

Minichromosome maintenance complex component 7 has an important role in the invasion of papillary urothelial neoplasia

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Received March 24, 2014; Accepted March 2, 2015

DOI: 10.3892/ol.2015.3333

Abstract. The aims of the present study were to investigate the expression of minichromosome maintenance complex component 7 (MCM7) and determine its association with tumor proliferation and invasion in pathological tumor (pT)a and pT1 papillary urothelial neoplasia. The MCM7, MCM3 and Ki67 proteins were detected in 154 cases of urothelial neoplasia using immunohistochemical analysis. The expression of MCM7 significantly increased ($P<0.001$) as the pathological stage and grade progressed between inverted papilloma, papillary urothelial neoplasm of low malignant potential (PUNLMP), pTa tumor and pT1 tumor. However, no statistically significant difference in MCM7 staining was observed between low-grade pTa tumors and PUNLMP ($P=0.2294$). In contrast to MCM7, MCM3 was highly expressed in all stages of urothelial neoplasia, with no statistically significant differences observed between the tumor types ($P=0.2993$, 0.3885 and 0.8489 for pTa tumors, PUNLMP and inverted papilloma, respectively). Furthermore, MCM7 expression was elevated with increased tumor grade and was positively correlated with Ki67 expression ($r_s=0.9106$, $P<0.001$). However, MCM3 expression was not correlated with MCM7 or Ki67 expression ($r_s=0.0734$, $P=0.3657$ and $r_s=0.0638$, $P=0.4318$, respectively). In conclusion, MCM7 overexpression may simultaneously promote tumor proliferation and invasion. Furthermore, it may be a reliable marker for the pathological differential diagnosis of pTa and pT1 papillary urothelial neoplasms; therefore, MCM7 expression may be used to predict tumor prognosis and behavior.

Introduction

Papillary urothelial neoplasia is the most common tumor of the bladder and ureter. Worldwide, urothelial bladder cancer incidence rates are 10 per 100,000 in males and 3 per 100,000

in females, and mortality rates are 6 per 100,000 in males and 1.3 per 100,000 in females (1). The majority of papillary urothelial neoplasia tissues are obtained from biopsies and transurethral resections of bladder and ureter tumors. Maintain the integrity and morphological structure of these tissues is challenging. However, the system proposed by the World Health Organization in 2004 (2), which was based on the morphological criteria of tumors, is without a specific marker. Therefore, it can be challenging to distinguish between the different stages and grades of papillary urothelial neoplasia, particularly between the pathological tumor (pT)a and pT1 neoplasia tissues. A number of markers have been extensively investigated in order to evaluate the differentiation grades. The results of tumor immunoreactivity analysis have suggested that several of these markers, including cluster of differentiation (CD)44, cytokeratin (CK)20, p53 and Ki67 (3-7), may be useful in the diagnosis of urothelial carcinomas.

Minichromosome maintenance (MCM) proteins participate in the initiation and elongation steps of DNA replication (8). In total, six of the MCM proteins (MCM2-MCM7) are highly conserved, share a 200-amino acid nucleotide-binding region and form a range of subcomplexes (9). For instance, the MCM4/MCM6/MCM7 trimers and MCM2-MCM7 hexamers serve as an ATPase and DNA helicase, initiating and elongating replication forks, respectively (8). MCM complexes are recruited temporally separated from their activation, in which the poised but inactive MCM complex is converted into an enzymatically active helicase. This helicase is involved in plasmid replication during late telophase and at the beginning of the G₁ phase of the cell cycle to ensure that initiation occurs only once in every cell division (10). The MCM complex is an important factor involved in DNA damage-dependent regulatory signals that control DNA replication. In addition, MCM7 interacts with the MYCN transcription factor and participates in the regulation of its own transcription (11,12). The knock-down of MCM7 disrupts checkpoint signaling and leads to a defect in an intra-S-phase checkpoint (13). Although MCM7 protein overexpression has been identified in other malignant tumors (14,15), it has not been previously reported in papillary urothelial neoplasia.

The present study analyzed the expression levels of MCM7 and MCM3 in different pathological stages and grades of papillary urothelial neoplasia tissues. In addition, the association of MCM7 and MCM3 with Ki67 in pTa and pT1 papillary urothelial neoplasias was investigated.

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Key words: minichromosome maintenance complex component (MCM)7, MCM3, urothelial neoplasia, papillary neoplasm

Table I. Expression levels of MCM7, MCM3 and Ki67 in urothelial neoplasia (mean \pm standard deviation).

| Tumor subtype | n | MCM7 | P-value ^a | MCM3 | P-value ^a | Ki67 | P-value ^a |
|--------------------|-----|-------------------|----------------------|------------------|----------------------|-------------------|----------------------|
| pT1 | 56 | 81.52 \pm 7.44 | | 85.98 \pm 4.99 | | 54.29 \pm 12.48 | |
| pTa | 78 | 51.35 \pm 20.03 | <0.001 | 85.06 \pm 5.13 | 0.2993 | 25.96 \pm 17.76 | <0.001 |
| High-grade | 46 | 64.80 \pm 12.69 | <0.001 | 85.22 \pm 4.83 | 0.4330 | 37.72 \pm 13.61 | <0.001 |
| Low-grade | 32 | 32.03 \pm 10.46 | <0.001 | 84.84 \pm 5.61 | 0.3406 | 9.06 \pm 3.83 | <0.001 |
| PUNLMP | 12 | 27.50 \pm 11.38 | <0.001 | 84.58 \pm 5.42 | 0.3885 | 7.17 \pm 3.35 | <0.001 |
| Inverted papilloma | 8 | 1.88 \pm 0.99 | <0.001 | 85.63 \pm 4.96 | 0.8489 | 1.75 \pm 0.71 | <0.001 |
| Total | 154 | 57.89 \pm 26.34 | | 85.39 \pm 5.07 | | 33.54 \pm 22.58 | |

^aCompared with the pT1. pT, pathological tumor; PUNLMP, papillary urothelial neoplasm of low malignant potential; MCM, minichromosome maintenance complex component.

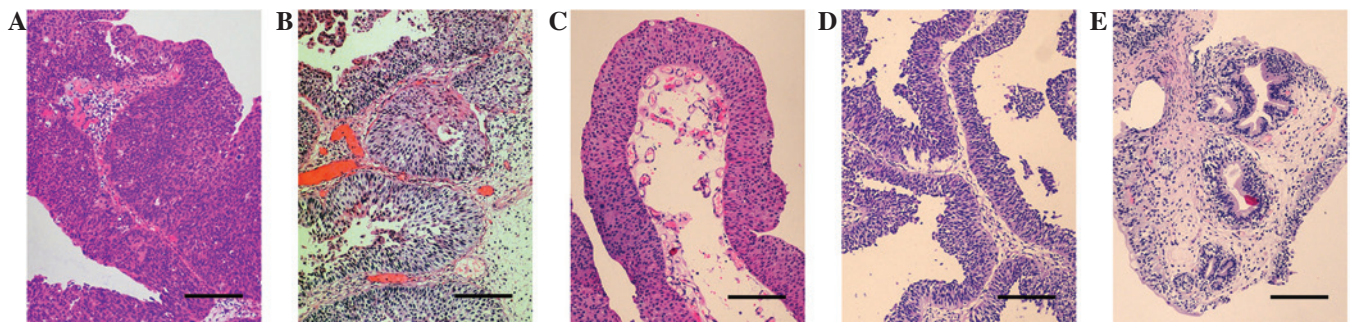


Figure 1. Urothelial neoplasia. Staining of (A) pT1 tumor, (B) high-grade pTa tumor, (C) low-grade pTa tumor, (D) papillary urothelial neoplasm of low malignant potential, and (E) inverted papilloma (A-E, magnification, \times 100). Bar, 125 μ m. pT, pathological tumor.

Materials and methods

Patients. In total, 134 pT1 and pTa papillary urothelial tumors obtained from patients admitted at the Second Hospital of Shandong University (Jinan, China) between 2007 and 2012 were selected for use in the present study. Of the patients, 105 were male, the median age was 70 years and the age range was 42-87 years. A total of 56 pT1 tumors (Fig. 1A) were selected, as well as 78 pTa tumors that included 46 cases of high-grade noninvasive carcinoma (Fig. 1B) and 32 cases of low-grade noninvasive carcinoma (Fig. 1C). In addition, 12 papillary urothelial neoplasms of low malignant potential (PUNLMP; Fig. 1D) 9 of which were male, with a median age of 62 years and age range of 38-82 years; and 8 inverted papillomas (Fig. 1E) 5 of which were male, with a median age of 67 years and age range of 36-81 years, were added to the study. The pathological samples included paraffin-embedded biopsies and transurethral resections of bladder and ureter tumors stored at the Second Hospital of Shandong University, which had been obtained from previous surgical resections. Paraffin-embedded control samples, which had been stored at the Second Hospital of Shandong University from previous surgical resections, included malignant mesothelioma tissues, as the positive tissue control for MCM7 and MCM3, normal tonsil tissues, as the positive tissue control for Ki67 and placental villi, as a negative tissue control for each antibody. All the experimental protocols were approved by the Medical Ethics Committee of the Second Hospital of Shandong

University and written informed consent was obtained from all patients.

Immunohistochemical analysis. The specimens were fixed with 10% neutral formaldehyde (Baibo Biosciences Co., Ltd., Jinan, China), conventionally dehydrated, embedded in paraffin and then sliced in 4- μ m sections using a microtome (RM2255; Leica Microsystems GmbH, Wetzlar, Germany). The antibodies used for immunohistochemical analysis were as follows: mouse monoclonal anti-human MCM7 (clone 47DC141; dilution, 1:100; catalog no. GTX22360; Abcam, Cambridge, MA, USA); a rabbit polyclonal anti-human MCM3 (clone 4F7; dilution, 1:100; catalog no. 15597-1-AP; Proteintech Group, Inc., Chicago, IL, USA); and mouse monoclonal anti-human Ki67 (clone MIB-1; dilution, 1:50; anti-human; catalog no. F726801F; Dako, Glostrup, Denmark) antibodies. First, the sections were deparaffinized and rehydrated, steamed in a 0.01 mol/l citrate buffer solution (pH 6.0; ZSGB-Bio Co., Ltd., Beijing, China) for 20 min and then cooled for 10 min. Next, the tissues were blocked by immersing the slides in a 3% solution of hydrogen peroxide in methanol for 10 min. The slides were then incubated with the primary antibodies in a humidity chamber for 45 min at room temperature, followed by incubation with the biotinylated secondary antibody (anti-mouse and anti-rabbit; Novolink Polymer Detection System; Novocastra; Newcastle; United Kingdom) in a humidity chamber for 40 min at 37°C. Subsequently, the slides were incubated with a streptavidin enzyme

Table II. Correlation between MCM7, MCM3 and Ki67 in urothelial neoplasia.

| | MCM7 | | MCM3 | | Ki67 | |
|------|--------|---------|--------|---------|--------|---------|
| | r_s | P-value | r_s | P-value | r_s | P-value |
| MCM7 | - | - | 0.0734 | 0.3657 | 0.9106 | <0.001 |
| MCM3 | 0.0734 | 0.3657 | - | - | 0.0638 | 0.4318 |

MCM, minichromosome maintenance complex component.

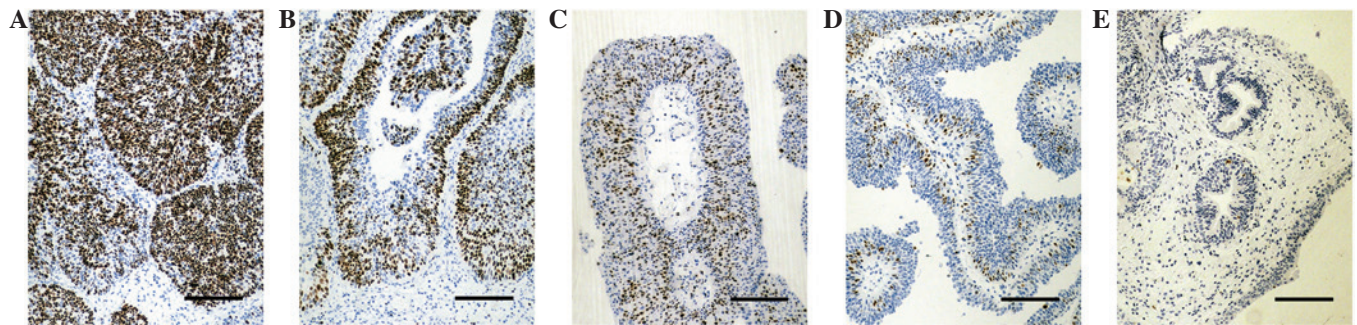


Figure 2. Minichromosome maintenance complex component 7 nuclear staining pattern in (A) pT1 tumor, (B) high-grade pTa tumor (C) low-grade pTa tumor, (D) papillary urothelial neoplasm of low malignant potential and (E) inverted papilloma (A-E, magnification, x100). Bar indicates 125 μ m. pT, pathological tumor.

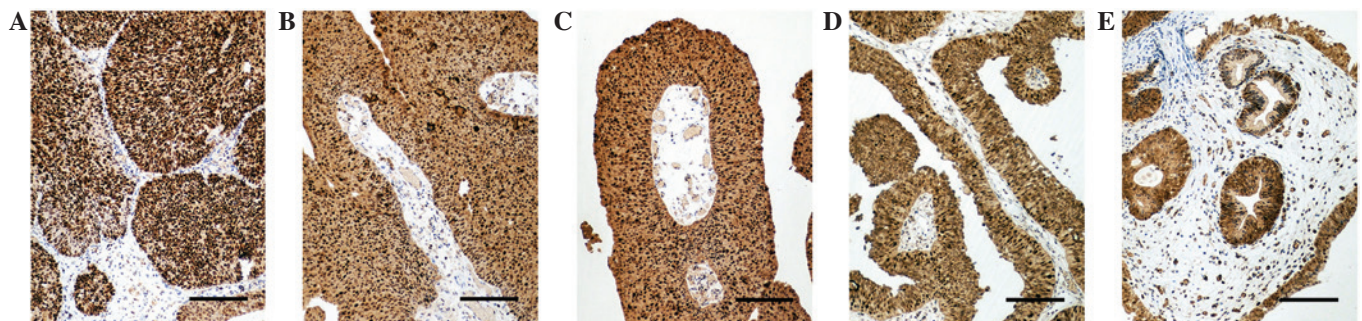


Figure 3. Minichromosome maintenance complex component 3 nuclear staining pattern in (A) pT1 tumor, (B) high-grade pTa tumor, (C) low-grade pTa tumor, (D) papillary urothelial neoplasm of low malignant potential and (E) inverted papilloma (A-E, magnification, x100). Bar, 125 μ m. pT, pathological tumor.

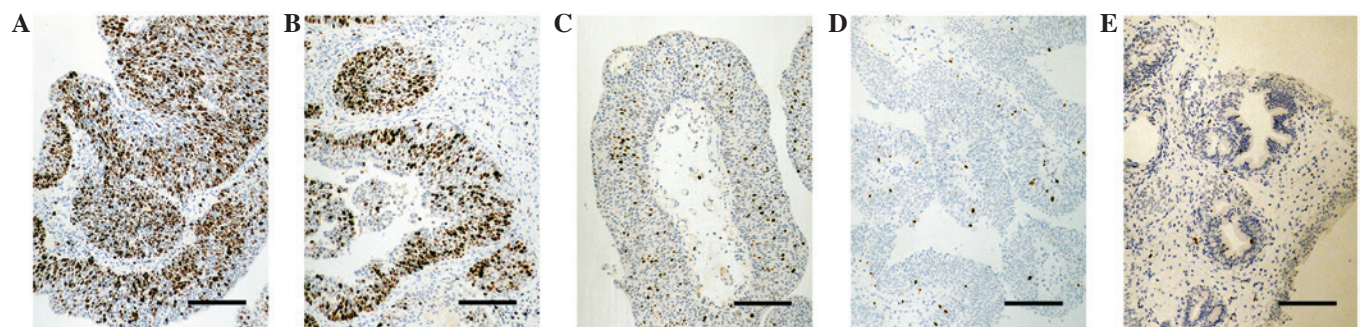


Figure 4. Ki67 nuclear staining pattern in (A) pT1 tumor, (B) high-grade pTa tumor, (C) low-grade pTa tumor, (D) papillary urothelial neoplasm of low malignant potential, and (E) inverted papilloma (A-E, magnification, x100). Bar, 125 μ m. pT, pathological tumor.

complex (Novolink Polymer Detection System) for 25 min. Finally, the slides were covered with 3,3'-diaminobenzidine tetrahydrochloride solution (ZSGB-Bio Co., Ltd.) for 15 min under a microscope (Eclipse Ci; Nikon Corporation, Tokyo,

Japan), and then counterstained with hematoxylin (Novolink Polymer Detection System) for 1 min. The expression levels of MCM3, MCM7 and Ki67 were assessed in tumor areas, including the basal, intermediate and surface urothelial cells.

The invasive areas were primarily observed in the pT1 tumors. In total, five fields selected in random were analyzed within a counting grid at a magnification of x400. The percentage of positive cells was expressed as the mean value of counted microvessels in the five fields.

Statistical analysis. Statistical analysis was performed using the SPSS 11.0 software package for Windows (SPSS, Inc., Chicago, IL, USA). The Mann-Whitney U-test was used to examine the differences in the MCM7, MCM3 and Ki67 expression levels between the pTa and pT1 papillary urothelial neoplasias. The statistical analysis of the correlation among MCM7, MCM3 and Ki67 expression levels in each sample was conducted with Spearman's rank correlation test. Data are expressed as the mean \pm standard deviation. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Immunohistochemical analysis. Nuclear staining patterns were demonstrated for MCM7 (Fig. 2), MCM3 (Fig. 3) and Ki67 (Fig. 4). The expression levels of MCM7, MCM3 and Ki67, as well as the pathological stage and grade of the 154 patients with papillary urothelial neoplasms are listed in Table I. MCM7 was highly and extensively expressed in the epithelial layers of the pT1 tumors, highly expressed in the basal and intermediate cells of the high-grade pTa tumors and moderately expressed in the basal cells of the low-grade pTa tumors and PUNLMP. MCM7 staining was limited to the basal cells of the inverted papillomas (Fig. 2). MCM3 was extensively expressed in the epithelial layers of pT1 tumors, pTa tumors, PUNLMP and inverted papillomas (Fig. 3). Ki67 was highly and extensively expressed in the epithelial layers of the pT1 tumors and highly expressed in the basal and intermediate cells of the high-grade pTa tumors. Ki67 was expressed in a small number of cells of the low-grade pTa tumors and PUNLMP. Ki67 staining was limited to the basal cells of the inverted papillomas (Fig. 4).

Expression of MCM7 in urothelial neoplasia. An increase in the expression of MCM7 was observed as the pathological stage and grade increased. MCM7 was highly and extensively expressed in the epithelial layers of the pT1 tumors (81.52 ± 7.44 ; Fig. 2A), highly expressed in the basal and intermediate cells of the high-grade pTa tumors (64.80 ± 12.69 ; Fig. 2B), and moderately expressed in the basal cells of the low-grade pTa tumors and PUNLMP (32.03 ± 10.46 and 27.50 ± 11.38 , respectively; Fig. 2C and D). MCM7 was expressed in low levels or individually in the basal cells of the inverted papillomas (1.88 ± 0.99 , Fig. 2E). A statistically significant difference in MCM7 expression was observed between pT1 tumors, pTa tumors, PUNLMP and inverted papillomas (Table I); however, no statistically significant difference was detected between the low-grade pTa tumors and PUNLMP ($P = 0.2294$). In addition, the expression of MCM7 in high-grade noninvasive carcinomas (64.80 ± 12.69) was significantly higher compared with that in low-grade noninvasive carcinomas (32.03 ± 10.46 ; $P < 0.001$).

Expression of MCM3 in urothelial neoplasia. MCM3 was extensively expressed in the epithelial layers of pT1 tumors, pTa tumors, PUNLMP and inverted papillomas (85.98 ± 4.99 ,

85.06 ± 5.13 , 84.58 ± 5.42 , 85.63 ± 4.96 , respectively; Fig. 3). However, no statistically significant difference in the expression of MCM3 was identified between the different pathological stages and grades (Table I).

Association of MCM7 and MCM3 with Ki67 in urothelial neoplasia. As the expression of the tumor cell proliferation index, Ki67, increased with increasing tumor grade, a significant difference was observed in the type of urothelial neoplasia, progressed from inverted papilloma to pT1 tumor ($P < 0.001$ Fig. 4). Furthermore, there was a significant positive correlation between MCM7 and Ki67 expression levels in the urothelial neoplasia tissues ($r_s = 0.9106$; $P < 0.001$); however, no significant correlation was detected between MCM3 and MCM7 or Ki67 ($r_s = 0.0734$, $P = 0.3657$; $r_s = 0.0638$, $P = 0.4318$; Table II).

Discussion

Previous studies have used molecular genetics to compare noninvasive (pTa) and superficially-invasive (pT1) carcinomas (2-4,6). In pTa tumors, *FGFR3* gene mutations are extremely common ($>70\%$), whereas *p53* gene mutations occur in $<5\%$ of cases. By contrast, pT1 tumors frequently exhibit *p53* mutations, while *FGFR3* gene mutations are reported in $\sim 30\%$ of cases (16,17). This indicates that different mechanisms are involved in the development of pTa and pT1 tumors. The latter are associated with a higher risk of recurrence and poorer survival rates. Therefore, a correct pathological diagnosis is important for clinical treatment and prognosis (18-20).

The present study identified a statistically significant and progressive increase in the expression of MCM7 with increasing grade and stage of papillary urothelial neoplasms. MCM7 was expressed at a low level or individually in the basal cells of normal urothelium and inverted papilloma, but overexpressed in urothelial carcinomas, particularly in pT1 tumors. The results in the present study suggested that MCM7 can promote the proliferation and even invasion of tumors. In addition, the overexpression of MCM7 with higher Ki67 expression in pT1 tumors indicated that the ratio of G₁ phase cells increased as the pathological stage and grade increased. The overexpression of MCM7 is therefore considered to induce aberrant DNA replication and in this process, the tumor invasion should be promoted. A significant difference in MCM7 expression was also observed between pT1 and pTa tumors ($P < 0.001$). Tumor invasion is known to be important for patient prognosis. Therefore, MCM7 in particular may be a reliable marker for the differential diagnosis of pTa and pT1 papillary urothelial neoplasms.

In addition, the current study revealed that MCM7 expression was significantly positively correlated with Ki67 expression in urothelial neoplasia tissues ($r_s = 0.9106$, $P < 0.001$). In previous studies, Ki67 was identified to be a potential prognostic marker that improved the risk stratification and a reliable indicator of biological aggressiveness in urothelial neoplasia (5,7). Therefore, MCM7 may have the potential to predict the prognosis and behavior of pTa and pT1 papillary urothelial neoplasms.

In conclusion, MCM7 was identified to be significantly associated with tumor grade and Ki67 expression. Therefore, MCM7 may be a useful marker for predicting the prognosis and behavior of pTa and pT1 papillary urothelial neoplasms.

Further investigation of MCM7 expression may aid in clarifying the molecular pathways involved in papillary urinary neoplasia.

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