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ORIGINAL RESEARCH

Bioinformatic Analysis of the Expression and Clinical Significance of the DNA Replication Regulator MCM Complex in Bladder Cancer

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Objective: The minichromosome maintenance (MCM) complex (MCM2, MCM3, MCM4, MCM5, MCM6, and MCM7), which regulates DNA replication and cell cycle progression, is essential for the development and progression of multiple tumors, but their role in bladder cancer development remains unclear. In the present study, the biological role and clinical significance of the MCM complex in bladder cancer were systematically elucidated.

Materials and Methods: We analyzed DNA mutations, mRNA expression and protein levels, protein–protein interaction (PPI) networks, functional enrichment, prognostic value of MCM2/3/4/5/6/7 in bladder urothelial carcinoma (BLC) and the connections between the immune cell infiltration and the overall survival of BLC patients with the MCM expression levels using Oncomine, Gene Expression Profiling Interactive Analysis (GEPIA), the Cancer Genome Atlas database (TCGA), Human Protein Atlas, UALCAN, STRING, cBioPortal, TIMER and GSCALite databases.

Results: The outcomes showed that the mRNA expression level of each member of the MCM complex was significantly correlated with histologic grade and tumor histology in BLC patients. Moreover, survival analysis showed that MCM/2/3/4/5/6/7 mRNA expressions were significantly associated with prognosis in patients with bladder cancer. Moreover, we experimentally validated the overexpression of the MCM2-7 complex in the BLC. Based on functional enrichment and PPI network analysis, the MCM complex might promote the progression of bladder cancer by activating DNA replication and accelerating cell cycle progression. In addition, MCM2/3/4/5/6/7 genes were also significantly associated with tumor immune cells infiltration and the drug sensitivity in BLC.

Conclusion: Our study suggests that the MCM complex especially MCM2/4/6/7 might be potential molecular therapeutic targets for BLC treatment and might be useful biomarkers for diagnosis and prognosis.

Keywords: bladder cancer, minichromosome maintenance complex, bioinformatics, potential biomarkers

Introduction

Bladder cancer (BC), a common urothelial carcinoma, is characterized by high morbidity and mortality.[1] BLC is more than four times more common in men than in women, with a worldwide incidence of 9.6 per 100,000 and 2.4 per 100,000. In men, BLC ranks the sixth of all newly diagnosed cancers and the ninth most deadly tumor worldwide.[2] Bladder cancer is divided into non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC). Lots of patients' futures developed into muscular invasion urothelial carcinomas with a very lower 5-year survival rate. Due to the high incidence and recurrence rates, it is necessary to investigate the relevant genetic pathways underlying the genesis and development of bladder cancer to look for a therapeutic target to improve the survival rates of patients with bladder cancer.

Genome instability is defined as the high frequency of mutations within the genome of a cellular lineage. Genomic variation, which helps regulate cancer genes, includes single-nucleotide variants, small insertions and deletions (indels), and copy number variants.[3] Dysregulation of DNA replication in cells contributes to tumor formation and progression. Minichromosome maintenance proteins (MCMs) were originally identified in Saccharomyces cerevisiae as necessary factors for the maintenance of extrasomal DNA.[4] It has been reported that MCMs are essential for tightly regulating DNA replication to maintain normal cellular processes, especially during DNA initiation as well as elongation. In many studies, genomic instability caused by MCM variants has been shown to be associated with cancer prognosis. Abnormal expression and activation of MCMs are often detected in various precancerous lesions and malignancies and contribute to genomic instability and uncontrolled cell cycle progression. The MCM2-7 complex proteins in the MCM family are related to each other and form a family of DNA helicases which implicated at the first committed step of DNA synthesis.[5]

Members of the MCM family, especially MCM2-7, are evolutionarily and functionally conservative throughout eukaryotes. MCM2-7 are the only proteins present throughout the cell cycle, while dividing.[6] All of them take part in the regulation of heterochromatin, gene expression, and developmental programs. The helicolytic enzyme activity can only be achieved when MCM2, MCM3, MCM4, MCM5, MCM6 and MCM7 form hex-mer, which is the basis of functional activity.[7] Previous studies have reported abnormal expression of some MCM2-7 members and their prognostic value on BLC. For example, by upregulating the expression of MCM5 in BLC cell lines, estrogen receptor β can promote tumor cell proliferation and invasion.[8] To date, the prognostic values of individual MCM2-7 members in the development and progression of BLC remain unclear.

In this research, with the use of multiple databases, we solved this problem by analyzing the expression and mutation status of members of the minichromosome maintenance complex of proteins (MCM2-7) in BLC, and their relationship with clinicopathological features, prognosis, tumor immune cell infiltration and the drug sensitivity based on the multiple databases. A complete and comprehensive analysis of MCM2-7 in BLC will be performed to further clarify the underlying molecular mechanisms involved in the development of BLC for developing novel prognostic biomarkers and effective therapeutic targets.

Materials and Methods

Ethics Statement

Our research protocol was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University. Data were retrieved from online databases, and tissue samples were obtained from patients who had given permission for their samples to be used in research and admitted to the Department of Urology in hospital.

ONCOMINE Database

Oncomine (https://www.oncomine.org) is the largest oncogene chip database and integrated data-mining platform designed to mine genetic information about cancer. With Oncomine, differential expression analysis of the MCM2-7 complex between cancerous tissue and adjacent tissue could be performed using a Student's t-test to generate a p-value.[9] The cut-off of p value and fold change were as follows: p value: 0.01, fold change: 1.5, gene grade: 10%, data type: mRNA.

UALCAN

UALCAN (http://ualcan.path.uab.edu) is an online tool based on CPTAC database and TCGA for gene expression profiling in cancer-free and cancerous tissues.[10] Differential expression analysis of the MCM2-7 complex was performed by querying the UALCAN portal. Differential mRNA expression in tumor histology was also achieved from the database. Using Student's t-test, $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ are considered statistically significant.

Human Protein Atlas

Immunohistochemistry-based expression data are collected from the Human Protein Atlas (https://www.proteinatlas.org). Immunohistochemical image analysis was used to directly compare the expression of MCM2-7 protein in normal human tissues and tumor tissues.

The Cancer Genome Atlas (TCGA) Database

There are more than 30 kinds of sequencing information and clinical data of human tumors in TCGA. Using genome sequencing, the genome variation map of human cancer was mapped, and a systematic analysis was conducted to understand the molecular mechanisms of cancer cell genesis and development, with the aim of obtaining new diagnosis and treatment methods. In our study, the clinical pathological parameters of 414 cases of patients with BLC were downloaded from UCSC XENA website (https://xena.ucsc.edu/). Data analysis was conducted with R software (R software, version 3.6.3). The clinical data are summarized in Table 1.

cBioPortal

cBioPortal (www.cbioportal.org) is an online website for exploring, visualizing and analyzing multidimensional cancer genomic dataset. In this study, we analyzed genomic maps of members of the MCM2-7 family containing mutations, protein expression z-scores (RPPA), mRNA expression z-scores (RNA Seq V2 RSEM), and putative copy number changes from GISTIC.

GEPIA Dataset

The GEPIA database is an online dataset for analyzing the RNA sequencing expression data based on TCGA and the Genotype-Tissue Expression (GTEx) projects. In our study, we obtained data related to MCM2-7 mRNA expression and clinical prognosis from GEPIA database.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Analysis

Functions and pathways of MCM2-7 mutations and 50 genes associated with MCM2-7 mutations were analyzed by GO and KEGG using R software. The functional roles of MCM2-7 complex mutations and 50 genes significantly associated

Table 1 Clinical Data Were Reviewed in 414 Patients with BLC from the TCGA

Characteristic	Levels	Overall
n		414
Gender, n (%)	Female	109 (26.3%)
	Male	305 (73.7%)
Age, n (%)	≤70	234 (56.5%)
	>70	180 (43.5%)
BMI, n (%)	≤25	153 (42%)
	>25	211 (58%)
Pathologic stage, n (%)	Stage I	4 (1%)
	Stage II	130 (31.6%)
	Stage III	142 (34.5%)
	Stage IV	136 (33%)
T stage, n (%)	T1	5 (1.3%)
	T2	119 (31.3%)
	T3	196 (51.6%)
	T4	60 (15.8%)
N stage, n (%)	N0	239 (64.6%)
	N1	46 (12.4%)
	N2	77 (20.8%)
	N3	8 (2.2%)
M stage, n (%)	M0	202 (94.8%)
	M1	11 (5.2%)

Abbreviation: BLC, bladder urothelial carcinoma.

with MCM2-7 mutations on the basis of three aspects were analyzed by GO function including cellular components, biological processes, molecular functions and Pathway analyses were performed using KEGG including the relevant pathways in biological processes.

STRING Database Analysis
STRING database (http://string-db.org/) was used as a cellular PPI network. The MCM complex and 50 neighbor genes interacted with each other and were performed using the database. The result of PPI network was downloaded from the webpage.

Reagents
TRIzol reagent was bought from CWBIO (Beijing, China), PrimeScript RT Master Mix and SYBR Green Premix reagents were purchased from TaKaRa (Japan).

Cell Lines and Cell Culture
The BLC cell lines (BIU-27, T24), which were obtained from the ATCC, were cultured in RPMI−1640 and DEMEM medium (GIBCO, Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Invitrogen; Thermo Fisher Scientific, Inc.)

Patients and Tumor Tissues
16 paired BLC tissues and paired-adjacent normal bladder tissues, which were pathologically confirmed by two independent pathologists were immediately frozen in liquid nitrogen after surgical removal in the First Affiliated Hospital of Nanchang University from 2021 to 2022 and stored in liquid nitrogen until use. The study protocol was approved by the hospital ethics committee and the informed consents were obtained from six participants in the study.

RNA and Quantitative Real-Time PCR
Total RNA from 16 for tumor tissues and 16 for paired adjacent normal tissues and BLC cell lines were extracted by Trizol. The 1 μg RNA from each sample was used for cDNA synthesis (Tiangen) according to the manufacturer's protocol. Primers used were listed as follows: GAPDH forward GTCTCCTCTGACTTCAACAGCG, GAPDH reverse: ACCACCCTGTTGCTGTAGCCAA; MCM2 forward: GCCTCTCCAAGGATCAGCAG, MCM2 reverse: GAGCCAG GCCTCTCTTGATG; MCM3 forward: TGGGGATTCATACGACCCCT, MCM3 reverse: GTGAGTCTGCCGT CTTTGGA; MCM4 forward: TCTCGTTGACAAGGTCCAGC, MCM4 reverse: ACTCGAATAGGCACAGCTCG; MCM5 forward: GACCTGGCCAAGCTGAAGAA, MCM5 reverse: GTTTCTCTGCAGCCTCTGCT; MCM6 forward: AGGTAGATGAGGGTGCTGGT, MCM6 reverse: GGCTTTGGGAGCAGACTCTT; and MCM7 forward: ATCCGGGGCATCAACAT, MCM7 reverse: GGTCAGTCCACTCACGG.

Statistical Analysis
Statistical analyses on the qRT-PCR were analyzed in the GraphPad prism 9.0.0. The data were analyzed with the Mann Whitney U-test and p value <0.05 was considered statistically significant.

GSCALite
GSCALite (http://bioinfo.life.hust.edu.cn/web/GSCALite/) is an important online website for the cancer genome analysis platform, which provides gene differential expression, overall survival, single nucleotide variation, copy number variation, methylation, pathway activity, miRNA regulation, normal tissue expression, and drug sensitivity. Furthermore, we utilized it to analyze pathway activity, miRNA network and the drug sensitivity of MCM2/3/4/5/6/7 in BLC.

TIMER
TIMER (http://cistrome.org/TIMER/) is a TCGA database visual website developed for analyses of tumor immunity.[11] The correlation between members of MCM2/3/4/5/6/7 and infiltration of immune cells in BLC was explored in the gene

module. The somatic copy number alteration (SCNA) module was used to associate genetic copy number alterations of MCM2/3/4/5/6/7 with the abundance of immune infiltrates.

Results

Overexpression of Different Members of the MCM Complex in Patients with BLC

We explore the different prognosis and potential therapeutic value of MCM members in patients with BLC. ONCOMINE database (www.oncomine.org), UALCAN (http://ualcan.path.uab.edu), and Human Protein Atlas (https://www.proteinatlas.org) were used to analyze mRNA expression and protein expression. The results are shown in Figure 1 and Table 2. The mRNA expression of the MCM complex in 20 types of cancer was detected for the first time and compared with that found in normal tissues using the Oncomine database. In multiple datasets, mRNA expression of MCM2/3/4/5/6/7 was significantly increased in BLC tissues. Table 2 shows the mRNA expression levels in different datasets. In Sanchez-Carbayo Bladder 2 Infiltrating Bladder Urothelial Carcinoma, compared with normal tissues, MCM2 over-expression was 4.918 times ($p = 5.75E-21$). While in Superficial BLC, the fold-change expression of MCM2 in adjacent normal tissues was 4.437 lower than in cancerous tissues ($p = 8.53E-16$).[12] In Lee Bladder infiltrating Bladder Urothelial Carcinoma, the fold change in MCM2 mRNA expression in tumor tissues compared with adjacent non-malignant tissues was 2.225 ($p = 1.47E-8$). In Dyrskjot Bladder 3 datasets, MCM3 in superficial BLC was found in BLC tissues compared with that in normal tissues with a fold change of 2.308 ($p = 4.44E-9$),[13] while Sanchez-Carbayo observed a 2.504-fold increase in MCM3 mRNA expression in BLC samples ($p = 1.93E-19$).[12] Similarly, in the Sanchez-Carbayo infiltrating bladder urothelial carcinoma dataset, a 2.90-fold increase in MCM4 mRNA expression was found in BLC tissues compared to normal tissues ($p = 1.32E-23$)[12] and in superficial BLC, MCM4 was found in BLC tissues compared with normal tissues with a fold change of 6.066 ($p = 1.48E-14$). In Dyrskjot Bladder 3 datasets, a 2.132-fold increase in MCM4 mRNA expression was found in BLC tissues compared with that in normal tissues ($p = 8.96E-8$). In Sanchez-Carbayo Bladder 2 Infiltrating Bladder Urothelial Carcinoma, compared with normal tissues, MCM5 over-expression was 4.683 times ($p = 1.87E-12$). While in Superficial BLC, the result demonstrates that MCM5 expression was higher in BLC tissues by 10.132-fold compared with normal tissues ($p = 4.46E-21$).[12] In Dyrskjot Bladder 3 datasets, compared with normal tissues, MCM6 expression was 5.318-fold hyper-expressed in cancer tissues ($p = 8.26E-6$). In Dyrskjot Bladder 3 Infiltrating Bladder Urothelial Carcinoma, compared with normal tissues, MCM7 over-expression was 7.628 times ($p = 3.01E-8$), while in Superficial BLC, MCM7 was unregulated 10.974-fold in carcinoma compared with its expression in normal bladder tissues ($p = 6.84E-14$).[13] Further, the mRNA expression of different MCM2-7 members was acquired from the R software running result based on TCGA database, which was different from ONCOMINE database. As is shown in Figure 2A-F, the mRNA expression of MCM2-7 members in BLC tissues was significantly higher than that in normal samples based on the TCGA database ($p<0.05$).

After examining the MCM2-7 mRNA expression in BLC, to further explore the protein expression level of MCM2-7 in BLC, we used the Human Protein Atlas database. As shown in Figure 2G-L, MCM2 protein was low expressed in normal bladder tissue, whereas high expression of MCM2 protein was observed in BLC tissues. MCM3 protein was low expressed in normal bladder tissue, whereas medium expression of MCM3 protein was observed in BLC tissues. The protein expression of MCM4 in normal bladder tissues was the same as that in BLC tissues. Medium expression of MCM5 was observed in normal bladder tissue, whereas high expression of MCM5 protein was observed in BLC tissues. MCM6/7 proteins were not detected in normal bladder tissues, whereas high expressions of them were observed in BLC tissues. To sum up, our results showed that transcriptional expressions of MCM2-7 were overexpressed and so were MCM2/3/5/6/7 protein expressions in patients bearing BLC. For further verification, the mRNA expression of MCM2-7 complex in BLC tissues and cell lines was detected by qRT-PCR. As are shown in Figure 3A, when compared with the normal human uroepithelial cell line (SV-HUC-1), the expression of the MCM complex was significantly overexpressed in 2 different BLC cell lines (T24 and BIU-87). As is shown in Figure 3B-G, in 16 pairs of BLC tissues and matched to adjacent tissues, MCM2/3/4/5/6/7 was upregulated in bladder cancer tissues compared with the corresponding non-carcinoma tissues.

These data above suggested that the protein expression of the MCM complex was significantly increased in BLC tissue compared to that in normal bladder tissue based on our experimental results and the public databases.

Analysis Type by Cancer	Cancer vs. Normal MCM2		Cancer vs. Normal MCM3		Cancer vs. Normal MCM4		Cancer vs. Normal MCM5		Cancer vs. Normal MCM6		Cancer vs. Normal MCM7	
Bladder Cancer	3		2		3		2		1		3	
Brain and CNS Cancer	5	1	4	1	1	1	4	1	3	1	3	1
Breast Cancer	8		2		10	1	1	1	2	1	1	
Cervical Cancer	4		3		4		4		4		4	
Colorectal Cancer	9		5		6		3		6		8	
Esophageal Cancer	2		1		2		1		2		3	
Gastric Cancer	1		1		3		1		1		2	
Head and Neck Cancer	6		1		5		1		3		5	
Kidney Cancer	2					2	4				2	
Leukemia		1	1	1	1	4	2		1	1		4
Liver Cancer	2		2		3		2		3		2	
Lung Cancer	8		2		8		4		5	1	3	
Lymphoma			4		2		3				3	
Melanoma			1		1				1			
Myeloma			1				1		1		1	
Other Cancer	3		2		3	1	8		5		3	1
Ovarian Cancer	3		1		3		1		3		4	
Pancreatic Cancer	1				1							
Prostate Cancer					2							
Sarcoma	9		6		10		5		9		5	
Significant Unique Analyses	65	2	39	2	67	9	46	2	49	4	52	6
Total Unique Analyses	402		446		453		436		450		424	

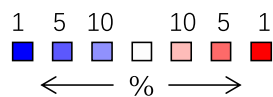1 5 10   10 5 1

←——— % ———→

Figure 1 Transcriptional expression of MCM2/3/4/5/6/7 in 20 different types of cancer diseases (ONCOMINE database). Difference of transcriptional expression was compared by Student's *t*-test. Cut-off of *p* value and fold change were as following: *p* value: 0.01, fold change: 1.5, gene rank: 10%, data type: mRNA.

Correlation Between mRNA Expression of MCM2/3/4/5/6/7 Members and Clinicopathological Characteristics of BLC Patients

We further investigated the relationship between MCM2/3/4/5/6/7 mRNA expression and its association with the clinicopathological parameters, including histologic grade and tumor histology from TCGA datasets (https://portal.gdc.cancer.gov/), in patients with BLC. As is shown in Figure 4A-F, the mRNA expressions of MCM2/4/5/6/7 members in high-grade tumor tissues were significantly upregulated compared with low-grade tumor tissues. We found that MCM4/7

Table 2 Significant Changes of MCM 2–7 Complex Expression in Transcription Level Between BLC and Normal Bladder Tissues (ONCOMINE)

	Types of BLC vs Normal	Fold Change	*p* value	*t*-Test	Ref
MCM2					
	Infiltrating Bladder Urothelial Carcinoma	4.918	5.75E-21	11.339	Sanchez-Carbayo Bladder 2
	Infiltrating Bladder Urothelial Carcinoma	2.225	1.47E-8	5.989	Lee Bladder
	Superficial Bladder Cancer	5.437	8.53E-16	10.737	Sanchez-Carbayo Bladder 2
MCM3					
	Superficial Bladder Cancer	2.308	4.44E-9	7.905	Dyrskjot Bladder 3
	Superficial Bladder Cancer	2.504	1.93E-19	12.809	Sanchez-Carbayo Bladder 2
MCM4					
	Infiltrating Bladder Urothelial Carcinoma	2.900	1.32E-23	12.311	Sanchez-Carbayo Bladder 2
	Superficial Bladder Cancer	6.066	1.48E-14	9.451	Sanchez-Carbayo Bladder 2
	Infiltrating Bladder Urothelial Carcinoma	2.132	8.96E-8	7.151	Dyrskjot Bladder 3
	Superficial Bladder Cancer	3.576	0.003	6.485	Blaveri Bladder 2
	Infiltrating Bladder Urothelial Carcinoma	3.497	0.006	6.743	Blaveri Bladder 2
MCM5					
	Superficial Bladder Cancer	11.132	4.46E-21	13.025	Sanchez-Carbayo Bladder 2
	Infiltrating Bladder Urothelial Carcinoma	4.683	1.87E-12	7.758	Sanchez-Carbayo Bladder 2
MCM6					
	Infiltrating Bladder Urothelial Carcinoma	5.318	8.26E-6	2.161	Dyrskjot Bladder 3
MCM7					
	Superficial Bladder Cancer	10.974	6.84E-14	3.033	Dyrskjot Bladder 3
	Infiltrating Bladder Urothelial Carcinoma	7.628	3.01E-8	2.426	Dyrskjot Bladder 3

Abbreviation: BLC, bladder urothelial carcinoma.

mRNA expressions have a positive correlation with tumor grades: with the increase in tumor grade, the mRNA expression tended to be higher. Similarly, as is shown in Figure 4G-L, the expression of MCM2/4/6/7 in the bladder papilloma was lower than that in the bladder non-papilloma, while no major differences in the amount of MCM3/5 mRNA expression were found between papillomas and non-papillomas. In conclusion, mRNA expression of MCM2/4/6/7 members was significantly correlated with the clinicopathological parameters of BLC patients.

Prognostic Value of mRNA Expression of MCM2/3/4/5/6/7 in BLC Patients

We used Survival plots (http://gepia.cancer-pku.cn/detaill) to analyze the prognostic values of the MCM2/3/4/5/6/7 mRNA expressions in BLC patients. The mRNA expressions in MCM2/3/4/5/6/7 members were significantly associated with prognosis in patients with BLC. As is shown in Figure 5A-F, our results showed that higher mRNA expression in MCM2/4/5/6/7 members was associated with poorer DFS (disease free survival) in BLC patients. Next, we further analyzed the relationship between mRNA expression of MCM2/3/4/5/6/7 members and prognosis of BLC patients. Higher mRNA expression of MCM2 (HR = 2 and p = 0.0027), MCM4 (HR = 1.8 and p = 0.015), MCM5 (HR = 1.5 and p = 0.012), MCM6 (HR = 2.3 and p = 0.0076) and MCM7 (HR = 1.7 and p = 0.0015) was significantly associated with shorter DFS in patients with BLC, while the higher expression of MCM3 mRNA was not associated with DFS in patients with BLC. As is shown in Figure 5G-L, our results showed that higher mRNA expression of MCM2 (HR = 2.3 and p = 0.024), MCM4 (HR = 2.2 and p = 0.042) and MCM6 (HR = 2 and p = 0.022) members was associated with poorer OS in BLC patients, while the higher expression of MCM3 (HR = 0.94 and p = 0.69), MCM5 (HR = 1.1 and p = 0.62) and MCM7 (HR = 1 and p = 0.95) mRNA showed no correlation with prognosis for BLC patients. These results suggested that MCM/2/3/4/5/6/7 mRNA expressions were significantly associated with prognosis in patients with BLC and might serve as useful clinical biomarkers for predicting disease-free survival and overall survival in patients with BLC.
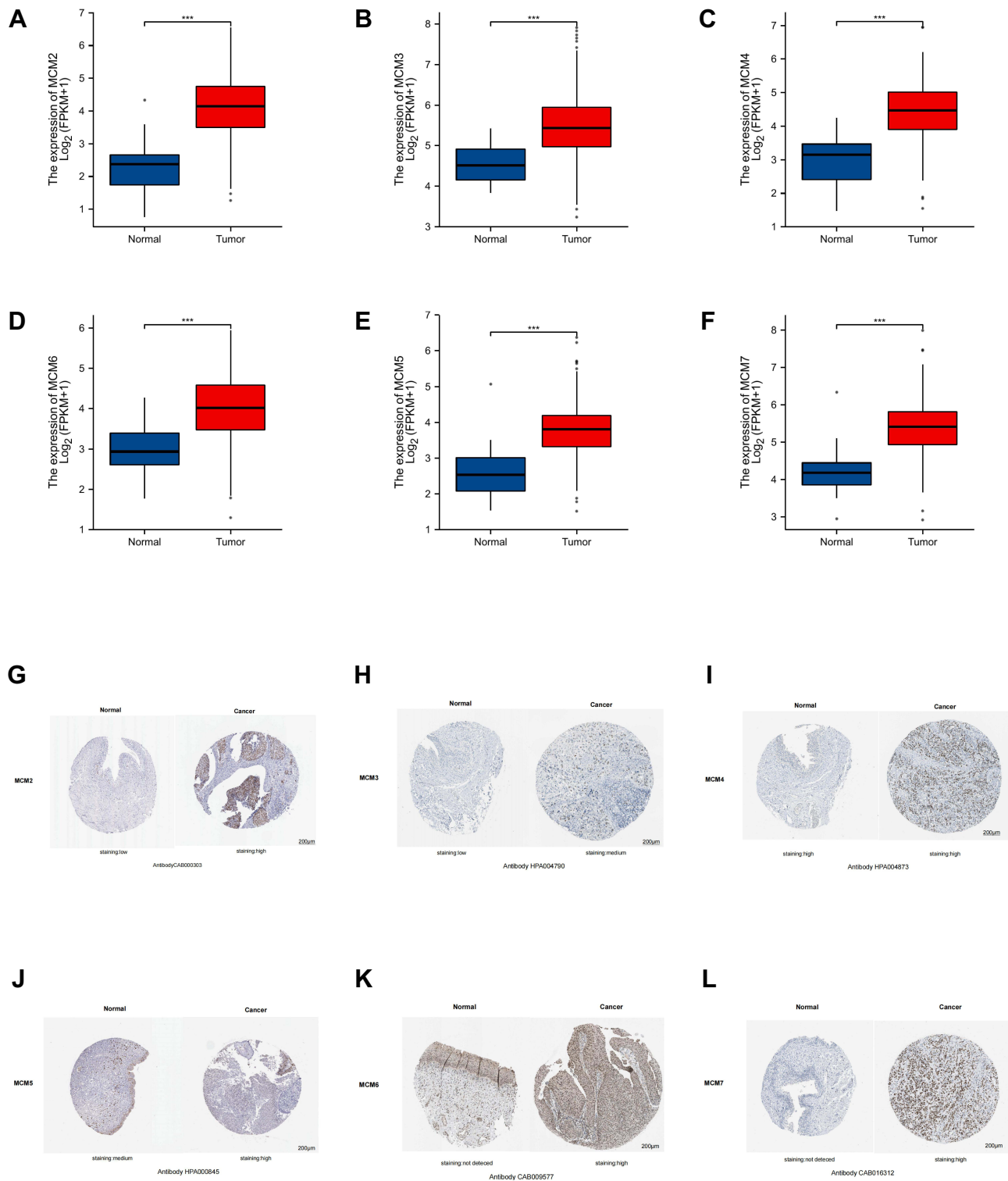
Figure 2 mRNA expression of distinct MCM2/3/4/5/6/7 members in BLC tissues and adjacent normal bladder tissues. mRNA expressions of MCM2/3/4/5/6/7 were found to be overexpressed in primary BLC tissues compared to normal samples (**A**–**F**). Immunohistochemistry images of MCM2/3/4/5/6/7 in bladder cancer (BLC) and normal bladder tissues (Human Protein Atlas). Low protein expressions of MCM2/3 were found in normal bladder tissues, while their medium and high protein expressions were observed in BLC tissues (**G** and **H**). Both normal bladder tissue and BLC tissues express high levels of MCM4 protein (**I**). Medium expression of MCM5 was observed in normal bladder tissue, whereas high expression of MCM5 protein was observed in BLC tissues (**J**). MCM6/7 proteins were not expressed in normal bladder tissues, whereas their high expressions were observed in BLC tissues (**K** and **L**). *** $p < 0.001$.
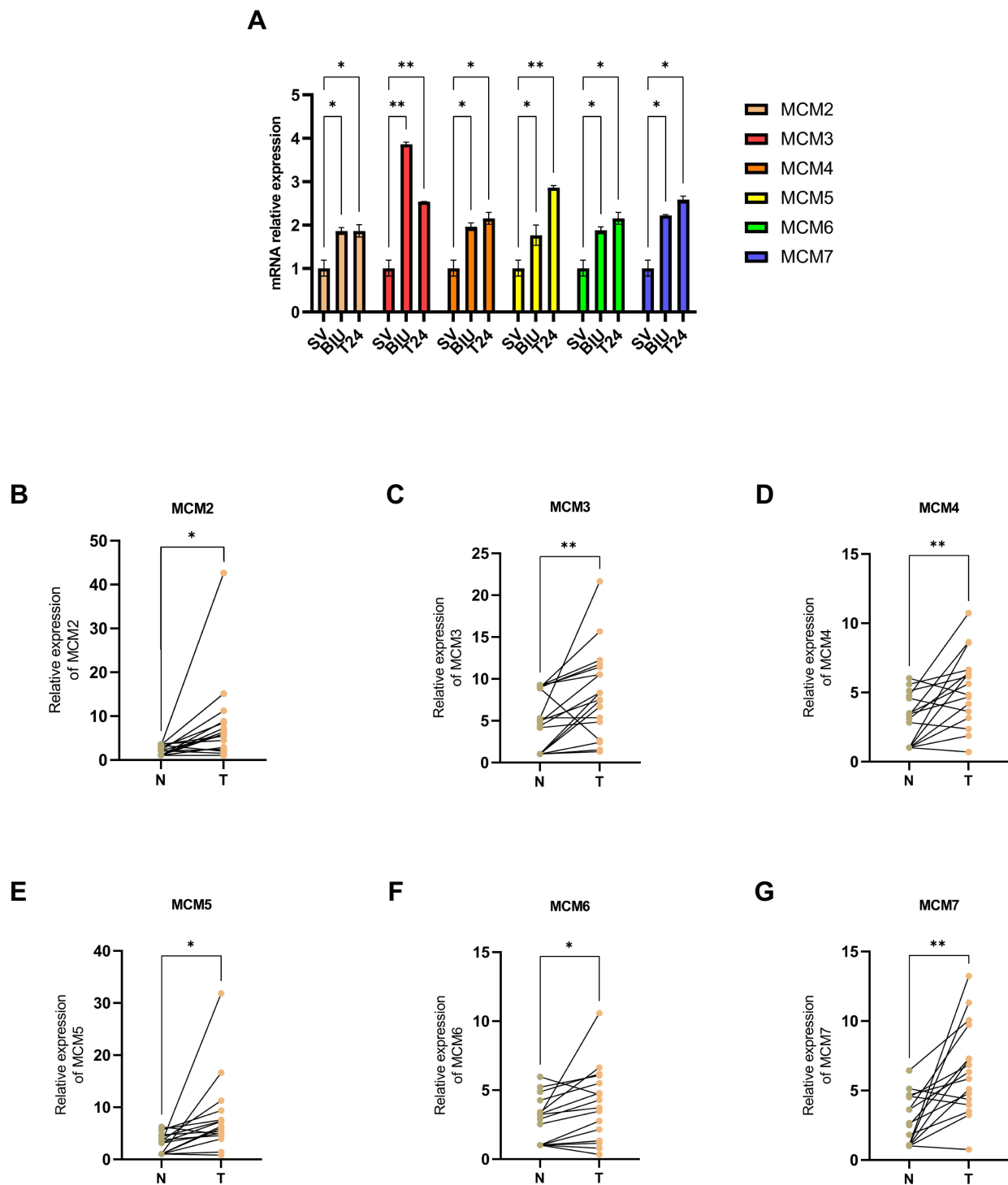
Figure 3 qRT-PCR was used to detect the mRNA expressions of MCM2/3/4/5/6/7 in normal human uroepithelial cell line (SV-HUC-1) and bladder cancer cell lines (BIU, T24) (**A**). mRNA expression of MCM2/3/4/5/6/7 was performed in BLC tissues and paired normal tissues (**B–G**). ns indicates not significant; *p <0.05; **p <0.01.

Diagnostic Value of mRNA Expressions of MCM2/3/4/5/6/7 in BLC Patients

We further investigated the relationship between MCM2/3/4/5/6/7 mRNA expression and diagnostic efficacy and value of distinguishing between benign and malignant lesions from UCSC XENA (https://xenabrowser.net/datapages/), in patients with BLC.[14] BLC (bladder urothelial carcinoma) and GTEX corresponding normal tissue data of TCGA were
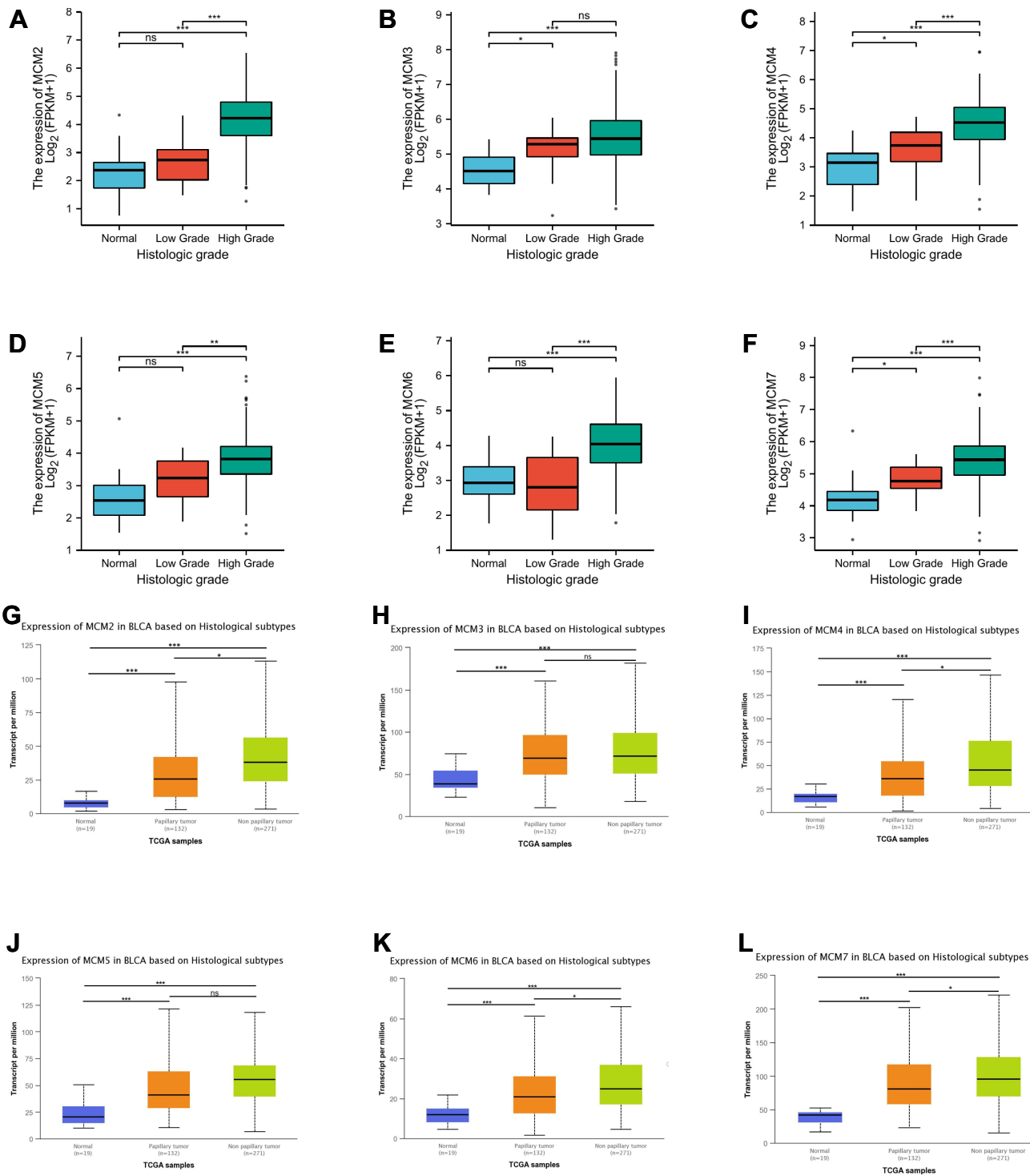
Figure 4 Relationship between mRNA expression of distinct MCM2/3/4/5/6/7 and individual histologic grade of BLC patients (**A-F**). The mRNA expressions of MCM3/4/7 were significantly related to patients' histologic grade (**B**, **C** and **F**). Relationship between mRNA expression of distinct MCM2-7 complex members and individual tumor histology of BLC patients (**G-L**). The mRNA expressions of MCM2/4/6/7 were significantly related to patients' histologic grade (**G**, **I**, **K** and **L**). ns indicates not significant; *p <0.05, **p <0.01, ***p <0.001.

extracted. As shown in Figure 6, in distinguishing the outcomes of benign and malignant, the predictive ability of MCM2 variable was relatively accurate (AUC = 0.918, 95% CI:0.874–0.962), the predictive ability of MCM3 variable had certain accuracy (AUC = 0.823, 95% CI:0.7666–0.880), the predictive ability MCM4 variable had certain accuracy
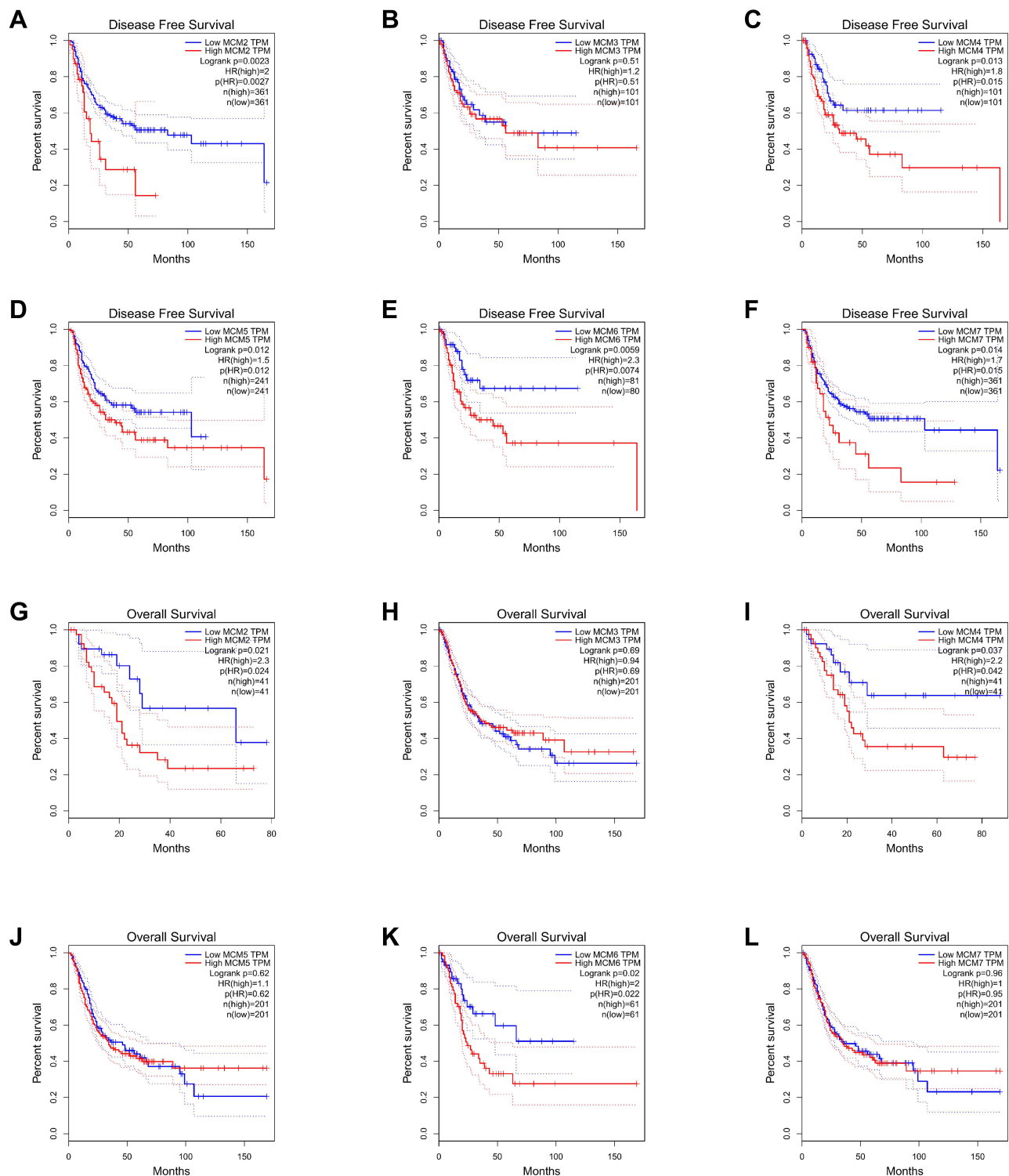
Figure 5 Prognostic value of mRNA expression of distinct MCM2/3/4/5/6/7 in BLC patients (**A-F**). The mRNA expression of MCM2/4/5/6/7 was associated with poor DFS (disease free survival) in BLC patients (**A** and **C–F**). Prognostic value of mRNA expression of distinct MCM2-7 members in BLC patients (overall survival) (**G-L**). The mRNA expression of MCM2/4/6 was associated with poor OS in BLC patients (**G**, **I** and **K**).

(AUC = 0.876, 95% CI:0.833–0.919), the predictive ability of MCM5 variable had certain accuracy (AUC = 0.863, 95% CI:0.796–0.929), the predictive ability of MCM6 variable had certain accuracy (AUC = 0.821, 95% CI:0.764–0.877), the predictive ability of MCM7 variable had certain accuracy (AUC = 0.822, 95% CI:0.7533–0.891). These results indicated
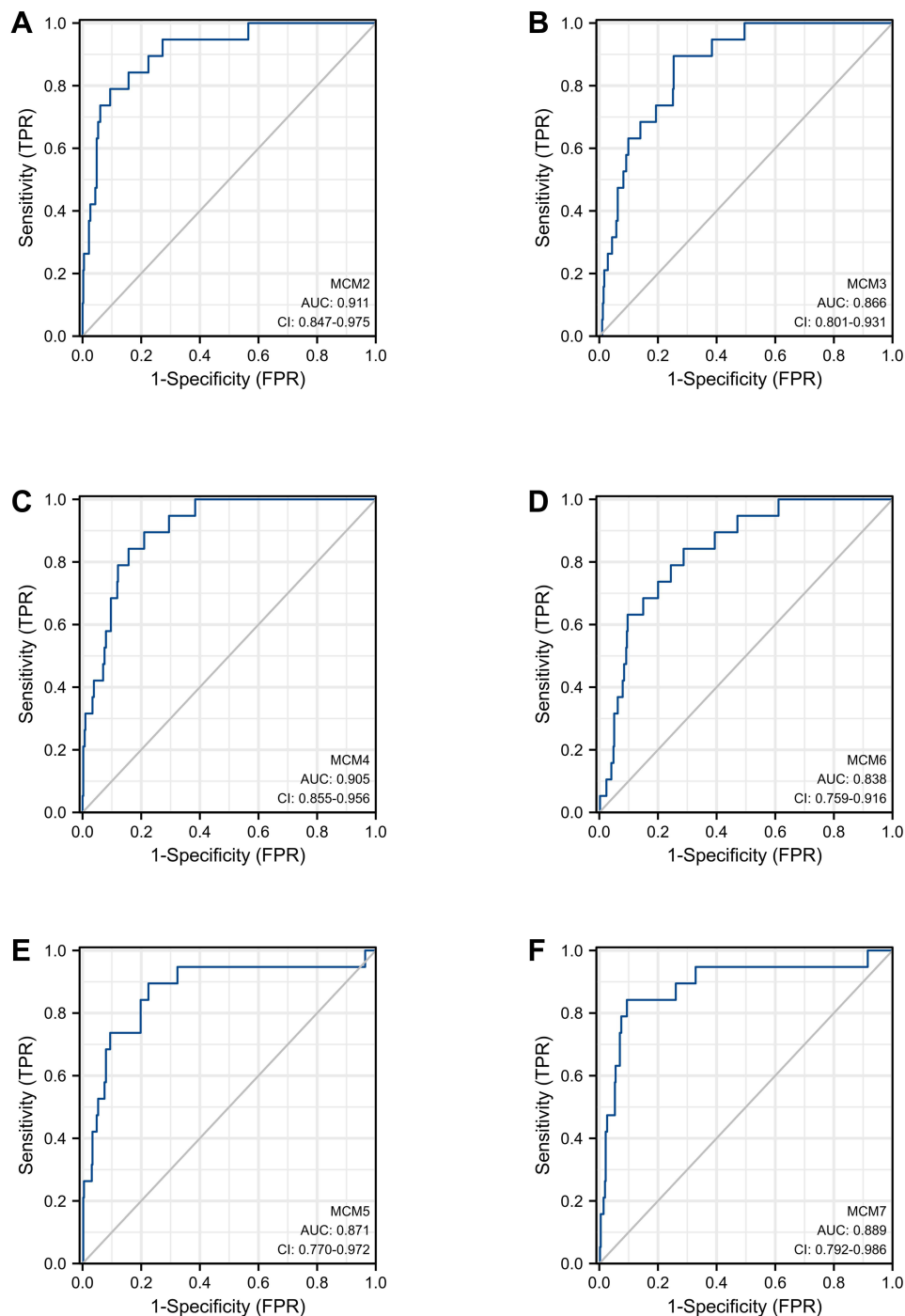
Figure 6 Receiver operating characteristic (ROC) curve for MCM2-7 mRNA expressions in normal bladder tissue and bladder cancer (BLC) (**A-F**).

that mRNA expressions of MCM2/3/4/5/6/7 were significantly associated with the diagnosis of BLC, and they might be useful as candidate biomarkers of diagnostic performance for BLC.

Genetic Mutations in the MCM Complex and Their Associations with OS and DFS of BLC Patients

We analyzed the association of MCM2-7 alterations with OS and DFS in patients with BLC. We interrogated The Cancer Genome Atlas BLC data set by using the cBioPortal web tool (http://www.cbioportal.org/). As is shown in Figure 7, high
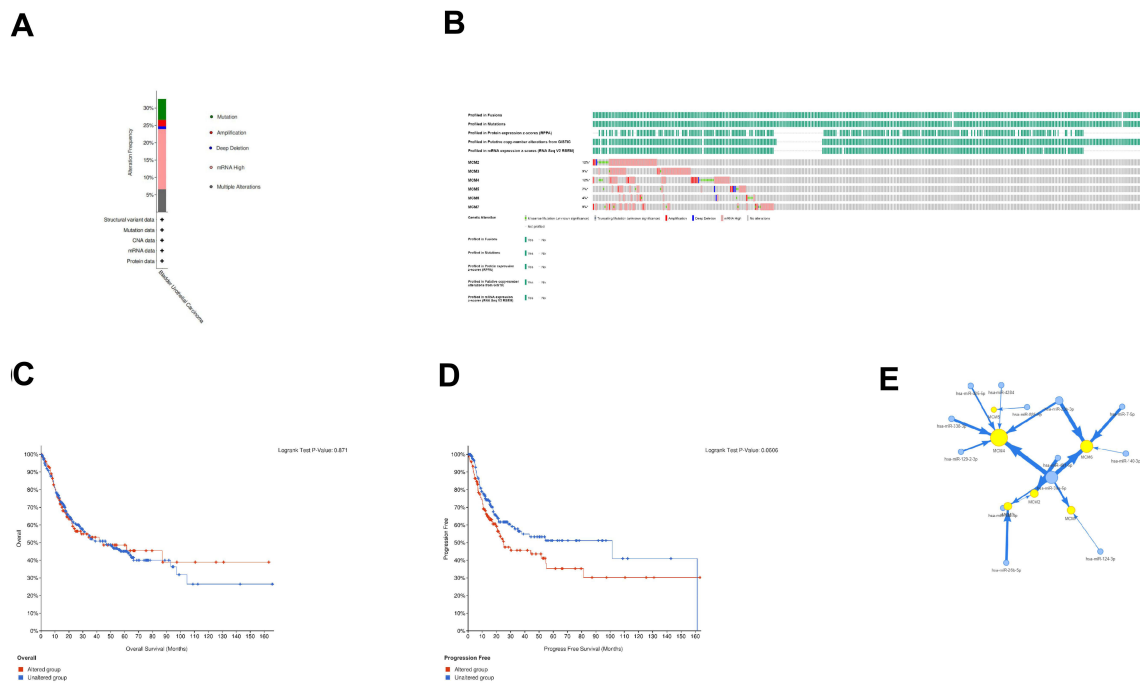
Figure 7 The genetic alteration of MCM complex in BLC (**A**). Alteration frequency of MCM2/3/4/5/6/7 based on the cBioProtal database (**B**). Kaplan–Meier plots revealed the overall survival and progression-free survival of BLC patients with or without MCM complex alterations (**C** and **D**). The network among MCM2/3/4/5/6/7 and miRNAs (**E**).

mutation rate was observed in BLC patients. MCM2/3/4/5/6/7 members were altered in 160 (39%) of queried patients and the mutation rate was 39%. MCM4, MCM2, MCM3 and MCM7 had the highest degree of variation, and the mutation rates were 16%, 14%, 10%, and 10%, respectively. Results from the Kaplan–Meier plot and *P* value showed that genetic variation in MCM2-7 was not statistically correlated with OS ($p = 0.871$) and DFS ($p = 0.442$) in BLC patients (Figure 7C and D). The results revealed that dysregulation of the MCM complex was not primarily attributed to DNA alterations. Further analysis revealed that MCM complex mRNA was regulated by a number of non-coding RNAs, which indicated that regulation of non-coding RNAs might play a vital role in alterations of the MCM complex (Figure 7E).

Predicted Functions and Pathways of the Changes in MCM2/3/4/5/6/7 and the 50 Most Frequently Altered Neighbor Genes in Patients with BLC

Functions of MCM2/3/4/5/6/7 as well as the 50 frequently altered neighbor genes were analyzed by GO and KEGG pathway enrichment analysis using limma packages of R software.[15] We constructed a protein interaction network including MCM2-7 complex as well as the 50 most frequently altered neighbor genes using the STRING interactive database (Figure 8A). These genes are able to interact with the Mcm2–7 complex. As is shown in Figure 8B, for predicting the functional roles of target host genes, we performed gene ontology enrichment analysis on the basis of three aspects including biological process, cell composition and molecular function. Biological processes such as GO:0006260 (DNA replication), GO:0006261 (DNA-dependent DNA replication), GO:0033260 (nuclear DNA replication), GO:0044786 (cell cycle DNA replication), GO:0071103 (DNA conformation change), were significantly regulated by MCM2/3/4/5/6/7 alterations in BLC. Cellular components, including GO:0098687 (chromosomal region), GO:0000793 (condensed chromosome), GO:0000775 (chromosome, centromeric region), GO:0000779 (condensed chromosome, centromeric region), GO:0000776 (kinetochore), were significantly associated with the MCM2/3/4/5/6/7 alterations. Molecular functions such as GO:0140097 (catalytic activity, acting on DNA), GO:0003678 (DNA helicase activity), GO:0003688 (DNA replication origin binding), GO:0004386 (helicase activity), GO:0043138 (3'-5' DNA helicase
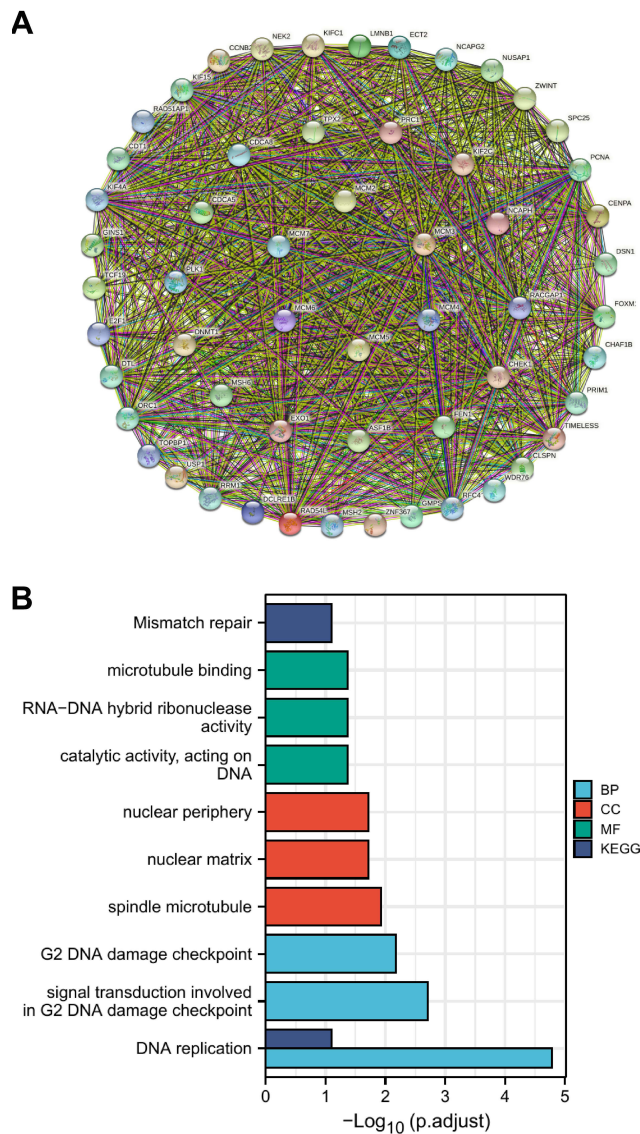
A

B

Figure 8 The network for MCM2/3/4/5/6/7 and the 50 most frequently altered neighbor genes (**A**). The enrichment analysis of MCM2/3/4/5/6/7 and the 50 most frequently altered neighbor genes associated with MCM complex alterations (**B**).

activity), were also affected by MCM2/3/4/5/6/7 mutations. KEGG analysis could identify the pathways and frequently altered adjacent genes related to MCM2/3/4/5/6/7 alterations function. In KEGG analysis, 5 pathways, including hsa03030 (DNA replication), hsa04110 (Cell cycle), hsa03430 (Mismatch repair), hsa03420 (Nucleotide excision repair), hsa04114 (Oocyte meiosis), were associated with functional changes in MCM2/3/4/5/6/7 mutations in BLC (Table 3). These data indicated that the MCM complex was involved in DNA replication, the cell cycle, DNA helicase activity, and mismatch repair.

Immune Infiltrations Analysis of the MCM Complex in BLC

Correlations between genes and immune infiltrations were estimated using TIMER. The abundance of CD8 + cell, neutrophil and dendritic cell showed positive associations with all the members of MCM 2/3/4/5/6/7. The abundance of CD4 + cell was not related to the expression of MCM 2/3/4/5/6/7, while the abundance of B cell was not related to the expression of the MCM complex except for MCM3. The abundance of macrophage showed positive associations with MCM 2/4/5/6 and showed no associations with MCM 3/7 (Figure 9A). To further reveal the somatic copy number alterations (SCNA) of the MCM complex in patients with BLC, we found that the SCNA of MCM3/6/7 was significantly

Table 3 GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) Functional Enrichment Analyses of MCM2/3/4/5/6/7 and Their 50 Associated Neighbor Genes

Ontology	ID	Description	Gene Ratio	Bg Ratio	p value	p Adjust	q Value
BP	GO:0006260	DNA replication	22/56	274/18,670	2.71E-26	2.24E-23	1.52E-23
BP	GO:0006261	DNA-dependent DNA replication	18/56	153/18,670	1.62E-24	6.67E-22	4.55E-22
BP	GO:0033260	Nuclear DNA replication	14/56	60/18,670	1.28E-23	3.52E-21	2.40E-21
BP	GO:0044786	Cell cycle DNA replication	14/56	71/18,670	1.74E-22	3.58E-20	2.44E-20
BP	GO:0071103	DNA conformation change	19/56	327/18,670	5.90E-20	9.74E-18	6.64E-18
CC	GO:0098687	Chromosomal region	23/56	349/19,717	4.56E-26	4.15E-24	2.06E-24
CC	GO:0000793	Condensed chromosome	14/56	223/19,717	1.42E-15	6.45E-14	3.21E-14
CC	GO:0000775	Chromosome, centromeric region	13/56	193/19,717	6.59E-15	2.00E-13	9.94E-14
CC	GO:0000779	Condensed chromosome, centromeric region	11/56	118/19,717	2.60E-14	5.92E-13	2.94E-13
CC	GO:0000776	Kinetochore	11/56	135/19,717	1.17E-13	2.14E-12	1.06E-12
MF	GO:0140097	Catalytic activity, acting on DNA	15/56	213/17,697	1.03E-16	1.25E-14	8.69E-15
MF	GO:0003678	DNA helicase activity	11/56	81/17,697	1.15E-15	6.96E-14	4.85E-14
MF	GO:0003688	DNA replication origin binding	8/56	24/17,697	4.22E-15	1.70E-13	1.18E-13
MF	GO:0004386	Helicase activity	12/56	163/17,697	9.74E-14	2.95E-12	2.05E-12
MF	GO:0043138	3'-5' DNA helicase activity	7/56	20/17,697	1.62E-13	3.91E-12	2.72E-12
KEGG	hsa03030	DNA replication	11/29	36/8076	8.33E-20	3.75E-18	2.80E-18
KEGG	hsa04110	Cell cycle	14/29	124/8076	1.22E-18	2.75E-17	2.06E-17
KEGG	hsa03430	Mismatch repair	4/29	23/8076	1.13E-06	1.70E-05	1.27E-05
KEGG	hsa03420	Nucleotide excision repair	3/29	47/8076	6.07E-04	0.007	0.005
KEGG	hsa04114	Oocyte meiosis	4/29	129/8076	0.001	0.01	0.007

Abbreviations: BP, biological process; CC, cellular component; MF, molecular function; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

correlated with the infiltrating levels of CD4+ T cells, neutrophils, and dendritic cells. The SCNA of MCM2 had a significant correlation with the infiltration level of CD4 + T cells and neutrophils, while that of MCM4 was in significant connections with the infiltrating levels of neutrophils. Meanwhile, the SCNA of MCM5 was significantly associated with dendritic cells, B cells, neutrophils, macrophages and CD8+ T cells (Figure 9B). Collectively, the MCM complex was closely related to the immune infiltration in patients bearing BLC. These findings indicate that the MCM complex could have a major impact on the immune status of patients with BLC.

Verification of the Drug Sensitivity of the MCM Complex

For drug susceptibility analysis, through the GSCALite platform we summarized the correlation between the MCM2/3/4/5/6/7 expressions and the sensitivity of Genomics of Drug Sensitivity in Cancer (GDSC) and Cancer Therapeutics Response Portal (CTRP) drugs (top 30) in pan-cancer (Figure 10). We found that the drug sensitivity of the MCM complex was significantly associated with chemotherapy resistance and further confirmed that the MCM complex was involved in multidrug resistance in BLC patients.

Pathway Enrichment of the MCM Complex in BLC

The pathways of the MCM complex were enriched with GSCALite. There were 10 pathways which were related to the function of MCM2/3/4/5/6/7 in BLC (Figure 11). The expressions of MCM2/3/4/5/6/7 were in a high level in apoptosis and the expressions of the MCM complex were also at a high level in cell cycle. The expressions of the MCM complex were almost inhibited in RAS/MAPK and hormone estrogen receptor (ER)-related pathways. These results indicated that the multiple signaling pathways and the biological function might be regulated by changes in the target genes.

Discussion

The minichromosome maintenance (MCM) complex is a group of six structurally related protein subunits that assemble to form hexamers.[16] The MCM proteins are believed to be closely associated with the development of tumor. MCM2-7
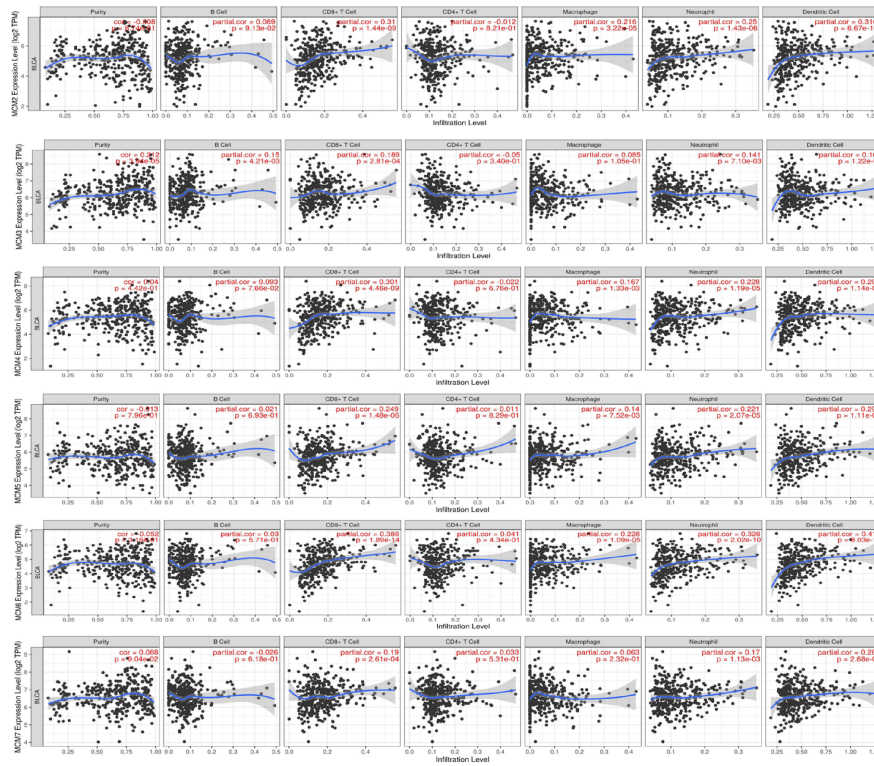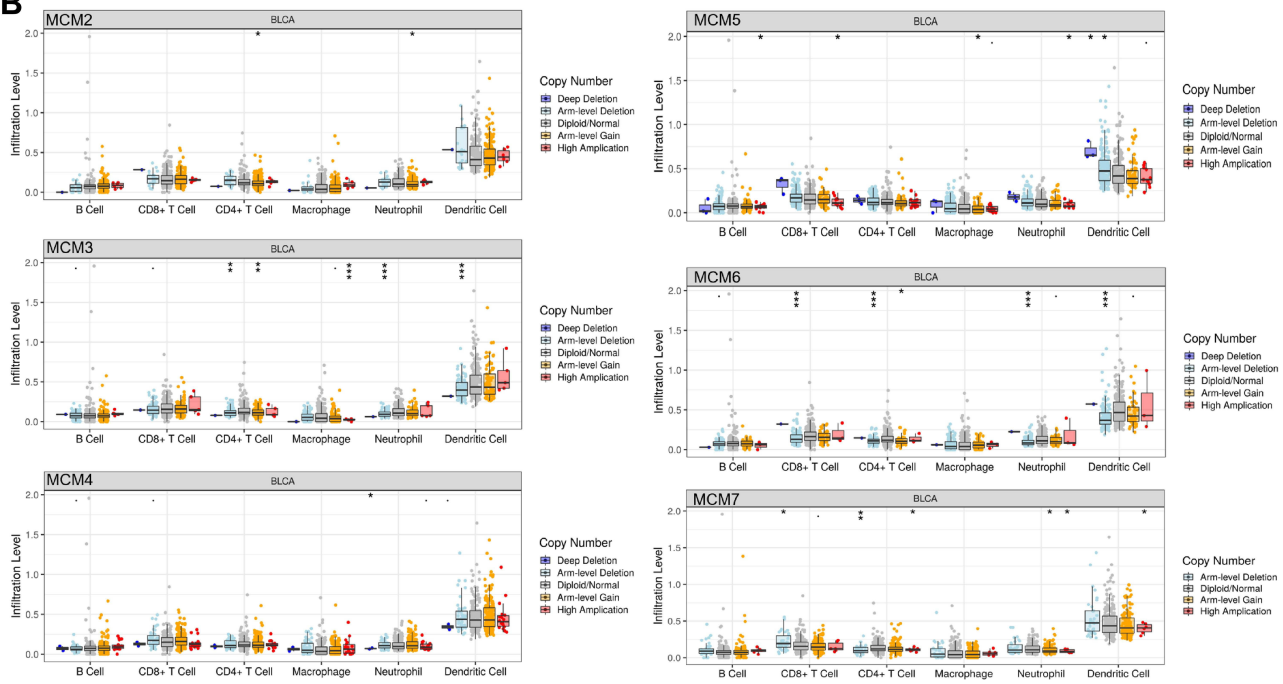
Figure 9 Association between the MCM2/3/4/5/6/7 expression level with immune infiltration level (**A**), and the comparison of tumor infiltration levels in BLC with different somatic copy number alterations for MCM2/3/4/5/6/7 (**B**). ns indicates not significant; $p \geq 0.05$; * $p < 0.05$; ** $p<0.01$; and *** $p <0.001$.

complex proteins can be found throughout the cell cycle, so they are useful as a universal biomarker for tumor growth. To date, dysregulation of the MCM complex has been widely observed in many types of cancer, such as common digestive tract tumors, gastric cancer,[17] liver cancer,[18] colon cancer,[19] prostate cancer,[20] kidney renal clear cell carcinoma,[21]
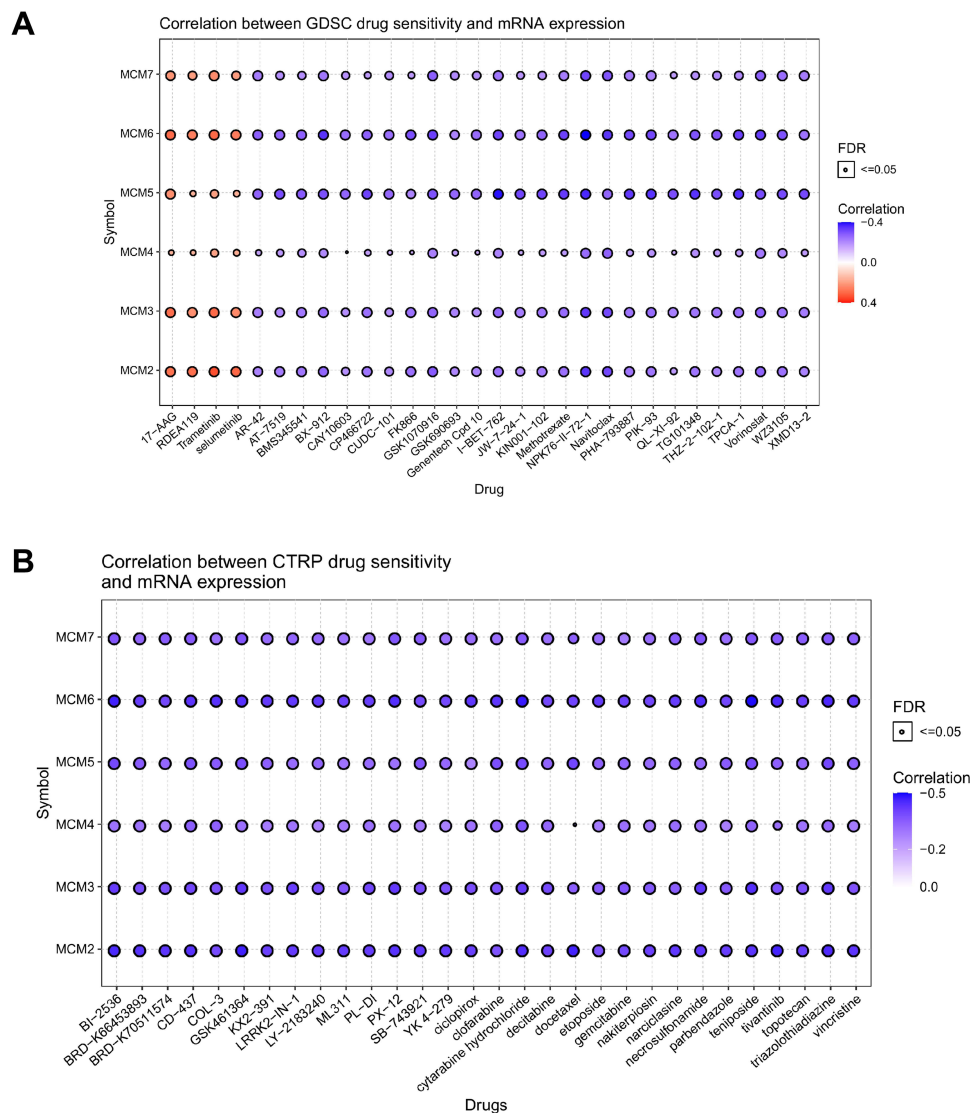
Figure 10 Drug resistance analysis of MCM2/3/4/5/6/7 based on GDSC (**A**) and CTRP (**B**) using the GSCALite web server.

glioma,[22] as well as ovarian cancer.[23] Compared with tumor proliferation index markers such as antigen Ki-67, the microchromosome maintenance proteins stand for potentially more accurate proliferation biomarkers for determining proliferation scores within tumors.[24] Although the role of MCM complex in oncogenesis and prognosis of several cancers has been partially confirmed, no comprehensive analysis of the MCM complex in BLC has been conducted in the literature to date. Hence, we systematically analyzed mRNA expression and protein levels, protein interactions, functional enrichment, diagnostic value, immune infiltration and prognostic value of the MCM complex in detail and provided experimental validation. In this study, the results showed that the mRNA levels of MCM complex were significantly increased in BLC tissues compared to those in normal bladder tissues from the TCGA database and GTEx database, while the protein levels of all MCMs except for MCM4 were increased, which indicated that MCM4 might be regulated by the non-coding RNA.

Our study revealed that the expression of MCM2 was significantly increased in BLC tissues and cell lines, which was associated with OS and DFS as well as MCM4/6. Burger M et al found that MCM2 was an independent predictor of recurrence in Ta/T1 BC, which predicted a risk of cancer recurrence in stage Ta/T1 BLC, and was more accurate than CK20, Ki67 and histological grade.[25] Kunal Kapoor et al[26] reported that immunocytochemical detection of MCM2 from urinary samples was detected to own a good sensitivity and specificity for the diagnosis of BLC and it might be used as
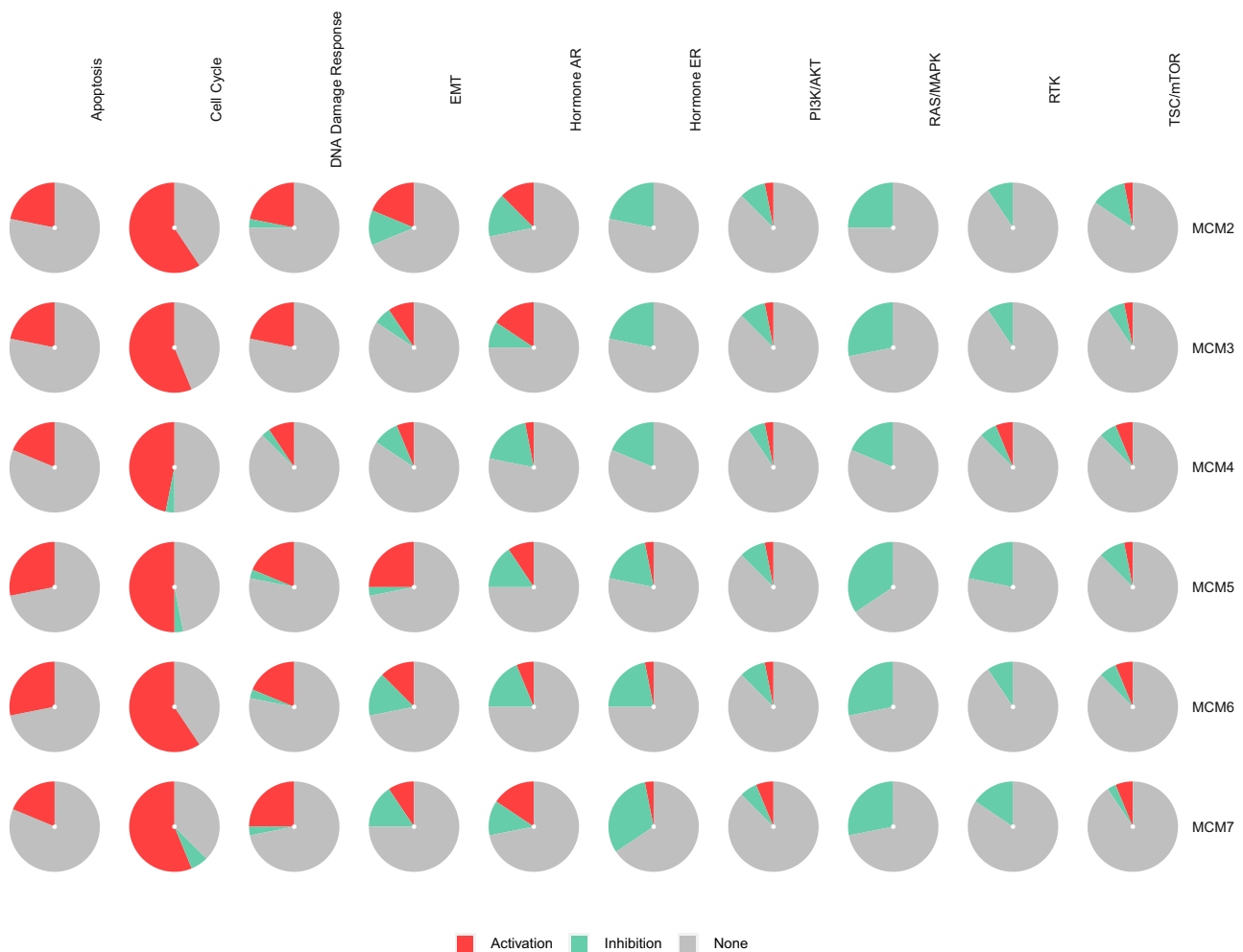
Figure 11 The pathway enrichment analysis of MCM2/3/4/5/6/7 genes with the GSCALite web server.

a potential biomarker for the diagnosis of BLC. Our study indicated that the variable MCM2 had a high accuracy in predicting the outcome of benign and malignant (AUC = 0.911, 95% CI = 0.847–0.975). High MCM2 expression was significantly correlated with poor OS and DFS, as well as histologic grade, suggesting an oncogenic role of MCM2.

Until now, MCM3 has been reported to be overexpressed in various tumors and its specific high expression in cancer cells makes it a promising biomarker for the detection of cancer,[27] but little is known about the expression and role of MCM3 in BLC. In our current study, the expression of MCM3 in BLC tissues was upregulated compared with the normal tissues. However, MCM3 expression had nothing to do with histologic grade in BLC patients. No statistically significant correlation was found between high MCM3 expression and DFS and OS in BLC. The reason for this phenomenon may be due to the fact that the MCM3 is regulated by miR-26p-5p according to the miRNA network (Figure 7E).

The MCM4 gene expression is downregulated in metastatic advanced BLCs and plays an essential role in replication and cell cycle.[28] However, the expression of MCM4 in BLC tissues was higher than that in normal tissues in our study, while the protein levels of all MCM4 were equal. The reason for this phenomenon may be that this could be involved with transcriptional as well as posttranscriptional regulatory mechanisms, such as the regulation of non-coding RNA.[29] The point mutation in MCM4 (F345I, G364R, and G486D) that disturbs MCM2-7 function causes genomic instability, which results in tumorigenesis.[30,31] Furthermore, high MCM4 expression was significantly associated with poor DFS, OS and tumor grade in patients with BLC, which seemed consistent with the role of MCM4 as an oncogene. In our study, we showed that patients with high MCM5 expression group showed significantly shorter DFS compared with lower MCM5 expression. The research found that the expression level of MCM5 was increased in muscle-invasive bladder cancer

compared with non-muscle-invasive bladder cancer and was relatively high in high-grade BLC.[32] Iawen Hsu et al[8] reported that estrogen receptor beta promoted the BLC cell proliferation and invasion by up-regulating the expression of MCM5. Furthermore, we demonstrated that MCM5 expression in BLC tissues was higher than that in normal bladder tissues, while an elevated expression was not associated with a shorter overall survival time among BLC patients. In an earlier proof-of-concept study, elevated Mcm5 levels in urine cell sediments were strongly predictive of bladder cancer.[33] MCM5 urine expression had moderate sensitivity to detect non-muscle invasive relapse and could correctly diagnose T1 stage relapse or high-grade tumor.[34] Immunocytochemistry with MCM5 antibodies from urine can improve the ability of urinary cytology to detect urothelial carcinoma of the bladder.[35] Another study also indicated that MCM5 was a novel biomarker for the diagnosis of ovarian cancer.[36] In predicting the outcome of benign and malignant, MCM5 had certain accuracy (AUC = 0.871, 95% CI: 0.770–0.972) based on our study, so that we could infer that MCM5 might be a promising early diagnostic biomarker for BLC.

There are few studies investigating the role of MCM6 in BLC. DNA mutation is a potent driver of tumorigenesis in replicating cells.[37] The MCM6 gene mutation value is the lowest in the MCM complex based on our study, so MCM6 is rarely reported in the literature for BLC. The expression of MCM6 was significantly increased in breast cancer compared with the corresponding paracancerous tissues,[38] which was similar to the PCR tests for the expression of MCM6 in BLC tissues we collected. Additionally, MCM4 and MCM6 expressions have been linked to the tumor stage.[39] Furthermore, patients with increased MCM6 expression had worse long-term survival and higher cumulative recurrence rates.[40] In our study, high MCM6 expression was significantly associated with poor OS and DFS in all BLC patients, and had a relatively high accuracy (AUC = 0.838, 95% CI: 0.759–0.916) in differentiating benign and malignant bladder tumors. The expression of MCM6/7 was closely correlated with the four main types of immune cells (CD8+ T cells, CD4+ T cells, neutrophils and dendritic cells). These results indicated that the MCM6/7 might play a key role in the prognosis and the immune status of BLC patients.

As an essential member of the MCM complex, MCM7 plays a crucial role in maintaining the initial stability of DNA replication,[41] and MCM7 participates in the degradation of MCM2-7 in a ubiquitin-dependent manner during termination.[42] By inhibiting the expression of MCM7, it could effectively inhibit the growth of BLC cells.[43] Kim et al found that cisplatin resistance in BLC was associated with up-regulation of MCM7 expression.[44] Moreover, it has been shown that MCM4 and MCM7 are better markers than Ki-67 for detecting esophageal cancers.[45] In our study, we demonstrated that MCM7 expression was higher in BLC tissues than that in normal tissues and that this expression was positively correlated with clinical tumor grade and poor DFS in BLC.

In this study, a bioinformatics method was used to analyze transcriptional levels, mutations, and their association with the main pathological parameters of BLC patients and to explore the potential biological role and prognostic value of the MCM complex in BLC, which brought important insights into the clinical application of the MCM complex as potential diagnostic biomarkers, prognostic factors and therapeutic targets. Clearly, this study has some limitations. First, the molecular mechanisms of the role of the MCM complex in BLC should be further explored via experiments in vivo and in vitro. In addition, since this study was a retrospective study, it required further investigation to support the results.

Conclusion

A comprehensive analysis of the role of the MCM complex in BLC is presented here, which takes into account the expression levels, genetic alterations, PPI, immune infiltration, drug sensitivity as well as prognostic factors. We found that higher expressions of the MCM2/4/6/7 were detected to be significantly associated with worse pathological characteristics of patients bearing BLC, including histologic grade, tumor histology, poor OS and DFS. The MCM complex has moderate-to-high accuracy in the differential diagnosis of benign and malignant bladder tumors, so they might become potential diagnostic markers for BLC. MCM2-7 were significantly associated with immune infiltration and chemotherapy resistance. In summary, the results of this study indicated that the MCM complex especially MCM2/4/6/7 might be potential molecular therapeutic targets for BLC treatment and might be useful biomarkers for diagnosis and prognosis.

Data Sharing Statement

The original contributions presented in this study are included in the article.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the Ethical Standards of the Institutional Ethics Committee of First Affiliated Hospital of Nanchang University and with the 1964 Helsinki declaration and its later amendments or comparable Ethical Standards. All patients provided informed consent.

Acknowledgments

We thank the two independent pathologists who confirmed the pathologic diagnosis of tumor tissues. Co-first authors: Chen Ru, Hu Bing, and Jiang Ming.

Funding

Disclosure

The authors declare no conflicts of interest in relation to this work.

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