

## ORIGINAL ARTICLE

## DNA replication regulation protein Mcm7 as a marker of proliferation in prostate cancer

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**Background:** Recent studies have shown that minichromosome maintenance (MCM) proteins (Mcm2–7) may be useful proliferation markers in dysplasia and cancer in various tissues.

**Aims:** To investigate the use of Mcm7 as a proliferation marker in 79 lymph node negative prostate cancers and compare it with Ki-67, a commonly used cell proliferation marker.

**Methods:** The percentage of proliferating cells (proliferation index; PI) was calculated for basal and luminal epithelial cells in benign prostate tissue, prostatic intraepithelial neoplasia (PIN), and epithelial cells in adenocarcinoma. The PI for each biomarker was correlated with the preoperative prostate specific antigen concentration, the Gleason score, surgical resection margin status, and the AJCC pT stage for each patient.

**Results:** The mean PIs for Ki-67 and Mcm7 were: benign luminal epithelium 0.7 and 1.2 and benign basal epithelium 0.8 and 8.2; PIN non-basal epithelium 4.9 and 10.6 and PIN basal epithelium 0.7 and 3.1; adenocarcinoma 9.8 and 22.7, respectively. Mcm7 had a significantly higher mean PI ( $p < 0.0001$ ) than Ki-67 for all cell categories except benign luminal epithelial cells. Mcm7 was a better discriminatory marker of proliferation between benign epithelium, PIN, and invasive adenocarcinoma ( $p < 0.0001$ ) than Ki-67. The drop in Mcm7 mean basal cell PI from benign epithelium to PIN epithelium was significantly larger than for Ki-67 ( $p < 0.0001$ ). Mcm7 had a significantly higher PI than Ki-67 at each risk level.

**Conclusion:** Mcm7 may be a useful proliferation marker in prostatic neoplasia and warrants further evaluation as a complementary tool in the diagnosis of PIN and prostate carcinoma.

Prostate cancer is the most common form of non-cutaneous malignancy in North American men.<sup>1</sup> It is the second leading cause of cancer related deaths in men after lung cancer. One of the challenges facing pathologists is distinguishing between the aggressive and indolent forms of this disease. One feature common to most neoplasms is their rapid proliferation rate compared with normal tissue.<sup>2</sup> The proliferative activity of many malignant tumours has been shown to correlate with progression and prognosis of disease.<sup>3</sup> There are several ways of measuring cell proliferation, but in processed tissue the technique of choice is immunohistochemical analysis, using antibodies to proteins such as proliferating cell nuclear antigens (PCNA) and Ki-67.<sup>4–6</sup> PCNA is a cyclin and an auxiliary protein of DNA polymerase  $\delta$ , which is essential for DNA replication and is also involved in DNA excision repair.<sup>7–10</sup> Ki-67 is a poorly characterised non-histone nuclear protein, which is expressed during the cell cycle in the G1, S, G2, and M phases, but not in the G0 phase.<sup>11, 12</sup> The original anti-Ki-67 antibody recognised Ki-67 only in fresh or snap frozen tissue. Subsequently, the MIB-1 antibody was developed, which recognises Ki-67 antigen in formalin fixed, paraffin wax embedded tissues.<sup>13</sup> The predictive value of PCNA and Ki-67 labelling as markers of prognosis has been studied extensively in prostate cancer with contradictory results.<sup>14–22</sup> Although most studies suggest that the Ki-67 labelling index (per cent of cells labelled) is predictive of patient outcome, there are variations in methods and reporting (ratio versus per cent), and no consensus on cutoff points, all of which make comparisons difficult.<sup>23</sup>

MCM (minichromosome maintenance) proteins are a family of six highly conserved and highly homologous proteins, which form a part of the pre-replication complex that licenses DNA replication.<sup>24, 25</sup> Recently, the role of MCM proteins (Mcm2–7) as markers of cell proliferation in human

tissues has been investigated.<sup>2, 26–32</sup> MCM proteins have been demonstrated in replicating cells, but not in quiescent, differentiated, or senescent cells, suggesting that they may be useful as markers of proliferation.<sup>26–27, 33, 34</sup> Immunohistochemical expression of Mcm2, Mcm5, and Mcm7 has been shown to be restricted to the proliferating regions of the normal epidermis, intestine, and lymphoid tissue.<sup>26, 29</sup> Increased expression has been noted in most solid tumours and premalignant proliferative states.<sup>26, 27</sup> The presence of the MCM proteins in dysplastic and malignant cells has suggested that these proteins might be clinically useful in the diagnosis of in situ and invasive carcinoma in the cervix and urinary bladder.<sup>2, 27, 30</sup> In one study, expression of Mcm2 in prostate carcinoma was shown to be a predictor of disease free survival after radical prostatectomy, independent of primary Gleason grade, surgical margin status, and neoadjuvant hormone treatment.<sup>31</sup> There is currently no detailed study on the role of Mcm7 as a proliferation marker in prostate cancer.

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Our study aims to compare the immunohistochemical staining patterns of normal prostate, prostatic intraepithelial neoplasia (PIN), and prostate cancer using Mcm7 and Ki-67.

**Abbreviations:** H&E, haematoxylin and eosin; MCM, minichromosome maintenance; PCNA, proliferating cell nuclear antigen; PI, proliferation index; PIN, prostatic intraepithelial neoplasia; PSA, prostate specific antigen

The proliferation index (PI) for each antigen was assessed relative to pathological and clinical variables.<sup>35</sup>

## MATERIALS AND METHODS

Seventy nine lymph node negative radical prostatectomy specimens were obtained between June 1999 and February 2001 from the department of pathology, Fletcher Allen Health Care Hospital, Vermont, USA. Each prostate gland was initially weighed, measured, and inked in different colours for orientation. After fixation in 10% neutral buffered formalin, apex and base sections were separated, cut into perpendicular sections, and totally submitted. The remaining prostate was then sectioned into 3 mm sections in a plane parallel to the rectal surface, cut into four quadrants, each to fit on a regular glass slide, and entirely submitted. The slides were stained with haematoxylin and eosin (H&E). Foci of tumour were outlined with ink and the images digitised with a flatbed transmission scanner (Umax, Dallas, Texas, USA). The images were then reoriented to form the equivalent of whole mount sections. The tissue was used in accordance with the guidelines for human tissue use approved by the University of Vermont institutional review board. Preoperative characteristics analysed included age of the patient at the time of surgery and serum prostate specific antigen (PSA) concentrations in ng/ml.

## Histology

H&E stained slides from the entire prostate were examined to identify foci of PIN and adenocarcinoma. The largest focus of cancer was identified by visual inspection of the images and was chosen for subsequent immunological studies. Tumours were assigned a Gleason score and determinations of the per cent of tumour were made by visual inspection from a reconstruction of the scanned images. Surgical resection margin status, capsular involvement, extracapsular extension, perineural invasion, and the AJCC pathological stage of the tumour were also determined.<sup>36</sup>

## Immunohistochemistry

Immunohistochemical analysis was performed on the largest (dominant) focus of cancer as described above, adjacent representative sections of PIN, and non-neoplastic prostatic tissue from the peripheral zone of the prostate. Slides prepared from 5 µm sections of the formalin fixed, paraffin wax embedded tissues were subjected to antigen retrieval with 10mM sodium citrate buffer for 15–20 minutes at 95–99°C. Endogenous peroxidase activity was blocked by incubation with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 15 minutes, followed by a protein block (DakoCytomation, Carpinteria, California, USA). The primary antibodies to Mcm7 (0.5 µg/µl) (Novacastra Laboratories Ltd, Newcastle upon Tyne, UK) and Ki-67 (1 µg/µl) (BioGenex, San Ramon, California, USA) were added and incubated at room temperature for one hour. Immunohistochemical analysis was performed on an auto-stainer (DakoCytomation), using a polymer based Envision+ detection system (DakoCytomation). Diaminobenzidine was used as the chromogen. An isotype matched IgG1 negative control was run on each section of the prostate and normal tonsil was used as the positive tissue control for each antibody.

## Assessment of immunostaining

Each slide was evaluated and scored independently by two pathologists in a blinded fashion. Counts were performed on high power (×40 objective) from the most intensely stained areas. At least 200 nuclei were counted from each area of interest, which included basal and luminal epithelial cells in benign prostatic tissue, basal and non-basal epithelial cells in areas of PIN, and the tumour cells from areas of adenocarci-

**Table 1** Clinicopathological features in 79 patients who underwent radical prostatectomy

Weight of prostate (g)*	44 (19)
% Tumour in prostate*	13.4 (12.4)
Total Gleason score	Number of cases (%)
5	10 (13%)
6	27 (34%)
7	31 (39%)
8	5 (6%)
9	6 (8%)
AJCC stage	
pT2a	10 (13%)
pT2b	34 (44%)
pT3a	30 (38%)
pT3b	4 (5%)

\*Mean (SD).

noma. The PI was computed as the percentage of cells with positive nuclear staining.

## Risk assessment

Risk assessment was calculated for each patient according to the S9921 system currently used in our institution, which is based on nomograms using PSA concentration, Gleason score, surgical resection margin, and the AJCC stage to predict the degree of anatomical extension of the tumour and the likelihood of recurrent prostate cancer after radical prostatectomy.<sup>35 36</sup>

High risk is defined as Gleason score  $\geq$  8, PSA  $>$  20 ng/ml, AJCC stage T3b, T4, or N1, and all cases with Gleason score  $\geq$  7, stage T3a, and positive surgical resection margins. Low risk is defined as Gleason score  $\leq$  6, PSA  $<$  10 ng/ml, and stage T1/T2. Medium risk is anything between high and low risk criteria.<sup>35 36</sup> Patients with a high risk have a greater than 50% chance of PSA failure within five years. Those with a low risk have less than a 15% chance of PSA failure by five years, and medium risk is in between these two.

## Statistical analysis

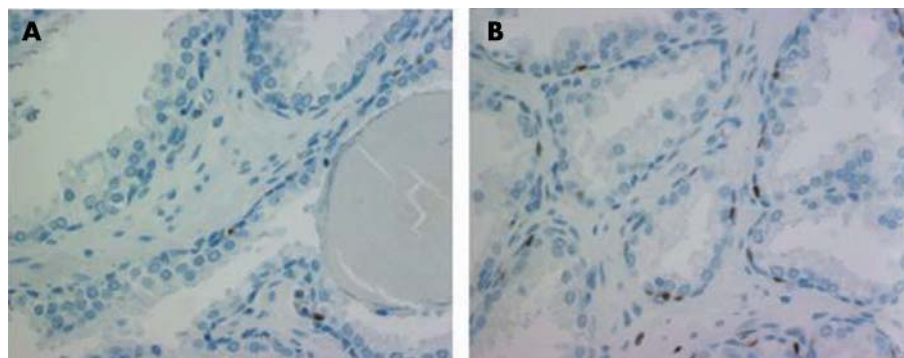
Repeated measures analysis of variance and paired *t* tests were used to compare PIs for Ki-67 and Mcm7 in all 79 cases. The relations between PI of carcinoma and the clinical variables were analysed using a bivariate analysis with Pearson correlation coefficients, two sample *t* tests, and one way analysis of variance for continuous, dichotomous, and categorical variables with more than two values, respectively.

## RESULTS

The patients' ages ranged from 45 to 72 years with a mean age of 60 years. The mean preoperative PSA concentration was 8.7 ng/ml (SD, 8.0), with a range of 0.8 to 41.1 ng/ml. Table 1 shows the distribution of composite Gleason scores and the pathological stage of the cancer specimens. The surgical resection margin was involved by adenocarcinoma in 27 cases. The capsule was involved by tumour in 48 cases with extracapsular extension of tumour in 31 cases. Sixteen cases showed extracapsular perineural invasion. Most of the cancers had a primary Gleason 3 pattern (57 of 79), followed by pattern 4 (19) and pattern 2 (three). This distribution of Gleason scores resulted in a statistical power too low for the detection of significant relations between Gleason score and PIs in our study.

## Immunostaining of the prostate

Table 2 shows the mean PIs for Ki-67 and Mcm7. Figures 1–3 show examples of immunostaining for both markers in the three tissue types. Only nuclear staining was considered positive. The Ki-67 PIs of luminal and basal cells of benign prostatic epithelium were 0.7 and 0.8 (fig 1A), whereas the



**Figure 1** Benign prostate: (A) immunohistochemical staining for Ki-67 showing low basal and luminal epithelial cell staining; (B) immunohistochemical staining for Mcm7 showing predominantly basal cell staining (original magnification,  $\times 400$ ).

corresponding Mcm7 indices were 1.2 for luminal cells and 8.2 for basal cells (fig 1B). PIN non-basal and basal cells had mean Ki-67 values of 4.9 and 0.7, respectively (fig 2A), and corresponding Mcm7 values of 10.6 and 3.1, respectively (fig 2B). Carcinomas had a mean Ki-67 PI of 9.8 (fig 3A) and a mean Mcm7 PI of 22.7 (fig 3B).

Rare stromal cells showed immunoreactivity with Mcm7. In a few cases, the central portions of lymphoid aggregates showed positive nuclear staining with Mcm7. Coarse, granular cytoplasmic positivity with Mcm7 was noted in mast cells in one case.

### Statistical analysis

The Mcm7 PI was significantly higher than the Ki-67 PI ( $p < 0.0001$ ) in all tissues except benign luminal epithelial cells ( $p = 0.23$ ). For both Ki-67 and Mcm7, the PI progressively increased from benign luminal epithelium, through PIN non-basal epithelium, to invasive cancer ( $p < 0.0001$ ; ANOVA). The mean increase in PI for carcinoma versus PIN non-basal epithelium and PIN non-basal epithelium versus benign luminal epithelium was significantly larger for Mcm7 than for Ki-67 ( $p = 0.0002$  and  $p < 0.0001$ , respectively). The drop in mean basal cell Mcm7 PI from benign foci to PIN foci was significantly larger ( $p < 0.0001$ ) than the drop in mean basal cell Ki-67 PI.

### Correlations between Ki-67 and Mcm7 PIs and clinicopathological variables

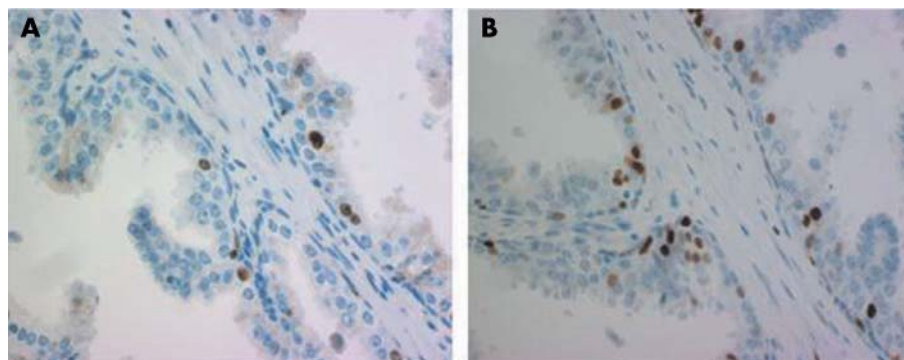
The relations between the Ki-67 and Mcm7 PIs for carcinoma, and the clinicopathological variables (age, serum PSA, weight of the prostate gland, Gleason score, per cent of tumour, surgical resection margin status, capsular invasion, extracapsular extension by tumour, perineural invasion, and the AJCC stage) were assessed using bivariate analyses. Neither Ki-67 nor Mcm7 showed a significant correlation with primary Gleason grade; however, the PI for Mcm7 showed a significant correlation with total Gleason score

( $p = 0.04$ ). The PI for Mcm7 also showed a significant association with tumour stage ( $p = 0.004$ ), and both markers showed a significant correlation with perineural invasion (Ki-67,  $p = 0.05$ ; Mcm7,  $p = 0.04$ ). The Ki-67 PI showed a significant association with surgical resection margin ( $p = 0.004$ ). Associations between Mcm7 PI and PSA concentration ( $p = 0.07$ ), prostate gland weight ( $p = 0.06$ ), and capsular involvement ( $p = 0.07$ ) were close to significance, as was the association between Ki-67 and tumour volume ( $p = 0.07$ ). All other associations were marginally significant.

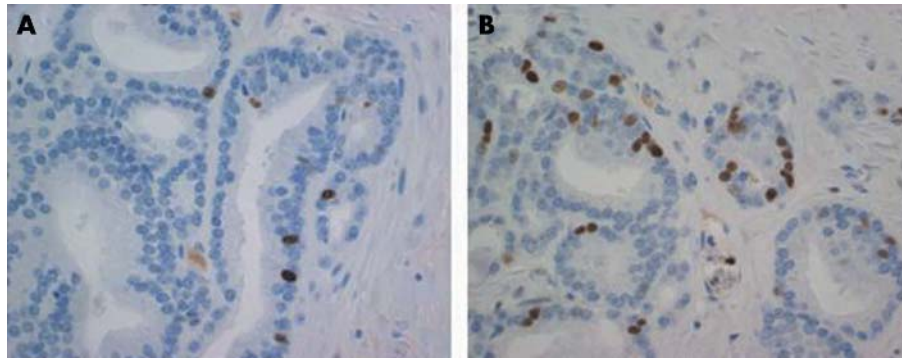
The PIs for Mcm7 and Ki-67 in carcinoma were highly correlated with each other ( $r = 0.54$ ;  $p = 0.0001$ ). Both Ki-67 ( $p = 0.01$ ) and Mcm7 ( $p = 0.007$ ) showed a significant linear increase in PI across the risk categories (low, medium, and high). However, the PI for Mcm7 was significantly higher than the PI for Ki-67 for each risk level: high,  $p = 0.002$ ; medium,  $p < 0.001$ ; and low,  $p = 0.002$  (fig 4).

### DISCUSSION

In eukaryotic cells, growth is controlled by cell cycle regulators, one of which is the minichromosome maintenance protein complex. MCM proteins are essential for DNA replication and help ensure that DNA replicates only once during each cell cycle.<sup>24 25</sup> Initiation of DNA replication begins with the association of a six subunit origin of replication complex, which determines the initiation site. This is followed by recruitment and assembly of several other initiation factors to form the pre-replication complex. The MCM family of proteins, Mcm2–7, are among those added to the pre-replication complex during late M to early G1 phase. This complex then ‘licenses’ the chromatin to replicate.<sup>24</sup> The presence of MCM proteins as part of the pre-replication complex and their dissociation from the chromatin during S phase make them especially good candidates as potential markers of replicating cells.<sup>34 37</sup> Several studies examining the expression of MCM proteins, predominately Mcm2 and



**Figure 2** Prostatic intraepithelial neoplasia: (A) immunohistochemical staining for Ki-67, showing a few non-basal cells staining; (B) immunohistochemical staining for Mcm7, showing more non-basal cells staining (original magnification,  $\times 400$ ).



**Figure 3** Adenocarcinoma of the prostate, Gleason score 3 + 4 = 7: (A) immunohistochemical staining for Ki-67; (B) immunohistochemical staining for Mcm7, showing increased epithelial cell staining compared with Ki-67 (original magnification, ×400).

Mcm5, in normal and malignant tissues have shown that these proteins have the potential to be indicators of proliferation in cell lines, in addition to histological and cytological specimens.<sup>2 26-32</sup> Furthermore, antibodies directed against Mcm5 and Cdc6, another protein that interacts with the origin of replication complex, identified abnormal precursor cells in both low grade and high grade squamous intraepithelial cells of cervical smears and biopsies with a higher sensitivity than either PCNA or Ki-67.<sup>27</sup> Similarly, the presence of Mcm5 protein expression in exfoliated transitional epithelial cells in the urine identified transitional cell carcinoma in patients with both newly diagnosed and recurrent disease with high sensitivity and specificity<sup>30 32</sup>

In our present study, a higher proportion of cells were positive for Mcm7 than for Ki-67 in each of the cell categories studied except benign luminal cells. Furthermore, the Mcm7 PI showed a larger difference between benign, PIN, and carcinoma than did the Ki-67 PI: the Mcm7 PI in tumour tissue (22.7%) was significantly higher than that of Ki-67 (9.8%). Other studies examining MCM expression in prostate cancer have shown a range of median staining from 10%<sup>31</sup> and 39% for Mcm2 and 38% for Mcm5.<sup>2</sup> Neither study showed data comparing their results with Ki-67.

“In our present study, a higher proportion of cells were positive for Mcm7 than for Ki-67 in each of the cell categories studied except benign luminal cells”

The Mcm7 PI was also significantly higher than the Ki-67 PI in PIN (10.6% v 4.9%), whereas there was no significant difference between these two markers in benign prostate luminal cells (1.2% and 0.7%, respectively). Similarly, the mean increases in PI for carcinoma versus PIN non-basal epithelium and for PIN non-basal epithelium versus benign luminal epithelium were significantly greater for Mcm7 than for Ki-67 (p = 0.002 and p < 0.0001, respectively).

Our finding that the Mcm7 PI was consistently higher than the Ki-67 PI in the same carcinoma and PIN lesions is similar

to results of Stoeber *et al* in lobular cells of premenopausal breast.<sup>34</sup> These authors suggested that Mcm7 may be present in cells licensed to proliferate in addition to those that are proliferating, whereas Ki-67 is expressed only in cells that are proliferating. Because the function of Ki-67 is still unknown, this is an interesting area that requires further study.

The intermediate PI seen in PIN, when compared with nodular hyperplasia and cancer using antibodies to PCNA and Ki-67, is thought to be evidence for the role of PIN as a premalignant lesion.<sup>38-40</sup> In our present study, the Ki-67 PI values in the three conditions (table 2) were similar to those described in the literature.<sup>41</sup> The PIs of both Ki-67 and Mcm7 in the PIN non-basal epithelium were between the benign and malignant PIs, in agreement with these earlier studies.

In benign prostatic epithelium the proliferative potential is restricted to the basal epithelium, as shown in previous studies with PCNA,<sup>42-44</sup> and other MCM proteins.<sup>2 31 26</sup> Not explained is the difference in basal cell PI of 8.2 using Mcm7 in our study compared with the PI of < 2 using Mcm2 in the study by Meng *et al*.<sup>31</sup> The reason for this difference in median PI values for the different MCM proteins in both normal and tumour tissue remains unclear, but it supports a direct comparison of the expression of these different MCM proteins on the same prostate tissue specimens.

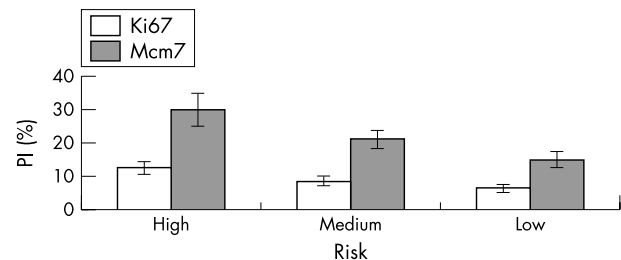
We noted a significant decrease (p < 0.0001) in the Mcm7 PI between the basal cells in benign prostatic tissue and those in PIN. This was not noted with Ki-67, but supports the findings of McNeal *et al*,<sup>43</sup> who reported a similar drop in basal cell PI from benign to PIN using PCNA.

Cell proliferation is a useful predictive factor and surrogate biomarker for malignant disease.<sup>38</sup> Categorisation of patients into risk assessment groups allows the patient and physician to have a more informed discussion regarding prognosis and treatment decisions. Although both Ki-67 and Mcm7 showed a significant linear increase in PI across risk categories (low to medium to high), at each risk level the mean PI for Mcm7

**Table 2** Mean proliferation index for Ki-67 and Mcm7 in the prostate (n=79)

Tissue	Proliferation index (%)	
	Ki-67*	Mcm7*
Benign luminal	0.7 (0.8)	1.2 (1.9)
Benign basal	0.8 (0.9)	8.2 (8.4)
PIN non-basal	4.9 (6.8)	10.6 (15.0)
PIN basal	0.7 (1.0)	3.1 (6.9)
Cancer	9.8 (9.5)	22.7 (18.5)

Values are mean (SD).  
PIN, prostatic intraepithelial neoplasia.



**Figure 4** Comparison between proliferation indices (PIs) for Ki-67 and Mcm7 at different relative risk levels, showing a significant linear increase in PI across the risk categories (low to medium to high). The Mcm7 PI is significantly higher than the Ki-67 PI at each risk level.

### Take home messages

- Using immunohistochemistry, the minichromosome maintenance protein Mcm7 correlated highly with Ki-67, an established marker for cell proliferation, and showed an improved ability to distinguish between benign prostatic epithelium, prostatic intraepithelial neoplasia (PIN), and adenocarcinoma
- Staining for Mcm7 increased with the progression of PIN to invasive cancer, supporting the notion that PIN is a premalignant lesion
- Further multivariate studies with longterm follow up and a larger cohort of patients with prostate cancer are needed to determine whether Mcm7 could be used as an independent prognostic marker of aggressive disease

was significantly larger than that for Ki-67. This provides an improved correlation between PI and risk category using Mcm7. Correlation with traditional clinical and pathological findings showed a significant correlation between Mcm7 PI and total Gleason score, perineural invasion and stage, and between Ki-67 PI and surgical resection margin and perineural invasion. In the study by Meng *et al.*,<sup>31</sup> patients with prostate cancer who had high Mcm2 expression had shorter disease free survival. However, they too noted a similar lack of correlation between Mcm2 PI and traditional prognostic factors, such as preoperative PSA, Gleason score, surgical margin status, and AJCC tumour stage. Clearly, additional studies are required to determine whether patients can be stratified into groups at risk for aggressive disease, metastatic potential, and response to treatment based on MCM protein staining.

In conclusion, Mcm7 correlated highly with Ki-67, an established marker for cell proliferation and demonstrated an improved ability to distinguish between benign prostatic epithelium, PIN, and adenocarcinoma. Trends in the staining patterns with Mcm7 support the progression of PIN to invasive cancer. Further multivariate studies with longterm follow up and a larger cohort of patients with prostate cancer will need to be performed to determine whether Mcm7 can be considered a suitable independent prognostic marker of aggressive disease.

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