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Immunohistochemical expression of minichromosome maintenance complex protein 2 predicts biochemical recurrence in prostate cancer: a tissue microarray and digital imaging analysis–based study of 428 cases^{☆,☆☆}

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Summary

Prostate cancer remains a major health problem in the United States. Established clinicopathologic parameters such as Gleason score, T stage, and prostate-specific antigen levels are currently the guiding tools for prognostication and disease management. The addition of biomarkers could increase the accuracy of these parameters for predicting disease progression, response to therapy, and survival. In this regard, the goal of this study was to evaluate minichromosome maintenance complex protein 2 and Ki-67 immunohistochemical expression as predictors of outcome in prostate cancer. For this purpose, 11 tissue microarrays were constructed using tumor and nontumor samples from 428 patients. Patients were divided into short-term (mean, 2.9 years) and long-term (mean, 14.1 years) follow-up groups. End points were biochemical recurrence for the short-term follow-up group and prostate cancer–related death for the long-term follow-up group. All men in the long-term follow-up group had biochemical recurrence at the time of recruitment. Expression of both markers was higher in tumor than in nontumor glands. Percentage of minichromosome maintenance complex protein 2 was associated with Gleason score in both groups. Percentage of Ki-67 was associated with Gleason score and pathologic stage only in the short-term follow-up group. Higher minichromosome maintenance complex protein 2 percentages were associated with biochemical recurrence in the short-term follow-up group. In the long-term

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follow-up group, neither minichromosome maintenance complex protein 2 nor Ki-67 levels predicted prostate cancer death. In conclusion, our results suggest that in patients treated by radical prostatectomy for clinically localized prostate cancer, immunohistochemistry for minichromosome maintenance complex protein 2 expression could be used to predict biochemical recurrence, independent of other known clinicopathologic factors.

Keywords

Prostate cancer; Biochemical recurrence; Cancer-specific death; MCM2; Ki-67

1. Introduction

Prostate cancer remains a major health problem in the United States. Currently, at the time of diagnosis, most cases present as localized disease [1] and are treated by radical prostatectomy, radiation therapy, or active surveillance. Established clinicopathologic parameters including clinical and pathologic stages, Gleason score, number of positive cores, and serum prostate-specific antigen (PSA) levels are the guiding tools for prognostication and disease management [2,3]. In addition, several biomarkers have been evaluated for their potential role in predicting disease progression, response to therapy, and survival. This wide array includes proliferation markers (eg, Ki-67), tumor suppression genes (eg, *p53*, *p27*, *NKX3.1*, *PTEN*, *Rb*), oncogenes (eg, *c-myc*, *EZH2*, and *HER2/neu*), members of the PI3K/AKT/mTOR pathway, and apoptosis regulators (eg, survivin and transforming growth factor β 1), among others [4,5].

Disturbances in cell proliferation and DNA replication are fundamental aspects of hyperplastic and neoplastic prostatic diseases. Recent studies suggest that measurement of tumor proliferation may provide relevant diagnostic and prognostic information [6-10]. In this regard, minichromosome maintenance complex protein 2 (MCM2), a protein involved in the initiation and regulation of eukaryotic DNA replication, has emerged as a promising prognosticator [8,11-13]. MCM2 is essential for DNA replication, representing the convergence point of several pathways related to cell proliferation. Thus, from the therapeutic view, *MCM* genes are ideal candidates for targeted therapy. In support of this hypothesis, recent studies using cancer cell lines have shown that it is possible to block *MCM* activity using small interfering RNAs [13,14]. Various investigators have also evaluated Ki-67, a well-known marker of cellular proliferation, as a prognostic biomarker in prostate cancer [4,5]. Although a number of studies have suggested that the proliferation index, as measured by Ki-67 staining, can add additional prognostic information beyond standard clinicopathologic variables, results for this marker are not entirely consistent [15-19]. In the present study, we measured MCM2 and Ki-67 immunohistochemical expression in 2 large series of patients with prostate cancer. Then, we evaluated whether MCM2 and Ki-67 immunohistochemical expression in cancer cells predicts biochemical recurrence and prostate cancer death.

2. Materials and methods

The current study was approved by the institutional review board at the Johns Hopkins School of Medicine.

2.1. Patients, tissue microarray construction, and outcome evaluation

A set of 11 tissue microarrays were built from tissue samples of 428 patients with prostate adenocarcinoma. Patients were uniformly treated by radical prostatectomy without adjuvant therapy at the Johns Hopkins Medical Institutions (Baltimore, MD). These cases

corresponded to 2 groups of patients, with either short-term or long-term follow-up. The first group included 199 patients treated between January 2000 and August 2001 who were observed for biochemical recurrence over a mean (SD) of 2.9 (1.8) years. The second group included 229 patients treated between January 1982 and December 1996 who subsequently experienced biochemical recurrence and were observed for prostate cancer death over a mean (SD) of 14.1 (5.1) years. Each patient was characterized by clinicopathologic features including pathologic stage, primary Gleason pattern, Gleason score, and preoperative PSA. *Biochemical recurrence* was defined as postoperative PSA greater than 0.2 ng/mL. *Prostate cancer death* was defined as death caused by disseminated prostatic carcinoma.

For every patient, 4 spots from each tumor and nontumor tissue were selected and placed in the tissue microarrays, using a previously described procedure [20]. For tumor spots, areas showing the largest tumor and/or highest Gleason score were selected.

2.2. Immunohistochemical staining

Two consecutive 5- μ m sections of each tissue microarray were stained for MCM2 and Ki-67 expression, as follows: (1) deparaffinization and rehydration of slides; (2) antigen retrieval: 20-minute steam in Antigen Unmasking solution (Vector Laboratories, Inc, Burlingame, CA) for MCM2 and 40-minute steam in Target Retrieval Solution (DAKO, Carpinteria, CA) for Ki-67; (3) Dual Endogenous Enzyme block (DAKO), 5 minutes; (4) protein detection: anti-MCM2 (BD Pharmingen, San Jose, CA), dilution 1:4000, 45-minute incubation at room temperature for MCM2 expression, and anti-Ki-67 (Zymed, South San Francisco, CA), dilution 1:400, overnight incubation at 4°C, for Ki-67 expression; (5) EnVision Plus Poly-HRP Immunohistochemical Detection System (DAKO), 30 minutes; (6) 3,3-diaminobenzidine (Sigma, St Louis, MO), 20 minutes; and (7) hematoxylin (DAKO), 1 minute.

2.3. Tissue microarray managing platform, image acquisition, and visual analysis

TMAJ, an open-source software (available at <http://tmaj.pathology.jhmi.edu/>), was used for managing the patients' information, tissue microarray construction data, and online image assessment. Stained tissue microarray sections were scanned using the Bacus Laboratories Image Scanning System (Bacus Laboratories, Inc, Lombard, IL). A total of 13 020 spots were exported as a composite image and uploaded to TMAJ. For tissue diagnosis and assessment of staining quality, Ki-67- and MCM2-stained spots were compared side by side with the corresponding hematoxylin and eosin-stained spot. Prostate cancer was graded using the Gleason system, according to the updated criteria published in 2005 by the International Society for Urological Pathology [21].

We excluded from the analyses spots showing significant folding, staining artifacts, or blurriness, as well as spots showing only prostatic intraepithelial neoplasia or gland atrophy. In addition, spots with prostate cancer showing more than 10% of benign glands were also excluded. The final data analysis included 2261 Ki-67-stained spots (1443 of nontumor glands and 818 of prostate carcinoma) and 1907 MCM2-stained spots (1219 of nontumor glands and 688 of prostate carcinoma).

Immunohistochemical expression of MCM2 and Ki-67 was evaluated using the image analysis system included in the Bacus Laboratories Image Scanning System platform. Briefly, the software was trained to separately recognize brown pixels (immunoreactivity) and blue pixels (counterstain). Threshold variables were adjusted until masked brown pixels visually correlated with immunopositive nuclei, and masked blue pixels correlated with areas of pure counterstain (Fig. 1). The percentages of areas staining positively were then estimated from the total area of brown and blue staining on each tissue microarray spot. This

output variable was named “immunostain percentage.” In addition, the optical density (color saturation) of the brown stain was also recorded under the denomination of “immunostain intensity.” Finally, to provide a weighted value for the expression levels of MCM2 and Ki-67, percentages and intensities for each spot were multiplied to create a “product score.” In the next section, “MCM2 and Ki-67 levels” refers to these 3 values (percentage, intensity, and product score).

2.4. Statistical analysis

Linear regression with robust variance estimation was used to estimate the association of MCM2 and Ki-67 levels with clinicopathologic features. Analyses were carried out to take into account the multiple tissue microarray spots per patient. To determine the association of MCM2 and Ki-67 with biochemical recurrence or prostate cancer death, we used survival analysis. First, we divided the distribution of MCM2 and Ki-67 immunohistochemical expression into tertiles (high) and then combined the lowest and middle tertiles to form the reference (low) group. Next, we generated survival curves for the groups with high and low immunohistochemical expression using the Kaplan-Meier method and compared them using the log-rank test. Finally, we estimated the association (hazard ratio) of MCM2 and Ki-67 immunohistochemical expression individually or in combination with biochemical recurrence or prostate cancer death. For this purpose, we used Cox proportional hazard models adjusted for pathologic stage, Gleason score, preoperative PSA, and surgical margins. A 2-tailed $P < .05$ was required for statistical significance. Data were analyzed using SAS version 9.1 (SAS Institute Inc, Cary, NC).

3. Results

3.1. Clinicopathologic features

The patients' mean (SD) age was 59.2 (6.7) years, with a median of 60 years (range, 37-75 years). Preoperative PSA ranged from 0.1 to 57.7 ng/mL, with a mean (SD) of 9.6 (8.2) ng/mL and a median of 7.5 ng/mL. Eighty-nine percent of the patients were white. Distribution by pathologic stage and Gleason score is shown in Table 1.

3.2. MCM2 and Ki-67 immunohistochemical expression

Staining for both MCM2 and Ki-67 was localized in the nuclei of prostate epithelial cells, both in nontumor and tumor glands. Staining was generally confined to the basal layer of the nontumor epithelium and evident in the epithelial cells of tumor glands. No differences were noted between the staining patterns of MCM2 and Ki-67 (Fig. 2).

3.3. MCM2 staining and clinicopathologic features

In the short-term follow-up group, the percentage of cells with MCM2 immunohistochemical expression was higher in tumor than in nontumor spots (Table 2). The percentage of cells staining positive for MCM2 increased with increasing Gleason primary pattern, Gleason score, and pathologic T stage, but not with preoperative PSA. Intensity of MCM2 staining was similar in tumor and nontumor spots, and no significant differences were noted by clinicopathologic features. The immunohistochemical expression score had the same patterns of association as the percentage of cells staining positive.

In the long-term follow-up group, the percent of cells staining positive for MCM2 was higher in tumor than in nontumor spots (Table 3). The percent of cells staining positive increased with increasing primary Gleason pattern and Gleason score, but not with preoperative PSA. The mean MCM2 staining intensity was similar between nontumor and tumor spots. Nevertheless, MCM2 staining intensity appeared to increase with preoperative PSA and decrease with increasing Gleason scores. MCM2 immunohistochemical expression

score was higher in tumor spots than in nontumor spots, and the score increased with increasing pathologic stage, especially with local metastasis, primary Gleason pattern, and Gleason score.

3.4. Ki-67 staining and clinicopathologic features

In the short-term follow-up group (Table 4), the mean percentage of cells staining positive for Ki-67, staining intensity, and immunohistochemical expression score were significantly higher in tumor spots when compared with nontumor spots. They also increased significantly with increasing pathologic stage, primary Gleason pattern, and Gleason score, but not preoperative PSA. In the long-term follow-up group (Table 5), the mean percentage of cells staining positive for Ki-67, staining intensity, and expression score were higher in tumor than in nontumor spots. However, Ki-67 expression did not change with increasing pathologic stage, primary Gleason pattern, Gleason score, or preoperative PSA.

3.5. MCM2 and Ki-67 as predictors of outcome

In the short-term follow-up group, 9 patients experienced biochemical recurrence. A higher percentage of cells staining positive for MCM2 (top tertile versus bottom and middle tertiles) was associated with a greater risk for biochemical recurrence (Fig. 3), whereas a suggestive positive association was observed for Ki-67. Percentage of Ki-67 did not appear to add predictive information to MCM2 percentage. Higher MCM2 and Ki-67 staining intensities were not associated with biochemical recurrence (Fig. 4). Finally, patients with higher MCM2 and, possibly, Ki-67 immunohistochemical expression scores had a higher risk for biochemical recurrence (Fig. 5).

In the long-term follow-up group, 41 patients died of prostate cancer. Similar risks were observed for high MCM2 and Ki-67 levels, either separately or in combination (Figs. 6-8). Table 6 shows the hazard ratios of cancer death in the long-term follow-up group by expression patterns of MCM2 and Ki-67. The risks of death were not significantly different between patients with low and those with high MCM2 and Ki-67 levels, for any of the evaluated parameters.

4. Discussion

This study evaluates the prognostic significance of MCM2 and Ki-67 immunohistochemical expression in prostate carcinomas for predicting biochemical recurrence and prostate cancer death. For this purpose, we used digital image analysis to estimate the percentage and intensity of stained areas in tissue microarray spots. Immunohistochemical expression of both markers was significantly higher in tumor glands when compared with nontumor epithelial cells, in agreement with previous studies [8,11,22,23]. Percentages of MCM2- and Ki-67-positive cells were associated with Gleason score, an expected finding, considering the association between proliferation rate and tumor differentiation. Despite the small number of men who recurred, higher percentages of MCM2 positive cells were associated with increased risks of biochemical recurrence. However, in men who already had biochemical recurrence, MCM2 and Ki-67 levels were not associated with prostate cancer death.

MCM2 is part of a multiprotein complex involved in DNA replication licensing. Its expression is maintained in all eukaryotic cells with proliferative capacity. During G1, MCM2 couples with other members of the same family (MCM1 and MCM3-7) to form the pre-Replication Complex. This pre-Replication Complex also includes the Origin Recognition Complex and the regulatory proteins Cdc6 and Cdt1. Once this complex is formed, DNA is licensed and replication begins. In this sense, MCM proteins are essential regulators of cell proliferation, acting as relay stations by integrating upstream growth

regulatory pathways with DNA replication. MCM2 proteins also ensure that this replication occurs only once during each cell cycle. MCM2 expression in prostate cancer has been evaluated previously, but results are inconsistent. Meng et al [11] reported that MCM2 levels were able to predict biochemical recurrence in a group of 92 patients treated by radical prostatectomy. However, they did not detect significant associations with traditional clinicopathologic predictors such as preoperative PSA, pT stage, Gleason score, regional lymph node involvement, or surgical margins. In contrast, Dudderidge et al [12] found that, in a series of 58 patients with prostate cancer, MCM2 levels were associated with Gleason score, presence of bone metastases, and overall survival. Majid et al [13] also identified higher levels of MCM2 in metastatic tumors when compared with primary prostate carcinomas.

In all the aforementioned studies, MCM2 expression was evaluated using “eyeball estimation.” Our study expands these previous results with a larger group of patients and using digital image analysis for the estimation of MCM2 levels. We also found a significant association between MCM2 percentages and Gleason score, suggesting a role for MCM2 in tumor progression and expected behavior. Higher percentages of MCM2-positive cells were also associated with a higher risk for biochemical recurrence. Because in our series, only 9 patients had biochemical recurrence during follow-up, our results need to be verified on a larger, prospective cohort. On the other hand, MCM2 percentages were not associated with cancer-specific mortality. This lack of association between MCM2 and prostate cancer death mortality could be real because investigators have argued that biochemical recurrence is not necessarily a surrogate for prostate cancer death [24]. However, the long-term follow-up group included only men who had experienced biochemical recurrence, limiting our conclusions to that specific population of patients. In that sense, once biochemical recurrence ensues, MCM2 levels detected in the primary tumor might not have a prognostic significance. Another explanation for this lack of association with outcome could be the differences between the percentages of positively stained cells. Indeed, the percentages of MCM2 (and Ki-67) stained cells were lower in the long-term follow-up group when compared with the short-term follow-up group. The tissue blocks used for building the long-term follow-up tissue microarrays were substantially older on average than those in the short-term-follow-up group. Thus, differences in block age or tissue fixation/processing may account for the lack of association with outcome in the long-term follow-up cohort. Finally, besides MCM2, other members of the MCM family were also evaluated as prognostic factors in prostate cancer. Recent studies have found that MCM7 levels were associated with Gleason score, pT stage, recurrence-free survival, and tumor progression [6,25,26].

Ki-67 is a basic, nonhistonic, DNA-binding protein encoded by the gene *MKI67* located in chromosome 10q25. In cells with proliferative capacity, Ki-67 is expressed throughout the cell cycle, reaching a peak during the M phase, but its exact function remains unclear. Ki-67 has been extensively studied as a prognostic factor in prostate cancer, yielding contradictory results, mainly due to unresolved interpretation/cutoff issues. The prognostic role of Ki-67 has been recently revisited in different clinical contexts (clinically insignificant, localized, and advanced prostate cancer treated by either salvage radiation or androgen deprivation therapies [6,9,17,27]) and specimen types (biopsies, radical prostatectomies, and transurethral resections [6,10,27-30]). Some investigators found a significant, independent association of Ki-67 immunohistochemical expression with well-established prognostic features (eg, Gleason score, PSA, and tumor stage), whereas others contradicted those findings [6,10,12,16,18,23,27-29,31]. In our series, we observed a significant association between Ki-67 levels and pathologic stage, primary Gleason pattern, and Gleason score, although these findings were limited to the short-term follow-up group. The lack of association between these parameters and Ki-67 in the long-term followup group seems intriguing and counterintuitive. A plausible explanation could be that, as mentioned before,

this group of patients included only men in whom biochemical recurrence was held constant. Thus, we only evaluated the association between Ki-67 levels and Gleason score that was independent of biochemical recurrence. A similar explanation could be given for our findings concerning Ki-67 levels and pathologic stage.

Regarding outcome, some research groups have found that Ki-67 levels were significant predictors of biochemical recurrence and survival [6,9,12,17-19,29-32]; whereas in other studies, such an association was not found [10,15,16]. In our cohort, only when Ki-67 was combined with MCM2 was a significant association with biochemical recurrence observed. A similar situation was reported by Malhotra et al [10], who found that only when Ki-67 was combined with other markers of cell proliferation (TOP2A and E2F1) was it useful for predicting outcome. Finally, as indicated previously, it is possible that the lack of the association of Ki-67 with death could be related to differences in tissue fixation/processing and block age.

Our results suggest that, when considered separately, MCM2 is a better prognosticator of biochemical recurrence than Ki-67. Perhaps, the potential for biological aggressiveness is related not only to the proportion of proliferating cells but also to the population of nonproliferating licensed cells. By licensing more cells into the proliferation cycle, MCM2 could initiate improper DNA synthesis, increasing the risk for mutations that might facilitate tumor progression. Two studies evaluating both MCM2 and Ki-67 in prostate cancer have been published previously. Using surgical specimens removed either for prostate cancer or prostatic hyperplasia, Ananthanarayanan et al [8] detected that, in malignant areas, levels of MCM2 were higher than Ki-67. They also found that MCM2 levels were lower in normal glands from prostates without cancer when compared with those from prostates with cancer. This suggests that increased MCM2 expression could be used as a marker for benign glands with field cancerization effect. Dudderidge et al [33] also found higher levels of MCM2 than Ki-67 in cancer glands. Interestingly, in that study, the fraction of nonproliferating licensed cells was associated with increasing Gleason scores and decreased disease-free survival time. However, only Ki-67 remained as an independent predictor of outcome in their series, trumping the prognostic value of MCM2 levels. Clearly, additional studies are required to define the clinical use that these immunostains might have for predicting tumor recurrence and disease-free survival.

One limitation of our study could be the use of percentage/intensity of stained areas instead of the more traditional approach of percentage/intensity of positive cells. However, by digitally adjusting the thresholds of brown and blue pixels, we have diminished the background noise and focused solely on stained areas. In addition, the digital estimation of the stained areas provided a less biased and more accurate evaluation of immunohistochemistry positivity.

In summary, using digital image-based analysis, we evaluated the immunohistochemical expression of MCM2 and Ki-67 in 2 large groups of patients with prostate cancer. We found that the expression of both markers was higher in tumor than in nontumor glands. For MCM2, we observed an association with primary Gleason pattern and Gleason score, both in the short-term and in the long-term follow-up groups. Ki-67 levels were associated with primary Gleason pattern, Gleason score, and pathologic stage only in the short-term follow-up group. Higher MCM2 levels were associated with biochemical recurrence. Once biochemical recurrence has occurred, neither MCM2 nor Ki-67 predicts tumor-specific mortality. Our results suggest that, in patients treated by radical prostatectomy for clinically localized prostate cancer, immunohistochemistry for MCM2 expression could be used to predict biochemical recurrence, independent of other known clinicopathologic factors.

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References

1. Siegel R, Ward E, Brawley O, Jemal A. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin.* 2011; 61:212–36. [PubMed: 21685461]
2. Makarov DV, Trock BJ, Humphreys EB, et al. Updated nomogram to predict pathologic stage of prostate cancer given prostate-specific antigen level, clinical stage, and biopsy Gleason score (Partin tables) based on cases from 2000 to 2005. *Urology.* 2007; 69:1095–101. [PubMed: 17572194]
3. Stephenson AJ, Scardino PT, Eastham JA, et al. Preoperative nomogram predicting the 10-year probability of prostate cancer recurrence after radical prostatectomy. *J Natl Cancer Inst.* 2006; 98:715–7. [PubMed: 16705126]
4. Netto GJ. Molecular diagnostics in urologic malignancies: a work in progress. *Arch Pathol Lab Med.* 2011; 135:610–21. [PubMed: 21526959]
5. Netto GJ, Epstein JI. Theranostic and prognostic biomarkers: genomic applications in urological malignancies. *Pathology.* 2010; 42:384–94. [PubMed: 20438413]
6. Laitinen S, Martikainen PM, Tolonen T, Isola J, Tammela TL, Visakorpi T. EZH2, Ki-67 and MCM7 are prognostic markers in prostatectomy treated patients. *Int J Cancer.* 2008; 122:595–602. [PubMed: 17943722]
7. Jhavar S, Bartlett J, Kovacs G, et al. Biopsy tissue microarray study of Ki-67 expression in untreated, localized prostate cancer managed by active surveillance. *Prostate Cancer Prostatic Dis.* 2009; 12:143–7. [PubMed: 18762814]
8. Ananthanarayanan V, Deaton RJ, Yang XJ, Pins MR, Gann PH. Alteration of proliferation and apoptotic markers in normal and premalignant tissue associated with prostate cancer. *BMC Cancer.* 2006; 6:73. [PubMed: 16545117]
9. Karnes RJ, Cheville JC, Ida CM, et al. The ability of biomarkers to predict systemic progression in men with high-risk prostate cancer treated surgically is dependent on ERG status. *Cancer Res.* 2010; 70:8994–9002. [PubMed: 21062978]
10. Malhotra S, Lapointe J, Salari K, et al. A tri-marker proliferation index predicts biochemical recurrence after surgery for prostate cancer. *PLoS One.* 2011; 6:e20293. [PubMed: 21629784]
11. Meng MV, Grossfeld GD, Williams GH, et al. Minichromosome maintenance protein 2 expression in prostate: characterization and association with outcome after therapy for cancer. *Clin Cancer Res.* 2001; 7:2712–8. [PubMed: 11555583]
12. Dudderidge TJ, McCracken SR, Loddo M, et al. Mitogenic growth signalling, DNA replication licensing, and survival are linked in prostate cancer. *Br J Cancer.* 2007; 96:1384–93. [PubMed: 17406359]
13. Majid S, Dar AA, Saini S, et al. Regulation of minichromosome maintenance gene family by microRNA-1296 and genistein in prostate cancer. *Cancer Res.* 2010; 70:2809–18. [PubMed: 20332239]
14. Shi YK, Yu YP, Tseng GC, Luo JH. Inhibition of prostate cancer growth and metastasis using small interference RNA specific for minichromosome complex maintenance component 7. *Cancer Gene Ther.* 2010; 17:694–9. [PubMed: 20539323]
15. Nariculam J, Freeman A, Bott S, et al. Utility of tissue microarrays for profiling prognostic biomarkers in clinically localized prostate cancer: the expression of BCL-2, E-cadherin, Ki-67 and p53 as predictors of biochemical failure after radical prostatectomy with nested control for clinical and pathological risk factors. *Asian J Androl.* 2009; 11:109–18. [PubMed: 19050681]
16. Aaltomaa S, Karja V, Lipponen P, et al. Expression of Ki-67, cyclin D1 and apoptosis markers correlated with survival in prostate cancer patients treated by radical prostatectomy. *Anticancer Res.* 2006; 26:4873–8. [PubMed: 17214354]

17. Khor LY, Bae K, Paulus R, et al. MDM2 and Ki-67 predict for distant metastasis and mortality in men treated with radiotherapy and androgen deprivation for prostate cancer: RTOG 92-02. *J Clin Oncol.* 2009; 27:3177–84. [PubMed: 19470936]
18. Khatami A, Hugosson J, Wang W, Damber JE. Ki-67 in screen-detected, low-grade, low-stage prostate cancer, relation to prostate-specific antigen doubling time, Gleason score and prostate-specific antigen relapse after radical prostatectomy. *Scand J Urol Nephrol.* 2009; 43:12–8. [PubMed: 18949633]
19. Gunia S, Albrecht K, Koch S, et al. Ki67 staining index and neuroendocrine differentiation aggravate adverse prognostic parameters in prostate cancer and are characterized by negligible interobserver variability. *World J Urol.* 2008; 26:243–50. [PubMed: 18392627]
20. Fedor HL, De Marzo AM. Practical methods for tissue microarray construction. *Methods Mol Med.* 2005; 103:89–101. [PubMed: 15542899]
21. Epstein JI, Allsbrook WC Jr, Amin MB, Egevad LL. The 2005 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. *Am J Surg Pathol.* 2005; 29:1228–42. [PubMed: 16096414]
22. Zhong W, Peng J, He H, et al. Ki-67 and PCNA expression in prostate cancer and benign prostatic hyperplasia. *Clin Invest Med.* 2008; 31:E8–15. [PubMed: 18312749]
23. Mitra AV, Jameson C, Barbachano Y, et al. Elevated expression of Ki-67 identifies aggressive prostate cancers but does not distinguish BRCA1 or BRCA2 mutation carriers. *Oncol Rep.* 2010; 23:299–305. [PubMed: 20043088]
24. Wright JL, Dalkin BL, True LD, et al. Positive surgical margins at radical prostatectomy predict prostate cancer specific mortality. *J Urol.* 2010; 183:2213–8. [PubMed: 20399459]
25. Padmanabhan V, Callas P, Philips G, Trainer TD, Beatty BG. DNA replication regulation protein Mcm7 as a marker of proliferation in prostate cancer. *J Clin Pathol.* 2004; 57:1057–62. [PubMed: 15452160]
26. Ren B, Yu G, Tseng GC, et al. MCM7 amplification and overexpression are associated with prostate cancer progression. *Oncogene.* 2006; 25:1090–8. [PubMed: 16247466]
27. Nagao K, Yamamoto Y, Hara T, et al. Ki67 and BUBR1 may discriminate clinically insignificant prostate cancer in the PSA range 4–10 ng/ml. *Jpn J Clin Oncol.* 2011; 41:555–64. [PubMed: 21233104]
28. Tolonen TT, Tammela TL, Kujala PM, Tuominen VJ, Isola JJ, Visakorpi T. Histopathological variables and biomarkers enhance the prognostic value of Ki-67 and minichromosome maintenance protein 7 as prognosticators in primarily endocrine-treated prostate cancer. *BJU Int.* 2011; 108:1430–8. [PubMed: 21592298]
29. Zellweger T, Gunther S, Zlobec I, et al. Tumour growth fraction measured by immunohistochemical staining of Ki67 is an independent prognostic factor in preoperative prostate biopsies with small-volume or low-grade prostate cancer. *Int J Cancer.* 2009; 124:2116–23. [PubMed: 19117060]
30. Berney DM, Gopalan A, Kudahetti S, et al. Ki-67 and outcome in clinically localised prostate cancer: analysis of conservatively treated prostate cancer patients from the Trans-Atlantic Prostate Group study. *Br J Cancer.* 2009; 100:888–93. [PubMed: 19293807]
31. Miyake H, Muramaki M, Kurahashi T, Takenaka A, Fujisawa M. Expression of potential molecular markers in prostate cancer: correlation with clinicopathological outcomes in patients undergoing radical prostatectomy. *Urol Oncol.* 2010; 28:145–51. [PubMed: 18848789]
32. Parker AS, Heckman MG, Wu KJ, et al. Evaluation of Ki-67 staining levels as an independent biomarker of biochemical recurrence after salvage radiation therapy for prostate cancer. *Int J Radiat Oncol Biol Phys.* 2009; 75:1364–70. [PubMed: 19464826]
33. Dudderidge TJ, Stoeber K, Loddo M, et al. Mcm2, Geminin, and KI67 define proliferative state and are prognostic markers in renal cell carcinoma. *Clin Cancer Res.* 2005; 11:2510–7. [PubMed: 15814627]

Fig. 1.

Image analysis of a tissue microarray spot. Spots were scanned and linked to the TMAJ platform for online review. For image analysis, the background threshold was adjusted until only the positive nuclei were visible. The progression from an unedited image (top left) to the one with only positive nuclei (bottom right) is shown. The percentage of the positive (brown) over the total (dark blue) area was estimated, as well as the intensity (saturation) of the brown staining. This process was repeated for every spot and for each one of the stains (MCM2 and Ki-67).

Fig. 2. Patterns of MCM2 and Ki-67 immunohistochemical expression. A and B, MCM2 immunohistochemical staining was limited to basal cells in nontumor prostatic glands. C and D, MCM2 staining is observed in cancer glands. E and F, Ki-67 immunohistochemical staining was limited to basal cells in nontumor prostatic glands. G and H, Ki-67 staining is observed in cancer glands. Left column: magnification $\times 4$. Right column: magnification $\times 20$.

Fig. 3. MCM2 and Ki-67 percentages as predictors of biochemical recurrence in the short-term follow-up group. Higher MCM2 percentages, either alone or in combination with Ki-67, were significantly associated with biochemical recurrence. The x-axis corresponds to follow-up in months. The y-axis corresponds to PSA recurrence-free survival.

Fig. 4. MCM2 and Ki-67 intensities as predictors of biochemical recurrence in the short-term follow-up group. Neither higher MCM2 nor Ki-67 intensities, separately or combined, were associated with biochemical recurrence. The x -axis corresponds to follow-up in months. The y -axis corresponds to PSA recurrence-free survival.

Fig. 5. MCM2 and Ki-67 product scores as predictors of biochemical recurrence in the short-term follow-up group. High MCM2 product scores, either alone or in combination with Ki-67, were significantly associated with biochemical recurrence. Patients with higher Ki-67 product scores showed a borderline significant tendency toward biochemical recurrence. The x-axis corresponds to follow-up in months. The y-axis corresponds to PSA recurrence-free survival.

Fig. 6. MCM2 and Ki-67 percentages as predictors of cancer-specific mortality in the long-term follow-up group. Neither higher MCM2 nor Ki-67 percentages, separately or combined, were associated with prostate cancer death. The *x*-axis corresponds to follow-up in months. The *y*-axis corresponds to cancer-specific survival.

Fig. 7. MCM2 and Ki-67 intensities as predictors of cancer-specific mortality in the long-term follow-up group. Neither higher MCM2 nor Ki-67 intensities, separately or combined, were associated with prostate cancer death. The x-axis corresponds to follow-up in months. The y-axis corresponds to cancer-specific survival.

Fig. 8.

MCM2 and Ki-67 product scores as predictors of cancer-specific mortality in the long-term follow-up group. Neither higher MCM2 nor Ki-67 immunohistochemical expression scores, separately or combined, were associated with prostate cancer death. The x-axis corresponds to follow-up in months. The y-axis corresponds to cancer-specific survival.

Table 1

Distribution of pathologic stage and Gleason score

	Short-term follow-up group (%)	Long-term follow-up group (%)
No. of cases	199	229
Pathologic stage		
T2, N0	131 (66)	23 (10)
T3a, N0	52 (26)	110 (48)
T3b, N0	12 (6)	41 (18)
Any T, N1	3 (2)	55 (24)
Missing	1 (1)	0 (0)
Gleason score		
6	110 (55)	52 (23)
7	67 (34)	115 (50)
8	22 (11)	62 (27)

Table 2

MCM2 immunoexpression in the short-term follow-up group

	No. of spots	Immunostain percentage ^a	Immunostain intensity ^b	Product score ^a
Tissue type				
Nontumor	746	1.4	39	53
Tumor	349	2.0	39	77
<i>P</i>		<.001	.43	<.001
Pathologic stage ^c				
T2, N0	223	1.9	39	69
T3a, N0	101	2.1	39	82
T3b, N0	19	5.3	39	206
Any T, N1	5	1.2	35	34
<i>P</i> trend value		.13	.99	.14
Primary Gleason pattern				
3	294	1.8	39	68
4	53	3.6	38	136
5	2	7.2	40	284
<i>P</i> trend value		.002	.95	.003
Gleason score				
6	191	1.8	39	67
7	124	1.9	38	71
8	34	5.5	40	219
<i>P</i> trend value		.002	.76	.003
Preoperative PSA ^d				
4 ng/mL	45	1.7	41	69
4-9 ng/mL	233	2.3	38	85
10 ng/mL	61	1.5	38	56
<i>P</i> trend value		.61	.11	.46

^aGeometric mean values.^bMean values.^cOne missing spot.^dTen missing spots.

Table 3

MCM2 immunoexpression in the long-term follow-up group

	No. of spots	Immunostain percentage ^a	Immunostain intensity ^b	Product score ^a
Tissue type				
Benign	473	0.9	36	31
Tumor	339	1.3	37	48
<i>P</i>		<.001	.44	<.001
Pathologic stage				
T2, N0	32	1.5	36	52
T3a, N0	166	1.1	36	39
T3b, N0	57	1.3	35	42
Any T, N1	84	1.9	40	73
<i>P</i> trend value		.11	.05	.04
Primary Gleason pattern ^c				
3	151	1.0	38	37
4	153	1.7	35	58
5	18	2.6	36	92
<i>P</i> trend value		.002	.09	.005
Gleason score				
6	65	0.8	40	32
7	181	1.4	37	49
8	93	1.9	34	61
<i>P</i> trend value		.008	.002	.04
Preoperative PSA ^d				
4 ng/mL	34	1.0	32	31
4-9 ng/mL	109	1.6	35	55
10 ng/mL	123	1.6	37	57
<i>P</i> trend value		.44	.03	.26

^aGeometric mean values.^bMean values.^cSeventeen missing spots.^dSeventy-three missing spots.

Table 4

Ki-67 immunoexpression in the short-term follow-up group

	No. of spots	Immunostain percentage ^a	Immunostain intensity ^b	Product score ^a
Tissue type				
Benign	768	0.5	28	12
Tumor	355	1.8	36	63
<i>P</i>		<.001	<.001	<.001
Pathologic stage ^c				
T2, N0	228	1.6	35	55
T3a, N0	100	2.0	37	73
T3b, N0	20	3.8	37	141
Any T, N1	6	2.5	40	100
<i>P</i> trend value		.004	.005	.002
Primary Gleason pattern				
3	297	1.7	35	58
4	56	2.8	37	104
5	2	1.4	39	53
<i>P</i> trend value		.04	.02	.02
Gleason score				
6	194	1.5	35	52
7	124	2.0	35	69
8	37	3.2	39	125
<i>P</i> trend value		.003	.01	.001
Preoperative PSA				
4 ng/mL	57	1.8	35	61
4-9 ng/mL	234	1.8	36	64
10 ng/mL	64	1.9	34	63
<i>P</i> trend value		.72	.36	.90

^aGeometric mean values.^bMean values.^cOne missing spot.

Table 5

Ki-67 immunoexpression in the long-term follow-up group

	No. of spots	Immunostain percentage ^a	Immunostain intensity ^b	Product score ^a
Tissue type				
Benign	675	0.3	23	6
Tumor	463	0.8	27	21
<i>P</i>		<.001	<.001	<.001
Pathologic stage				
T2, N0	40	0.8	29	24
T3a, N0	221	0.7	27	18
T3b, N0	84	0.8	26	19
Any T, N1	118	1.0	28	27
<i>P</i> trend value		.24	.89	.28
Primary Gleason pattern ^c				
3	207	0.7	27	17
4	208	1.0	27	26
5	22	0.7	26	16
<i>P</i> trend value		.23	.92	.29
Gleason score				
6	87	0.7	27	18
7	243	0.8	27	21
8	133	0.9	27	23
<i>P</i> trend value		.44	.96	.48
Preoperative PSA ^d				
4 ng/mL	45	0.7	26	19
4-9 ng/mL	139	0.8	27	22
10 ng/mL	171	0.8	27	22
<i>P</i> trend value		.78	.99	.80

^aGeometric mean values.^bMean values.^cTwenty-six missing spots.^dOne hundred eight missing spots.

Table 6

Hazard ratios of cancer-specific death in the long-term follow-up group by MCM2 and Ki-67 expression

Parameter	Adjusted hazard ratio (95% confidence interval) ^a
Immunostain percentage	
High MCM2	0.8 (0.3-2.2)
High Ki-67	1.3 (0.6-3.0)
High MCM2 or high Ki-67	1.4 (0.6-3.4)
High MCM2 and high Ki-67	0.8 (0.2-2.8)
Immunostain intensity	
High MCM2	0.9 (0.4-2.5)
High Ki-67	1.5 (0.6-3.9)
High MCM2 or high Ki-67	0.8 (0.3-2.0)
High MCM2 and high Ki-67	2.5 (0.8-7.2)
Immunostain product score	
High MCM2	0.7 (0.3-1.9)
High Ki-67	1.2 (0.5-2.8)
High MCM2 or high Ki-67	0.6 (0.2-1.6)
High MCM2 and high Ki-67	1.6 (0.7-3.6)

NOTE. *High* is defined as greater or equal to the third tertile.^aParameters are adjusted for pathologic stage, Gleason score, preoperative PSA, and surgical margins.