis of Oncomine, HCCDB, and TCGA databases, MCM6 transcription and posttranscriptional levels were significantly upregulated in HCC. In subgroup analyses based on disease stage, tumor grade, age, gender, ethnicity, and body weight, the MCM6 levels were significantly higher in HCC patients than in healthy people. Furthermore, the eBioPortal databases revealed that the frequencies of alterations of MCM6 might be one reason for its ectopic expression and induce a low survival rate in HCC patients with MCM6 alteration. The UALCAN databases also indicated that HCC patients with high expression of MCM6 had a poorer survival rate.
Figure 6: Continued.
Bar chart of cellular component categories.

Figure 6: Continued.
Bar chart of molecular function categories

![Bar chart of molecular function categories](image)

Figure 6: Continued.
MCM6 is mainly involved in primary bile acid biosynthesis, valine, leucine and isoleucine degradation, arginine biosynthesis, DNA replication, fatty acid degradation, and homologous recombination by mining-related functional networks through the KEGG pathway. As we know, MCM6 is a DNA replication license regulator that plays a key role in the cell cycle progression. It has been reported that MCM6 could interact with other MCM proteins (MCM2, MCM3, MCM4, MCM5, and MCM7) to assemble into MCM complex, regulating the DNA replication to accelerate cell cycle [6]. However, the effect of MCM6 on cell metabolism, such as primary bile acid, amino acid, and fatty acid, remains unclear [13]. We speculate that MCM6, like a molecular switch, has a divergence in these critical nodes, with one branch forming MCM complex to directly accelerate DNA replication and another branch regulating cell metabolism to provide the necessary materials and energy in DNA replication.

Furthermore, we found several genes associated with MCM6. The level of NDUFAF1, LDHD, and SERPING1 were negatively correlated with MCM6 expression levels. NDUFAF1, also known as NADH ubiquinone oxidoreductase complex assembly factor 1, is an essential assembly mitochondrial protein for the stability of complex I (NADH:ubiquinone oxidoreductase), which may play a key role in energy metabolism [14]. LDHD, lactate dehydrogenase D, is responsible for human D-lactate metabolism [15]. However, the function of LDHD in HCC progression is still unclear.

SERPING1, serpin family G member 1, plays a key role in hereditary angioneurotic edema through classical complement pathway activation [17], decreased in breast cancer [18], prostate cancer [19], and lung cancer [20]. Therefore, our analysis showed that MCM6 might repress the tricarboxylic acid cycle by inhibiting the expression of NDUFAF1, inhibit aerobic glycolysis by inhibiting the expression of LDHD, and regulate the classical complement pathway activation by regulating the expression of SERPING1.
Figure 7: Continued.
Figure 7: Identification of MCM6 expression, function, and mechanism in HCC. (a) IHC staining of MCM6 expression in HCC and corresponding paracancer tissues from 30 HCC patients. T: tumor; N: nontumor tissues. (b) Kaplan–Meier survival analysis for HCC patients with high or low MCM6 expression based on KM-plot database ($P < 0.05$, log-rank test). (c) The MTT analysis and (d) clone formation analysis among HepG2 and SNU-368 cell lines with vector, MCM6 shRNA or not. (e) The GSEA for MCM6 in KEGG_Notch_signaling_pathway. (f) The expression of MCM6, N-cadherin, E-cadherin, NCID, Notch1, Hey1, and Hes1 in HepG2 and SNU-368 cell lines with vector, MCM6 shRNA or not. **$P < 0.01$ and ***$P < 0.001$. 
and chromosome motility, inhibited the chromosome bridge phenotype from interacting with sister chromatids [22]. ZWINT knockdown could attenuate mitotic arrest mediated by the microtubule inhibitor nocodazole, resulting in cell death [23]. MCM2 could interact with MCM3-7 to form a hexameric ring-shaped complex around DNA, which could promote DNA replication to accelerate the cell cycle [24]. Several previous studies indicated that MCM2 could be an important biomarker of multiple cancer types, such as oral squamous cell carcinoma [25], cervical carcinoma [26], and medulloblastoma [27]. Hence, MCM6 not only interact with MCM2 to form a DNA replication license complex to directly activate DNA replication, but also interacts with DTL as well as ZWINT to accelerate the cell cycle. We also confirmed that MCM6 was significantly upregulated in HCC samples, which was in accordance with previous studies [28, 29]. Moreover, we found that MCM6 can interact with Notch pathway to enhance the proliferation level in HCC cells.

Notch pathway is a classical and important signaling in multiple cancer types, such as breast cancer, HCC, lung cancer, ovarian cancer, cervical cancer, gastric cancer, and renal carcinoma [30]. This signaling could also cross talk with other pathways, including PI3K/AKT, NF-κB, integrin, and miRNAs, to significantly drive cell progression [31]. Previous study has indicated that Notch signaling is critical to cancer cell proliferation, apoptosis escaping, angiogenesis, EMT cascade, and stemness maintaining [32]. Because of Notch pathway is upregulated in HCC patients, the targeted treatment for anti-Notch pathway in HCC patients will need considerable on the upstream of Notch pathway, which are important to reduce the major side effects [33]. Our study indicated that MCM6 could activate the Notch pathway. The MCM6 might be a significant target for anti-Notch treatment in HCC patients.

5. Conclusion
This study indicates that MCM6 is highly expressed in HCC compared to normal liver tissues and is correlated with multiple networks. MCM6 is an excellent biomarker for predicting the prognosis of HCC. However, current research focuses on the role of MCM6 in DNA replication. The role of MCM6 in tumor metabolism is required to confirm the effect of MCM6 on tumor metabolism and transcriptional regulation in HCC and other cancers. Moreover, MCM6 is a carcinogenic role in activating Notch pathway to promote HCC cell proliferation, which may be a new therapeutic target for HCC patients.

Data Availability
The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval
This study was approved by the ethics committee of Three Gorges Hospital of Chongqing University with the written consent of all patients.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Authors’ Contributions
Conception and design were contributed by Liangjuan Hou and Xin Zeng. Collection and assembly of data were performed by Chune Zhao and Yukun Li. Data analysis and interpretation were performed by Xuan Li and Juan Zou. Manuscript writing was contributed by Liangjuan Hou and Xin Zeng. Paper revision was performed by Gang Liu. Final approval of manuscript was performed by all the authors. Liangjuan Hou and Xin Zeng contributed equally to this work.

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