Minichromosome Maintenance Protein 2 Expression in Prostate: Characterization and Association with Outcome after Therapy for Cancer[1]

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ABSTRACT

The minichromosome maintenance (MCM) proteins are highly conserved proteins essential for initiating and regulating eukaryotic DNA replication. Recent studies have demonstrated the potential use of MCM proteins as markers of proliferation. We characterized the pattern of Mcm 2 staining in benign and malignant prostate tissues and examined the role of Mcm 2 expression in disease-free survival after surgery in men with localized prostate cancer.

Tumors from 92 patients who underwent radical prostatectomy for prostate cancer (median follow-up of 54 months) were examined for Mcm 2 expression by immunohistochemistry using a monoclonal antibody. Prostate tissue from five men without histopathological evidence of prostate cancer was also stained for Mcm 2. Mcm 2 expression was quantified by calculating a labeling index, and patients were grouped according to degree of staining. An analysis of the association between Mcm 2 expression with traditional clinicopathological characteristics of prostate cancer was carried out. A Cox proportional hazards analysis was performed to determine whether Mcm 2 staining was a significant independent predictor of disease-free survival.

Mcm 2 expression is low (<2%) and limited to the basal cell layer in nonmalignant prostate glands. Mcm 2 expression is consistently increased in malignant glands and is significantly associated with disease-free survival in univariate ($P = 0.002$) and multivariate ($P = 0.01$) analyses. Patients with high Mcm 2 expression exhibited shorter disease-free survival. Mcm 2 expression was not associated with any traditional clinical or pathological factors and therefore is an independent predictor of survival in these patients with prostate cancer.

These data support evidence that Mcm 2 may serve as a novel proliferation marker in the prostate. Mcm 2 expression is an independent predictor of disease-free survival after definitive local therapy and has potential as a molecular marker for clinical outcome in prostate cancer.

INTRODUCTION

The MCM[3] proteins are a family of six proteins (Mcm 2–7) involved in the initiation and regulation of DNA replication. First discovered in yeast, MCM proteins possess highly conserved gene sequences and bind to DNA sites where the origin recognition complex and Cdc 6 proteins have sequentially bound, together forming the prereplicative complexes (1–3). Subsequently, the chromatin is competent or "licensed" for replication. The MCM proteins then dissociate from chromatin irreversibly during S Phase, ensuring that DNA replication occurs once and only once during each cell cycle (4).

In all of the eukaryotic cells examined to date, the MCM proteins are essential for DNA replication (3). Studies (5–8) of the MCM and Cdc 6 proteins have revealed that they are found only during the cell cycle. The presence of MCM proteins in proliferating cells, but not quiescent or differentiated cells, suggests a role as a novel cell proliferation marker (8). In addition, initiation of genome replication represents the convergence point of the complex, multiple, and redundant regulatory pathways. Therefore, the members of the conserved prereplicative complex can be seen as relay stations coupling growth regulatory pathways with DNA replication, thereby serving as biomarkers for proliferation. These hypotheses have been studied clinically in both the cervix and bladder, demonstrating a potentially important role of the MCM proteins in cancer; *e.g.*, Williams *et al.* (9) demonstrated that identification of abnormal, proliferating cells by positive MCM staining in cervical smears was extremely sensitive in detecting both low-grade and high-

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[3] The abbreviations used are: MCM, minichromosome maintenance; PSA, prostate-specific antigen; PCNA, proliferating cell nuclear antigen.

grade premalignant states. This may lower the 20–40% false-negative rate previously reported with the standard Pap smear screening test. Similarly, MCM expression in exfoliated transitional epithelial cells in the urine was able to detect transitional cell carcinoma in patients with both newly diagnosed and recurrent disease with high sensitivity and specificity (10).[4] In a study of various tissues, Freeman *et al.* (11) found increased staining for MCM proteins in cells of dysplastic and malignant areas. This aberrant expression of the proteins involved in regulating DNA replication is postulated to be linked to abnormal controls of proliferation and reflects persistence in the cell cycle. Thus, it appears that MCM expression may be altered in many states of abnormal growth including malignancies.

Prostate cancer remains the most common malignancy and is the second leading cause of cancer-related deaths in American men (12). Advances in the diagnosis and treatment of this disease have increased opportunities for a cure. Nevertheless, traditional predictors of outcome, such as PSA at diagnosis, tumor stage, and tumor grade, have limitations, and none are able to reliably predict outcome for an individual patient. Previous studies have addressed molecular and pathological markers of disease progression in patients with prostate cancer, including Ki-67, PCNA, p53, Bcl-2 expression, DNA ploidy, and microvessel density (23–26). Although these markers may provide some improvement in predicting outcome (13, 14) and models that incorporate these factors have been developed, none is perfect for the individual patient. The need for additional molecular and pathological markers in prostate cancer still exists.

MCM protein expression has not been characterized previously in the prostate. Thus, the goals of the current study were to characterize the distribution and pattern of Mcm 2 expression in normal, hyperplastic, and malignant prostate tissue and to determine whether this novel proliferation marker is useful in predicting outcome after therapy for patients undergoing surgery for prostate cancer.

MATERIALS AND METHODS

Patients and Tissues

Nonmalignant Prostate Tissues. Prostate tissue from five men who underwent transurethral resection of the prostate was used to study Mcm 2 expression in normal (nonmalignant) tissue. There was no histopathological evidence of prostate cancer in any of these specimens.

Prostate Cancer Tissue. Prostate tissue was obtained from 92 specimens removed at the time of radical retropubic prostatectomy. All surgery was performed at University of California San Francisco-affiliated hospitals between 1990 and 1999. Cases of prostate cancer were newly diagnosed, and surgery was the primary treatment modality chosen by the patients. Preoperative characteristics analyzed included age at the time of surgery, serum PSA level, clinical tumor stage, and biopsy Gleason grade. Twenty-nine patients with high-risk disease characteristics were treated with neoadjuvant hormonal therapy before surgery. This consisted of 3 to 4 months of complete androgen blockade with a combination of luteinizing hormone-releasing hormone agonist and oral antiandrogen. No patient was taking finasteride. Patients undergoing surgery had clinically localized disease and no preoperative evidence of metastatic spread to the bones or lymph nodes. In all of the cases, pelvic lymph node dissection was performed at the time of surgery. Prostatectomy specimens were processed in the usual fashion with fixation of fresh tissue in 10% neutral buffered formalin and embedding in paraffin. The prostate gland was step sectioned at 3-μm intervals perpendicular to the rectal surface, graded according to the method of Gleason (15), and staged according to the tumor-node metastasis classification (16). Patients were followed with serial serum PSA determination every 4 months. Disease recurrence was defined as two consecutive serum PSA levels ≥0.2 ng/ml.

Table 1 Clinical characteristics of cohort

	No.
All patients	92
Age (yr)	63.5 (range, 44.8–74.7)
Preoperative PSA (ng/ml)	Median 8.2 (range, 0.9–103)
0–4	18 (20%)
4–10	39 (44%)
10–20	19 (22%)
>20	12 (14%)
Biopsy Gleason grade	
Sum < 7	64 (72%)
Sum = 7	13 (14%)
Sum > 7	13 (14%)
Primary grade 1–3	71 (79%)
Primary grade 4 or 5	19 (21%)
Clinical stage	
T_{1b}	1 (1%)
T_{1c}	7 (7%)
T_{2a}	17 (21%)
T_{2b}	37 (40%)
T_{2c}	16 (17%)
T_3	13 (14%)
Neoadjuvant hormonal treatment	29 (32%)
Adjuvant therapy	5 (5%)
Follow-up (months)	Median 54.3 (0.9–127)

Antibodies and Immunohistochemical Staining. A His_6-tagged fragment of hsMcm2 was expressed in *Escherichia coli* and purified on Ni-NTA agarose. Balb/c CBA F1-crossed mice approximately 3 months of age were immunized by injection of 100 μg of bacterially expressed fusion protein emulsified with an equal volume of Titremax Gold (CytRx). The immunization was repeated three times at 2- to 3-month intervals, and the mice were rested for 6 months. An additional 100 g of fusion protein in PBS/0.3% SDS was injected i.p. at 6 and 3 days before sacrifice. Hybridoma cell lines were established by fusing splenocytes from immunized animals with the myeloma line Sp2/0-Ag14 by polyethylene glycol treatment using conventional procedures. Lines were screened by a modified blot procedure using the bacterial expression fusion protein (17) and cloned a minimum of three times before use for antibody production. Antibody subtyping was performed using the Roche Diagnostics IgG Isotyping Kit following the manufacturer's instructions. Specificity of the anti-Mcm2 monoclonal antibody applied in this study was established by immunoblot and immunofluorescence assays. This antibody can be obtained from Dr.

[4] K. Stoeber, *et al.*, manuscript in preparation.

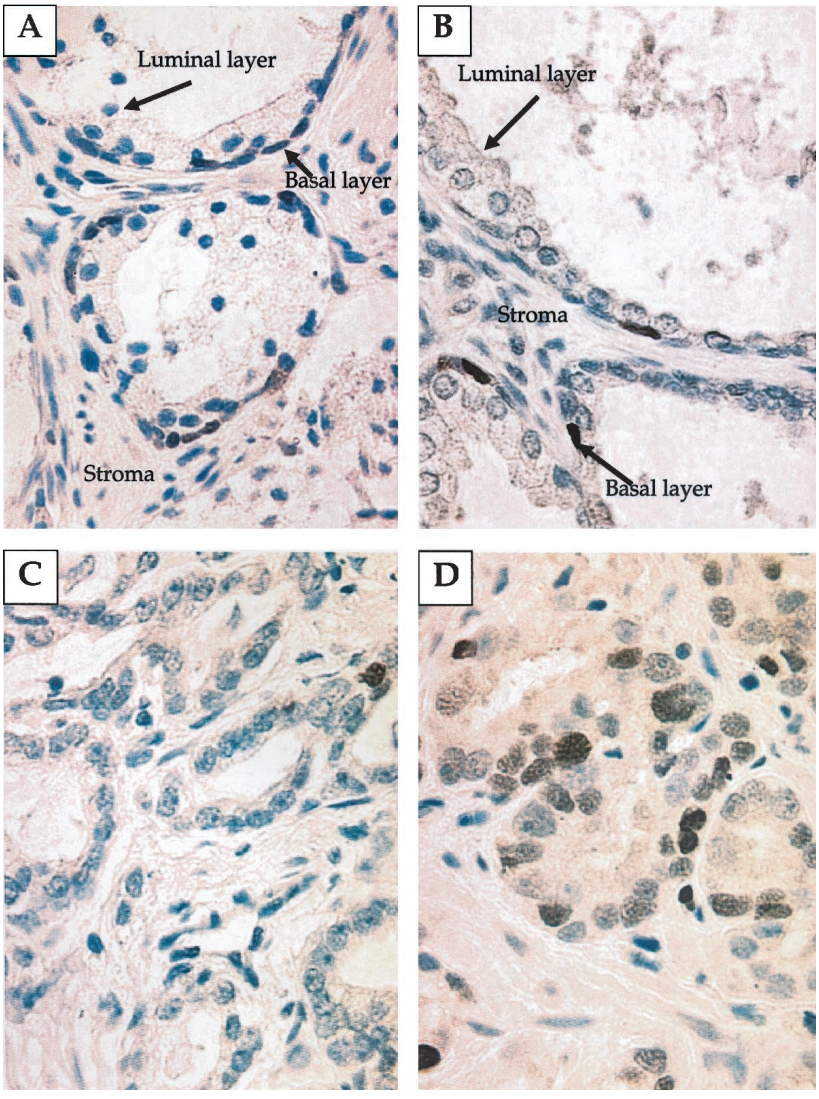

Fig. 1 Photomicrographs of paraffin-embedded tissue sections of the prostate gland stained with antibody to Mcm 2. *A*, normal prostate gland. Area was chosen to demonstrate basal epithelial cell staining. *B*, hyperplastic glands. *C*, adenocarcinoma (low staining). *D*, adenocarcinoma (high staining). Original magnification, ×400.

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Representative prostate tissue sections that displayed both adenocarcinoma and normal glands on H&E staining were chosen for immunohistochemical analysis. These blocks were sectioned at 5-μm thickness, and unstained sections were mounted on positively charged glass slides. The tissues were deparaffinized in Histoclear (National Diagnostics, Atlanta, GA) and rehydrated with 95% and 100% ethanol. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide-methanol for 20 min. Slides were then washed with PBS followed by distilled water. Heat-mediated antigen retrieval was performed in a 10 mM citrate buffer (pH 6.0) and microwave processor for 10 min at the highest power setting. After allowing the sections to cool for 30 min and washing with PBS, nonspecific activity was blocked by incubation in normal sheep and donkey serum (each diluted 1:80 in PBS) for 1 h. The sections were then incubated overnight at 4°C in a humidified chamber with the primary Mcm 2 mouse monoclonal antibody (diluted 1:250 in PBS). The slides were washed in PBS and incubated with biotinylated sheep antimouse secondary antibody (Amersham, Piscataway, NJ) at 1:200 in PBS for 1 h at room temperature. After washing in PBS, preformed avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) was applied for 1 h at room temperature. Sites of activity were visualized using 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) as the chromagen. Sections were counterstained with hematoxylin and mounted in Permount (Fischer, Pittsburgh, PA).

Assessment of Immunostaining. Each slide was evaluated twice by one investigator and recorded blind in relation to the clinical information. An experienced pathologist reviewed a subset of the slides, and good concordance in reading was observed. Light microscopy was used to evaluate the localization and degree of the immune reaction. The entire section was assessed at low (100×) and high (400×) power, and the areas of prostate adenocarcinoma with the most intense staining were analyzed. Nuclear staining was considered representative of Mcm 2 expression; cytoplasmic staining was not considered positive. At least 500 nuclei were counted/case. The fraction of

nuclei staining positive for Mcm 2 was calculated for each specimen, and the median value (10%) was chosen as the cutoff to stratify patients before any statistical analysis. Additionally, areas with benign prostate glands by histology were analyzed, and the degree of Mcm 2 staining was quantified.

Statistical Methods. Ninety-two patients who underwent radical prostatectomy for localized prostate cancer were included in this analysis. Descriptive statistics were calculated to characterize the patients with comparisons between subsets analyzed using a χ^2 statistic for categorical variables, ANOVA methods for continuous variables, and the Mann-Whitney nonparametric test for medians. The probability of remaining disease-free was estimated using the Kaplan-Meier product limit method. Time until disease recurrence was measured from the date of surgery until the first of two consecutive PSA values of 0.2 ng/ml or greater. For patients without biochemical failure, follow-up was measured from the date of surgery to the date of most recent PSA value. Univariate comparisons of the disease-free interval were tested using the log-rank statistic. Variables determined to be significant with a probability value less than 0.1 were considered as potential predictors in the multivariate analysis. Histological grade was considered as primary Gleason grade, Gleason sum, or a combination of the two for any single analysis because of the association among the three variables. Grade was also analyzed when grouped in three categories: primary Gleason 1–3 and Gleason sum <7, primary Gleason 1–3 and Gleason sum ≥7, and primary Gleason 4 or 5. Cox proportional hazard model using a stepwise forward approach was performed to identify independent predictors of outcome.

RESULTS

Patients. A total of 92 men with prostate adenocarcinoma and no evidence of metastatic disease treated with radical retropubic prostatectomy were identified. The clinical characteristics of these patients are listed in Table 1. Of note, a significant fraction had high-risk disease features: 36% had PSA ≥ 10 ng/ml, 28% had Gleason sum ≥ 7, and 14% had clinical T_3 disease.

Mcm 2 Staining in Prostate. Immunostaining with the Mcm 2 antibody was characterized in areas of prostate with normal and hyperplastic glands on histology. The cells exhibiting Mcm 2 staining were confined to the basal, proliferating layer of the prostate epithelium (Fig. 1*A*). The luminal, differentiated cells in nonmalignant glands were rarely positive for Mcm 2 staining. Overall, in areas of normal glands and hyperplastic glands (Fig. 1*B*), basal epithelial immunostaining with Mcm 2 was less than 2%. Staining was uniformly confined to the nuclear compartment, with rare cytoplasmic staining. Stromal cells were not stained with Mcm 2. The findings were consistent in specimens from patients with and without prostate carcinoma.

In areas of prostate adenocarcinoma, immunoreactivity was observed in a greater fraction of cells (Fig. 1, *C* and *D*). In all of the specimens, the percentage of cells positive for Mcm 2 in areas of tumor ranged from 1–41% (median, 10%). Prostate cancer is characterized by the loss of the basal cell layer (18). Therefore, Mcm 2 staining was evident in the luminal epithelial cells of malignant glands. Again, stromal cells were not positive for Mcm 2 staining.

Table 2 Relationship between clinicopathological findings and Mcm 2 immunostaining[a]

	Total (n = 92)	Percentage of cells staining positive for Mcm 2	
		<10%	≥10%
Preoperative PSA			
<10 ng/ml	61	40 (66%)	21 (34%)
≥10 ng/ml	31	23 (74%)	8 (26%)
Stage			
Organ confined	52	37 (71%)	15 (29%)
Nonorgan confined	40	29 (73%)	11 (28%)
Grade			
Primary Gleason 1–3	72	50 (69%)	22 (31%)
Primary Gleason 4 or 5	20	16 (80%)	4 (20%)
Gleason sum < 7	50	39 (78%)	11 (22%)
Gleason sum = 7	24	16 (67%)	8 (33%)
Gleason sum > 7	18	11 (61%)	7 (39%)
Lymph node metastases			
Absent	87	62 (71%)	25 (29%)
Present	5	4 (80%)	1 (20%)
Surgical margin			
Negative	68	47 (69%)	21 (31%)
Positive	24	19 (79%)	5 (21%)
Neoadjuvant therapy			
No	63	46 (73%)	17 (27%)
Yes	29	20 (70%)	9 (30%)

[a] No association between Mcm 2 staining and clinicopathological variables ($P > 0.05$ in all of the cases).

Relationship between Mcm 2 Staining and Clinicopathological Characteristics. The relationship between Mcm 2 staining and standard preoperative and pathological variables is shown in Table 2. There was no statistically significant association between the fraction of Mcm 2 staining (10% cutoff) and preoperative PSA, tumor stage, primary Gleason grade or Gleason sum, presence of positive lymph nodes, and surgical margin status. There was a trend toward association between Mcm 2 staining and tumor grade when primary grade and Gleason sum were combined to classify grade ($P = 0.05$). Repeat analyses were unchanged when Mcm 2 expression was grouped in thirds.

Mcm 2 Expression and Disease-free Survival: Univariate and Multivariate Analyses. At 5 years after surgery, the probability of disease-free survival for the entire cohort was 72% (Fig. 2*A*). Table 3 summarizes the results of the univariate analysis for the entire cohort of patients. Preoperative PSA less than 10 ng/ml, primary Gleason score 1–3, Gleason sum <7, the combination of primary Gleason score 1–3 and Gleason sum <7, not receiving neoadjuvant hormonal therapy, and negative surgical margins were all predictors of improved disease-free survival. Pathological stage, as reflected by seminal vesicle invasion, did not reach statistical significance. In addition, Mcm 2 staining <10% was significantly associated with improved survival (Fig. 2*B*).

Results from the multivariate analysis using a forward stepwise Cox proportional hazards regression model is also summarized in Table 3. In this multivariate analysis, only neoadjuvant hormonal treatment, Mcm 2 staining, surgical margin

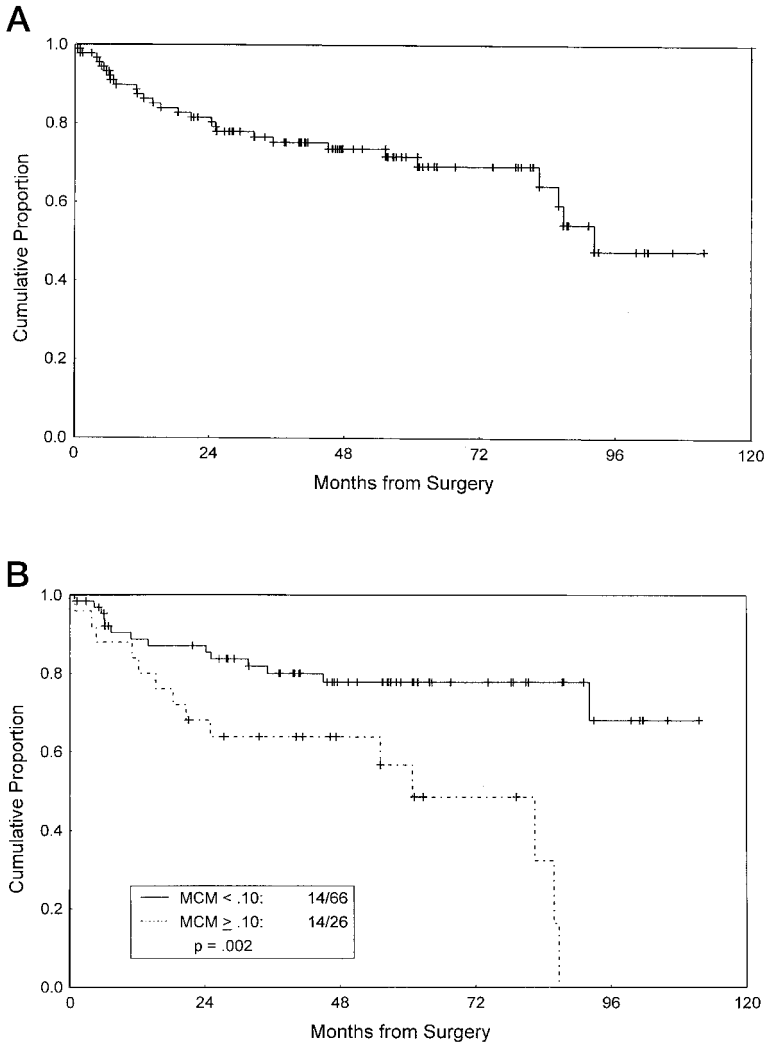

Fig. 2 *A,* Kaplan-Meier curve showing PSA-free survival in all of the patients. *B,* Kaplan-Meier curve showing PSA-free survival stratified by Mcm 2 staining. Fractions indicate the number of failures of the total number in the patient subset.

status, and primary Gleason grade were significant independent predictors of disease-free survival. If the three-category combination of Gleason primary score and sum was substituted for the primary grade, there was no difference in the likelihood ratios (−93.18 and −93.11, respectively), and the same variables were considered to be independent predictors of outcome. Because a single model could not be preferred, the simpler model using primary grade was presented in Table 3.

DISCUSSION

The MCM proteins have been demonstrated to be important regulators of eukaryotic DNA replication (2). As such, they have been postulated to be potential markers of proliferation. Studies have shown that MCM expression indicates the presence of cells in the cell cycle (5–8). Loss of MCM expression reflects cellular differentiation or quiescence. Only recently (8, 9, 10, 19) has MCM expression been examined in human tissue samples and used to define the proliferative compartments of both normal and abnormal tissues. In addition, the ability to detect dysplastic and malignant cells by altered prereplicative protein expression has suggested clinical utility in detection and diagnosis of preneoplastic and neoplastic states.

Freeman *et al.* (11) previously reported limited data regarding MCM expression in prostate tissue. In two samples of normal prostate, median labeling indices for Mcm 2 and Mcm 5 were 16% and 11%, respectively. We found a much lower fraction of cells expressing Mcm 2 in our normal samples. In addition, histologically normal areas of prostate specimens harboring cancer elsewhere also had a lower labeling index, typically less than 2%. Nevertheless, in both studies, Mcm 2 expression was restricted to the basal epithelial layer of nonmalignant prostatic glands. This finding is consistent with the hypothesis that the basal cell layer actively proliferates and is the progenitor of luminal cells (20, 21). Therefore, it is expected that terminally differentiated, luminal prostate epithelial cells do not express MCM proteins. Areas of glandular hyperplasia also demonstrate Mcm 2 expression limited to the proliferating basal epithelial cells. Thus, although altered cellular growth is likely to result in benign hyperplasia, the normal pattern of MCM expression is maintained with basal localiza-

Table 3 Univariate and multivariate Cox regression analysis of risk factors in predicting disease-free survival

Factor	Variables	Univariate analysis *P*	Multivariate model *P*	Multivariate model HR (95% CI)[a]
Preoperative PSA				
	<10	0.05	Not significant	
	≥10			
Primary grade[b]				
	Gleason 1–3	0.004	0.03	2.5 (1.1, 5.7)
	Gleason 4 or 5			
Gleason sum[b]				
	<7	0.02	Not significant	
	≥7			
Surgical margins				
	Negative	0.02	0.003	2.6 (1.1, 5.9)
	Positive			
Mcm 2 staining				
	<10%	0.002	0.01	3.9 (1.8, 8.8)
	≥10%			
Neoadjuvant therapy				
	No	0.0001	0.0001	5.2 (2.2, 12.3)
	Yes			

[a] In the multivariate model, the significant independent predictors are indicated with their associated hazard ratio (HR) and confidence interval (CI).
[b] Primary Gleason grade, Gleason sum, and a combination of the two were considered in separate multivariate models. Primary grade and a combination of primary grade and Gleason sum resulted in the same final multivariate model predicting outcome. The simpler model using primary grade is presented; see text.

tion and loss of expression with the luminal phenotype. It is interesting to note that cells of the stroma were uniformly negative for Mcm 2 staining. This likely reflects the low proliferative rate of stromal elements, such as fibroblasts, and suggests that states of hyperplasia are not necessarily dependent on increased cellular proliferation (22).

Prostate adenocarcinoma is characterized by the loss of the basal epithelial cell layer and the presence of single-cell layered glands (18). Thus, in areas of cancer, the increased MCM staining was found in the luminal epithelial cells. We noted a wide range of staining in the 92 specimens. Importantly, such staining was not associated with traditional predictors of malignant potential and risk for disease progression, including Gleason grade and tumor stage. This suggests that MCM expression may reflect a unique characteristic of the malignant cell and, therefore, could provide independent prognostic information. We found that the level of Mcm 2 expression, as determined by immunohistochemistry and quantification with a labeling index, can provide information with respect to disease-free survival in prostate cancer patients undergoing radical prostatectomy. Patients with higher Mcm 2 expression exhibited a significantly increased risk of treatment failure after radical prostatectomy when compared with patients exhibiting lower Mcm 2 expression. Mcm 2 expression was independent of prognostic features including preoperative PSA, tumor stage, histological grade, lymph node status, surgical margin status, and pathological stage. In a multivariate analysis, the Mcm 2 labeling index was found to be an independent prognostic indicator of disease-free survival when considered along with histological grade, neoadjuvant hormonal treatment, and surgical margin status. Similar to the present study, Freeman *et al.* (11) found a higher median labeling index (39%) in three prostate carcinoma samples. In contrast to the present study, however, there was a suggestion of correlation between MCM staining and tumor grade.

Neoadjuvant androgen deprivation was used in 29 patients before radical prostatectomy. The use of such treatment was associated with a significantly worse disease-free survival after surgery. This likely reflects the high-risk disease characteristics of patients receiving combination therapy. It is important to note, however, that the level of Mcm 2 expression was not associated with the use of neoadjuvant androgen deprivation (Table 2) and that the Mcm 2 labeling index remained a significant independent predictor of outcome in a multivariate analysis that included the use of androgen deprivation before radical prostatectomy. These data support the hypothesis that Mcm 2 expression may provide important prognostic information with respect to prostate cancer prognosis, above that obtained from traditional clinical predictors of disease-specific outcome.

Although many other biomarkers, such as PCNA, Ki-67, p53, and Bcl-2, have been examined in prostate cancer, the MCM proteins and other members of the replicative complex possess several advantages (23–26). Staining for PCNA can be associated with technical challenges and is limited to fixed, paraffin-embedded tissues. In addition, PCNA detects cells undergoing DNA repair as well as those cells that are proliferating (27). Ki-67 is able to accurately assess the fraction of proliferating cells and may have prognostic value in prostate cancer; however, to date, the biological function of the antigen is unknown and it may not be required for proliferation (28). Oncogenes and tumor suppressor genes are often altered in malignancy and have been associated with outcome in prostate cancer. These proteins function in complex and redundant pathways. A major advantage of studying the MCM proteins is that the replicative complex is necessary for DNA replication and represents a convergence point for many signaling pathways (19). Moreover, the presence and functions of these proteins have been well characterized in several *in vitro* systems (3). Thus, the role of MCM proteins in prostate cancer may not be

limited to that of prognostic marker but may direct avenues of research and therapeutic interventions.

Additional studies in larger numbers of patients are required to determine whether routine staining for MCM proteins yields clinically useful information. This may be particularly important in patients with high-risk disease features, such as extracapsular extension and positive surgical margins. In these patients, controversy currently exists regarding the role of adjuvant therapy such as postoperative radiotherapy, because up to half of these men have no evidence of disease recurrence without additional therapy (29). It may be possible to stratify patients according to degree of MCM staining to determine which patients are appropriate candidates for early adjuvant therapy or entry into clinical trials for novel therapeutic modalities. MCM staining may also have utility in early identification of prostatic intraepithelial neoplasia, the precursor lesion of prostate cancer, and additional staining in prostate biopsies should be performed. Another area in which MCM staining may have interesting implications includes those men with metastatic disease. A molecular marker such as ours may help select the appropriate type and timing of therapy as well as identify those patients who will respond to treatment, and these aspects deserve further investigation. Finally, similar to the study in transitional cell carcinoma of the bladder, biochemical analysis of prostate epithelial cells shed into the seminal fluid could provide an early, noninvasive assay for prostate cancer.

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