High MCM6 Expression as a Potential Prognostic Marker in Clear-cell Renal Cell Carcinoma

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Abstract. *Aim: Minichromosome maintenance (MCM) proteins are involved in initiation of DNA replication and cell-cycle progression. Loss of MCM function results in genomic instability and causes carcinogenesis. Among MCM genes, the role and prognostic value of MCM6 expression in clear-cell renal cell carcinoma (ccRCC) has not been elucidated. Materials and Methods: We assessed the mRNA expression level of MCM6 using the Gene Expression Profiling Interactive Analysis database and investigated MCM6 protein expression by immunohistochemistry in 238 ccRCC cases. Results: High MCM6 expression was significantly associated with increasing tumor size, pT, stage, tumor necrosis, and metastasis. Furthermore, high MCM6 expression was significantly associated with shorter overall and disease-free survival, and was an independent unfavorable prognostic marker. Regarding patients with metastasis, high MCM6-expressing ccRCC conferred significantly shorter survival than for those with low expression. Conclusion: A high MCM6 expression level may be a promising biomarker to predict tumor progression, metastasis, and survival in patients with ccRCC.*

Clear-cell renal cell carcinoma (ccRCC) is the most common type of RCC, accounting for 70-80% of all RCC cases (1, 2). Patients with ccRCC generally have worse outcomes compared to those with other RCC types (3). At diagnosis, one-third of patients with ccRCC already exhibit metastasis (4). Surgical resection is the most effective treatment for

localized ccRCC, and its overall 5-year survival rate is up to 97.4% for stage I ccRCC and 89.9% for stage II (5). However, 30-35% of patients who undergo nephrectomy experience relapse and metastasis. Metastatic and inoperable ccRCC is refractory to conventional chemotherapy and radiotherapy and has a 5-year survival rate of approximately 20% (6, 7). Therefore, the identification of potential biomarkers to predict disease progression and prognosis remains crucial.

The minichromosome maintenance (MCM) protein complex consists of six highly-conserved proteins (MCM2-7) and plays an essential role in the initiation of DNA replication (8). MCM proteins are expressed only during the cell cycle and are down-regulated and dissociated from chromatin in quiescent cells. Thus, they are considered useful proliferation markers (9, 10). In addition, the elevation or depletion of MCM levels causes genomic instability and leads to carcinogenesis (11). MCM6 expression has been evaluated in various human cancer types, including breast cancer, hepatocellular carcinoma, endometrial adenocarcinoma, craniopharyngioma, meningioma, chondrosarcoma, pancreatic adenocarcinoma, and colorectal cancer (8, 12-15). In those studies, MCM6 was found to be overexpressed in cancer tissue and was associated with important clinicopathological factors. However, to the best of our knowledge, this is the first study to evaluate MCM6 protein expression and its prognostic significance in ccRCC.

The aim of this study was to assess the MCM6 expression level in ccRCC, evaluate its clinicopathological significance, and determine whether MCM6 can be used as a potential prognostic and predictive marker of ccRCC.

Materials and Methods

Public database analysis. We assessed the Gene Expression Profiling Interactive Analysis (GEPIA; <http://gepia.cancer-pku.cn>) database to evaluate the RNA-Seq mRNA expression of MCM6 in 523 ccRCC samples and 100 normal samples from the Cancer Genome Atlas and Genotype-Tissue Expression (GTEx) projects.

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Figure 1. Distribution of minichromosome maintenance 6 (MCM6) gene expression in clear-cell renal carcinoma tissue and normal tissue. MCM6 gene was significantly up-regulated ($p < 0.01$) in clear-cell renal cell carcinoma [mean value of $\log_2(\text{TPM}+1)$]. TPM: Transcripts per million.

Patient data and histological review. We included 238 cases of ccRCC who underwent nephrectomy between May 2000 and July 2017 at Yeungnam University Medical Center. This study was approved by the Ethics Committee of the Yeungnam University College of Medicine (2020-06-009). Written informed consent was exempted by the Ethics Committee due to the retrospective nature of the study. Clinical, pathological, and follow-up data were obtained from the patients' medical records. Cases that involved pre-operative therapy, insufficient clinicopathological data, or tissue unavailability for immunohistochemical staining were excluded from the analysis. The clinicopathological data included data regarding patient sex, age, tumor size, tumor necrosis, tumor hemorrhage, lymphovascular invasion (LVI), TNM (tumor, node, metastasis) stage, metastasis/recurrence, and nuclear grade (based on the 2016 World Health Organization classification of Tumors of the Urinary System and Male Genital Organs (2). Tumor grading and staging were performed according to the eighth American Joint

Figure 2. Distribution of minichromosome maintenance 6 (MCM6) gene expression in different stages of clear-cell renal cell carcinoma.

Committee on Cancer TNM Staging System (16). Overall survival (OS) was defined as the period from the date of diagnosis to the date of death. Disease-free survival (DFS) was evaluated as the period from the date of diagnosis to the date of first recurrence, distant metastasis, or last follow-up date. The OS and DFS data were obtained from the medical charts or the microdata integrated service of South Korea.

Tissue microarray construction. Tissue microarrays (TMAs) were constructed using paraffin-embedded blocks of ccRCC tissues. Two to four 1.5-mm tissue cores that represented the tumor areas of patients were included in the TMA. Non-neoplastic kidney, lymph node, gastric mucosa, breast cancer, and normal liver parenchyma tissues were included as a control in each TMA section. One section from each TMA was stained with hematoxylin and eosin and assessed to confirm the histological presence of representative ccRCC features.

Immunohistochemical analyses and scoring. The 4- μm -thick tissue sections were deparaffinized and rehydrated. Immunohistochemistry for MCM6 was performed using an automated Benchmark platform (Ventana Medical Systems, Tucson, AZ, USA). Antigen retrieval was carried out using the Cell Conditioning Solution (mild cc1; Ventana Medical Systems), and the samples were then incubated with MCM6 antibody (ERP17686, rabbit monoclonal, 1:1,000; Abcam, Cambridge, UK) for 40 min at room temperature. Stains were visualized using an UltraView universal DAB detection kit (Ventana Medical System). Negative controls were performed in the absence of primary antibody. The slides were then scanned using a Leica Aperio ScanScope (Leica Biosystems, Vista, CA, USA) system at 40 $\times$ magnification, and the images were captured using a Leica ImageScope.

MCM6 expression was defined as positive when the tumor cells had moderate or strong nuclear immunoreactivity. Manual counting of MCM6-expressing cells was performed on a computer monitor using the scanned images. We selected two to four hotspots (with a minimum of 500 tumor cells) for each case, and all tumor nuclei

Figure 3. In the Gene Expression Profiling Interactive Analysis data, high minichromosome maintenance 6 (*MCM6*) gene expression tended to confer better overall (A) ($p=0.35$) and worse disease-free (B) ($p=0.22$) survival than low expression.

Figure 4. Immunohistochemical analysis of minichromosome maintenance 6 (*MCM6*) expression. A: *MCM6* expression was found only rarely in tubule, not in glomeruli in normal kidney ($\times 20$). B: Low *MCM6* expression ($\times 10$). C: High *MCM6* expression ($\times 10$).

and *MCM6*-positive tumor cells were counted by clicking on the images to yield a continuous score from 0 to 100.

Statistical analysis. To identify the optimal cutoff value that would demonstrate the highest accuracy in predicting outcomes, receiver operating characteristic curve analysis was used. Quantitative variables were compared using Student's *t*-test. Categorical variables were compared using Fisher's exact test. OS and DFS curves were assessed using the Kaplan-Meier method and compared using the log-rank test. Multivariate Cox regression analyses were performed using pathological variables that were recognized as significant in the univariate survival analyses. Additionally, hazard ratios (HRs) and relevant 95% confidence intervals (CIs) were assessed. All statistical analyses

were conducted using SPSS software version 25.0 (IBM, Armonk, NY, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

mRNA expression of *MCM6* gene. In the GEPIA data, the *MCM6* gene was found to be up-regulated in the ccRCC samples when compared to normal kidney samples (Figure 1). The *MCM6* gene expression level did not differ significantly between stages; however, it was found to be a slightly more up-regulated in stage IV ccRCC samples when compared to other stages (Figure 2). Patients with RCCs

Table I. Relationships between minichromosome maintenance 6 (MCM6) expression and clinicopathological factors.

Variable	MCM6 expression, n (%)		
	Low (n=191)	High (n=47)	p-Value
Gender			
Male	135 (78.0)	38 (22.0)	0.161
Female	56 (86.2)	9 (13.8)	
Tumor size			
≤4 cm	112 (84.8)	20 (15.2)	0.017
<4-≤7 cm	55 (77.5)	16 (22.5)	
<7-≤10 cm	16 (72.7)	6 (27.3)	
>10 cm	8 (61.5)	5 (38.5)	
Nuclear grade			
1	27 (81.8)	6 (18.2)	0.001
2	86 (83.5)	17 (16.5)	
3	62 (86.1)	10 (13.9)	
4	16 (53.3)	14 (46.7)	
Lymphovascular invasion			
No	180 (81.8)	40 (18.2)	0.058
Yes	11 (61.1)	7 (38.9)	
Necrosis			
No	166 (83.4)	33 (16.6)	0.006
Yes	25 (64.1)	14 (35.9)	
Hemorrhage			
No	27 (77.1)	8 (22.9)	0.617
Yes	164 (80.8)	39 (19.2)	
T Status			
pT1	161 (83.9)	31 (16.1)	0.016
pT2	11 (68.8)	5 (31.3)	
pT3	19 (63.3)	11 (36.7)	
N Status			
pN0	17 (70.8)	7 (29.2)	0.320
pN1	0 (0)	1 (100)	
Metastasis			
No	181 (83.0)	37 (17.0)	0.001
Yes	10 (50.0)	10 (50.0)	
Stage			
I	155 (83.8)	30 (16.2)	0.001
II	13 (81.3)	3 (18.8)	
III	11 (84.6)	2 (15.4)	
IV	12 (50.0)	12 (50.0)	

with high *MCM6* gene expression tended to have better OS and shorter DFS than those with lower expression ($p=0.35$ and $p=0.22$) (Figure 3).

Patient cohort and clinical characteristics. The patient cohort included in this study consisted of 238 patients (173 males and 65 females). The mean age was 57.6 years (range=26-93 years), and the tumor size ranged from 9 to 14.5 cm (mean=4.4 cm). The mean follow-up time was 93.9 months (range=1-217 months). During the follow-up period, 20 patients (8.4%) experienced tumor recurrence or metastasis and 26 patients (10.9%) died.

Immunohistochemical analysis of MCM6 expression. MCM6 was found to be rarely expressed in non-neoplastic renal tubular epithelium (<1%), and immunoreactivity was not observed in the glomerulus (Figure 4A). Notably, the MCM6 expression level in ccRCC samples (Figure 4B) ranged from 0% to 83% (mean=9.8%). The cutoff value for MCM6-positivity with the highest accuracy was 20%. The global predictive accuracy (area under the receiver operating characteristics curve) was found to be 0.709% (95% confidence interval=0.588-0.830; $p<0.0001$). We therefore defined samples with high MCM6 expression as samples in which more than 20% of the tumor cells exhibited nuclear staining. Overall, 47 out of 238 (19.7%) ccRCC samples showed high MCM6 expression (Figure 4C).

MCM6 expression is correlated with advanced clinicopathological factors. High MCM6 expression was significantly correlated with increasing tumor size ($p=0.025$) and nuclear grade ($p=0.001$), and with tumor necrosis ($p=0.006$). MCM6 was found to be significantly overexpressed in patients with pT2 and pT3 when compared to those with pT1 ($p=0.016$). In addition, a high MCM6 expression level was associated with metastasis ($p=0.001$) and increasing stage ($p=0.004$). Patients with LVI also tended to have higher MCM6 expression but the differences were not statistically significant ($p=0.058$). No significant association was observed between high MCM6 expression and sex, tumor hemorrhage, or lymph node metastasis. The results are detailed in Table I.

MCM6 expression is associated with poor outcome. The Kaplan-Meier curve showed that patients with high MCM6 expression had a significantly worse OS and DFS (both $p<0.0001$) compared to those with low MCM6 expression (Figures 5 and 6). In univariate analyses, OS and DFS were found to be shorter in patients with ccRCC with LVI, tumor necrosis, metastasis, and high MCM6 expression (all $p<0.0001$). Moreover, survival was found to worsen as pT stage increased. Patients with stage III or IV ccRCC were revealed to have a similar survival time of only a few months, which was significantly shorter compared to that of patients with stage I or II ccRCC. In multivariate analyses, high MCM6 expression and stage III and IV ccRCC were independent predictors of poorer OS and DFS. Additionally, the presence of metastases was found to be an independent prognostic factor for poorer DFS. The data are summarized in Table II and III.

Discussion

DNA replication is a key process in cell proliferation and growth (17). MCM proteins are key regulators of DNA replication, and dysregulation of MCM proteins may cause developmental defects or cancer (13). The up-regulation of MCM genes has been observed in many human cancer types

Table II. Univariate and multivariate analyses of the associations between clinicopathological factors and overall survival.

Variable	Comparison	Univariate analysis		Multivariate analysis	
		HR (95% CI)	p-Value	HR (95% CI)	p-Value
LVI	Yes vs. no	17.248 (8.016-37.114)	<0.0001	1.569 (0.558-4.412)	0.393
Necrosis	Yes vs. no	5.101 (2.426-10.727)	<0.0001	1.798 (0.642-5.036)	0.264
T Status	pT1 vs. pT2	10.076 (2.371-42.822)	0.002	2.101 (0.341-12.957)	0.424
	pT1 vs. pT3	50.509 (18.755-136.025)	<0.0001	1.986 (0.504-7.820)	0.327
Metastasis	Yes vs. no	26.741 (12.337-57.959)	<0.0001	5.326 (0.704-40.292)	0.105
Stage	I vs. II	6.752 (0.611-74.654)	0.119	6.914 (0.621-77.045)	0.116
	I vs. III	129.726 (26.649-631.509)	<0.0001	208.776 (9.686-626.395)	<0.0001
	I vs. IV	108.983 (25.153-472.203)	<0.0001	19.537 (0.410-109.443)	0.016
MCM6 expression	High vs. low	4.893 (2.327-10.288)	<0.0001	5.326 (0.704-40.292)	0.105

CI: Confidence interval; HR: hazard ratio; LVI: lymphovascular invasion; MCM6: minichromosome maintenance 6. Statistically significant *p*-values are shown in bold.

Table III. Univariate and multivariate analyses of the associations between clinicopathological factors and overall survival.

Variable	Comparison	Univariate analysis		Multivariate analysis	
		HR (95% CI)	p-Value	HR (95% CI)	p-Value
LVI	Yes vs. no	17.529 (8.140-37.750)	<0.0001	6.592 (0.837-5.767)	0.110
Necrosis	Yes vs. no	5.363 (2.549-11.284)	<0.0001	1.426 (0.569-3.571)	0.449
T Status	pT1 vs. pT2	9.379 (2.212-39.763)	0.002	3.610 (0.504-25.855)	0.201
	pT1 vs. pT3	55.121 (20.137-150.883)	<0.0001	1.832 (0.421-7.961)	0.420
Metastasis	Yes vs. no	38.159 (17.263-84.350)	<0.0001	12.923 (1.569-106.410)	0.017
Stage	I vs. II	6.399 (0.579-70.712)	0.130	6.218 (0.557-69.424)	0.138
	I vs. III	107.492 (22.000-525.201)	<0.0001	163.052 (27.081-981.712)	<0.0001
	I vs. IV	142.793 (32.732-622.930)	<0.0001	15.034 (1.343-168.317)	0.028
MCM6 expression	High vs. low	5.017 (2.387-10.542)	<0.0001	4.458 (1.755-11.324)	0.002

CI: Confidence interval; HR: hazard ratio; LVI: lymphovascular invasion; MCM6: minichromosome maintenance 6. Statistically significant *p*-values are shown in bold.

and was associated with histological grade, TNM stage, or survival (9). However, some controversial results have been reported in colorectal cancer and lung adenocarcinoma (18). Gou *et al.* reported in a meta-analysis that MCM2, MCM5, and MCM7 were associated with worse outcomes in human cancer (18). In addition, several studies have also assessed the clinicopathological significance of MCM protein expression, and its role as a proliferation marker in comparison to Ki-67. Notably, the majority of those studies were mainly conducted on MCM2 protein expression among the MCM family members. The results of those studies showed that high MCM2 expression was a marker of poor prognosis and MCM2 was proven to be a more reliable and useful proliferation marker than Ki-67 in many types of human cancer (8, 9, 19-21). The prognostic value of Ki-67 in RCC is controversial and inconsistent. Some studies have reported that a high Ki-67 index is correlated with an unfavorable prognosis in RCC (22-24). In contrast, other studies found that the Ki-

67 index was not associated with prognosis (25, 26). The aforementioned studies used different cutoff values for positive Ki-67 expression, ranging between 5 and 50%. Additionally, tumor proliferation markers, including Ki-67, are not routinely used to predict prognosis in RCC.

High MCM6 expression was associated with poor survival and higher histological grade in endometrioid endometrial adenocarcinoma and low-grade chondrosarcoma (15, 27). Patients with high MCM6 expression level in non-small cell lung cancer, mantle cell lymphoma, and craniopharyngioma were also found to have worse survival and an increased risk of death (15, 28, 29). In breast cancer, high MCM6 expression was also associated with a higher histological grade (14). Notably, in hepatocellular carcinoma, MCM6 was found to be the best diagnostic and prognostic marker when compared to the other MCM proteins (30).

There have been a few studies that have investigated the expression of MCM proteins in RCC but the majority of

Figure 5. Survival curves for the whole patient cohort according to minichromosome maintenance 6 (MCM6) protein expression. A: Cumulative overall survival. B: Disease-free survival. High MCM6 expression was significantly associated with worse survival.

Figure 6. Survival curves according to minichromosome maintenance 6 (MCM6) protein expression in patients with metastasis of clear-cell renal cell carcinoma. Those with high MCM6 expression had significantly worse overall (A) and disease-free (B) survival.

those studies were on the MCM2 expression in RCC. In addition, they included all histological types of RCC – ccRCC, papillary RCC, chromophobe RCC, transitional cell carcinoma, and even oncocytoma (21, 31-33). These studies suggested that MCM2 is a useful proliferation marker, and its expression is associated with nuclear grade (21, 31, 33). In a study using gene-expression data, the overexpression of MCM genes was found to be an independent prognostic factor of RCC (34). That study was also conducted on all histological types of RCC. High MCM5 expression in RCC was associated with lymph node metastasis, distant metastasis, clinical stage, and a shorter OS (32).

In the present study, high MCM6 expression in ccRCC was found to be correlated with aggressive clinicopathological factors and especially associated with tumor progression (as reflected by increasing tumor size, pT stage, stage, and metastasis). Similar to the GEPIA results, the frequency of high MCM6 expression was not significantly different between stage I, II, and III ccRCC, but was a little up-regulated in stage IV ccRCC. This suggests that high MCM6 expression may be associated with metastasis rather than disease stage, as there was no pT4 case included in the present study. Furthermore, in ccRCC with metastasis, patients with high MCM6 expression had a significantly shorter OS (mean=48.1 months) and DFS (mean=13.7 months) than those with low MCM6 expression

(mean of 74.5 months and 52.2 months) (Figure 6). This might support our hypothesis that MCM6 plays an oncogenic role in tumor proliferation and progression, and suggests that it might be a promising potential prognostic marker for ccRCC. The MCM6 labeling index with a cutoff value of 20% was found to be a highly significant predictor of aggressive clinicopathological factors, metastasis, and poor survival. However, further studies with a larger number of ccRCC cases are required to validate the cutoff value.

In conclusion, our study is the first to assess MCM6 expression and its prognostic value in ccRCC. As such, we revealed that high MCM6 expression was significantly associated with aggressive clinicopathological factors and was an independent adverse prognostic factor. Thus, the assessment of MCM6 expression in patients with ccRCC as a routine pathological practice might provide useful information in regard to aggressive behavior. Additionally, MCM6 expression might be a useful predictive marker of metastasis and poor survival. However, future studies are needed to verify the cutoff value of MCM6 expression and to investigate whether MCM6 can serve as a potential therapeutic target.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

MJG carried out experiments, analyzed data, and prepared the article. NRJ and JAB reviewed pathological data, carried out experiments and analyzed data. YWK and PHS prepared clinical data.

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