

Minichromosome Maintenance Protein 2 Expression in Normal Kidney and Renal Cell Carcinomas: Relationship to Tumor Dormancy and Potential Clinical Utility¹

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ABSTRACT

Purpose: A major problem in the management of patients with renal cell carcinoma is predicting tumor behavior. In the search for more accurate markers of prognosis, tumor cell proliferation has been investigated. These studies have mostly used antibodies directed against Ki-67 or proliferating cell nuclear antigen and have given conflicting results, findings that are likely because of a combination of specificity and methodological differences. Minichromosome maintenance (Mcm) proteins are a series of closely related proteins that are components of the prereplicative complex. The Mcm proteins are essential for initiating eukaryotic DNA replication and serve as useful markers of proliferating cells.

Experimental Design: The aims of this study were to determine the frequency and pattern of Mcm2 expression by immunohistochemistry in normal kidney ($n = 10$) and renal tumors [$n = 56$; clear cell $n = 36$; chromophil (papillary) $n = 7$; oncocytoma $n = 5$; and transitional cell carcinoma $n = 8$], compare its sensitivity to the established proliferation marker Ki-67, examine for differences in tumors derived from stable and labile epithelial cell populations in the kidney, and assess the relationship of Mcm2 proliferation to clinicopathological characteristics of kidney tumors. In addition, to additionally investigate the issue of tumor dormancy we wished to assess the relationship between Mcm2 labeling index (LI) and the angiogenic factors angiopoietin-1 (Ang) and Ang-2.

Results: In normal tissues, Mcm2 nuclear labeling was identified in both glomeruli (LI median 0.35%; range 0–1.7) and renal tubules (LI median 0.3%; range 0.1–2.9%). In tumors Mcm2 labeling was predominantly at the periphery with LIs ranging from 0.2–91.5%, which was significantly greater than Ki-67 LI (0.2–40.5%; $P < 0.001$). Mcm2 LI was also significantly higher in tumors derived from a labile epithelium (transitional cell carcinomas) than a stable epithelium (renal cell carcinomas; $P = 0.013$). A significant association was also demonstrated between Mcm2 LI and tumor grade ($P = 0.0006$), and angiogenic phenotype (defined by Ang expression; $P = 0.03$) but not with patient age ($P = 0.84$), patient sex ($P = 0.25$), tumor size ($P = 0.74$), or stage ($P = 0.33$). Furthermore, although not significant, a survival analysis demonstrated that 100% of patients with a low Mcm2 LI survived compared with 84% of those with a high Mcm2 LI over the follow-up period (up to 53.2 months; $P = 0.14$).

Conclusions: This is the first study examining Mcm2 protein in normal and tumor kidney samples, and the first to perform histological subgroup analysis. It shows that Mcm2 is a superior marker to Ki-67 in the assessment of cell cycle entry in histological archival material and that normal kidney has a subset of cells within the glomerular and tubular compartments that are in cycle. It demonstrates that the frequency of cells in cycle in tumors formed from stable or labile epithelial populations mirrors that in the nonneoplastic epithelium. This study additionally demonstrates that the number of cells in cycle in tumors is limited by the angiogenic phenotype and supports animal models that show angiogenesis determines the likelihood of tumor dormancy. Additional study to confirm the clinical utility of Mcm2 as a prognostic marker is now indicated.

INTRODUCTION

RCCs⁴ account for only 1–3% of visceral malignancies but pose a significant health problem with >23,000 new cases diagnosed annually in the United States alone. The clinical course of RCC is notoriously unpredictable with numerous instances of spontaneous regression of metastases yet frequent recurrences in many who survive 10 or more years. A major problem in the management of patients with RCC is predicting tumor behavior. Stage is the most useful determinant, with tumor grade and histology being of lesser value (1–3). The

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⁴ The abbreviations used are: RCC, renal cell carcinoma; Mcm, minichromosome maintenance; Ang, angiopoietin; LI, labeling index; pre-RC, prereplicative complex; TCC, transitional cell carcinoma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Table 1 Clinicopathological data for the kidney tumors studied

Histology	Sex		Age (years)		Size (mm)		Grade ^a			
	M ^b	F	Median (range)	<i>n</i>	Median (range)	1	2	3	4	
Clear cell	16	20	65 (35–79)	36	60 (30–132)	2	15	17		
Chromophil	5	2	60 (53–75)	7	60 (30–110)		5		1	
Oncocytoma	1	4	70 (54–78)	5	29 (25–68)					
TCC	4	4	79 (55–92)	8	30 (18–70)		2	6		

^a Fuhrman (RCC) or WHO grading (TCC).

^b M, male; F, female; *n*, number.

tumor cell proliferation index using a variety of markers has been reported to give clinically important information in renal tumors with some markers such as Ki-67 and proliferating cell nuclear antigen providing additional prognostic information above that of stage (4–11). Nevertheless, not all of the studies have shown that tumor cell proliferation correlates with clinical course (5–15), findings that are likely because of inherent problems with Ki-67 (16) and proliferating cell nuclear antigen (17) as markers, together with the techniques and cutoffs used in analyses. The function of Ki-67 is still unknown. Indeed the molecule appears not to be essential for cell proliferation (18), and its expression can be altered by nutrient deprivation (19), a common occurrence in tumors that is likely to confound analysis.

Recently, antibodies to the Mcm proteins have become available (20). The Mcm proteins are a family of closely related proteins that show close sequence homology and are essential for DNA replication in all eukaryotic cells. Similar proteins, with marked conservation of sequence, have been identified in *Xenopus*, mice, and humans (21). The Mcm proteins form a pre-RC and bind to DNA at sites at which the origin recognition complex and Cdc6 proteins have sequentially bound. Once the pre-RC is formed, DNA replication commences, followed by disassociation of the pre-RC, which limits DNA replication to once per cell cycle. Unlike the origin recognition complex, the Mcm proteins are present only during the cell cycle and, thus, are specific markers of proliferating cells (20, 22). The aims of this study were to assess the pattern and frequency of Mcm2 expression in normal and neoplastic renal tissues (of different histological types), examine for differences in Mcm2 in renal tumors derived from stable and labile epithelial populations, and using correlation analysis with other clinicopathological factors, investigate the potential prognostic utility of Mcm2 in assessing kidney tumors by performing a Phase I prognostic study (23). In addition, to additionally investigate the issue of tumor dormancy and survival in assessing kidney tumors we have also assessed the relationship in kidney tumors between Mcm2 and the angiogenic factors Ang-1 and Ang-2.

MATERIALS AND METHODS

Tumors and Patients. RCCs (*n* = 56) and histologically normal kidney tissue (*n* = 10; taken from uninvolved morphologically normal kidney tissue distant from tumor) were derived from patients undergoing surgery at the Christchurch Hospital, Christchurch, New Zealand. All of the tissues were derived from patients having given informed consent in accordance with Canterbury Ethics Committee guidelines (approval number 96/

02/008). Histological subtypes included 36 clear cell carcinomas, 7 chromophil (papillary) carcinomas, 5 oncocytomas, and 8 TCCs. Grading of RCCs and TCCs was performed according to the Fuhrman grading (24) and WHO systems, respectively (25). The clinicopathological characteristics of the patient series are contained in Table 1. There were 5 cancer-related deaths over the follow-up period (median 22 months; range, 5.7–53.2) in RCCs.

Immunohistochemistry and Assessment of Labeling Indices. Formalin-fixed paraffin-embedded sections (4 μm) were cut onto silane coated slides, deparaffinized through graded alcohols, and washed in water before placing in Tris/EDTA buffer [50 mM Tris/2 mM EDTA (pH 9)] and performing microwave pretreatment for 10 min. The slides were allowed to cool before being placed in PBS. Parallel slides were then incubated for 1 h with primary monoclonal antibodies to Mcm2 (antibodies to Mcm2 are described in Ref. 26; available from Dr. N. Coleman, Hutchison/MRC Research Center, Cambridge, United Kingdom) and Ki-67 (clone MIB-1; Dako, Medbio Enterprises Ltd., Christchurch, New Zealand) before following with a standard streptavidin-biotin-peroxidase-diaminobenzidine immunohistochemical technique (Dako, Medbio Enterprises, Ltd.). Negative controls were performed by omitting the primary antibody. A LI for Mcm2 and Ki-67 was derived by dividing the number of positively stained cell nuclei by the total number of nuclei counted in areas of maximal proliferation that were identified at medium magnification (×20–40 objective lenses) over a total area of 0.25 mm². As with our and other studies on microvessel counting (27, 28), this method of selection was used, because these hot spots are the tumor areas that are most likely to be of biological significance; a median of 3212 (range, 1097–4185) cells were counted for each tumor.

Angiogenic Factor Expression. In 25 clear cell carcinomas the abundance of the angiogenic factors Ang-1 and Ang-2 mRNA was measured using the RNase protection assay. Riboprobes were generated from cDNA (Dr. G. D. Yancopoulos, Regeneron Pharmaceutical Inc, Tarrytown, NY) and hybridized to total RNA isolated from snap-frozen tissue after surgical resection as described previously (29). Total RNA (20 μg) was loaded for each sample, and to control for any intraexperimental losses, known amounts of transcribed GAPDH sense probe were also added to each sample and assayed (30). Scanning laser densitometry was used to quantitate mRNA levels with signal standardized against the sense GAPDH control spike signal to generate arbitrary units. tRNA (20 μg) was used as a negative control for each experiment.

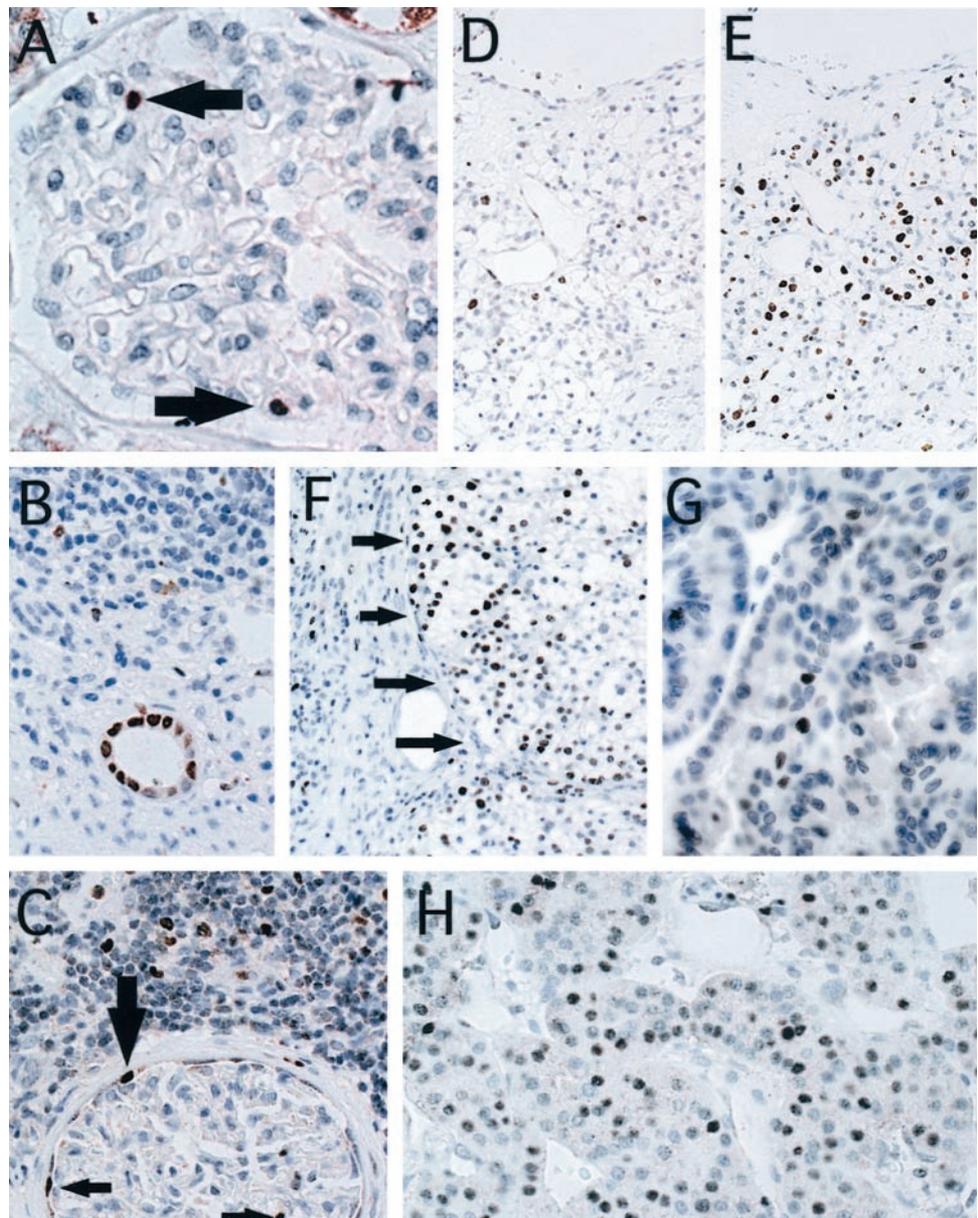


Fig. 1 Immunohistochemistry for Mcm2 and Ki-67 in normal and neoplastic kidney. *A*, staining for Mcm2 in occasional nuclei within the renal glomerulus (arrows). Mcm2 immunopositivity of nuclei (arrows) in a regenerating renal tubule (*B*) and in Bowman's capsule (*C*) in chronically inflamed kidney at the periphery of a tumor. Comparison between Ki-67 (*D*) and Mcm2 (*E*) in parallel sections from the same tumor area. Mcm2 expression is identified mostly at the invasive edge of tumors (arrows; *F*). Mcm2 staining in a chromophil (papillary) carcinoma (*G*) and a benign oncocytoma (*H*).

Statistical Analysis. Spearman rank correlation coefficients were used for studying the association between continuous variables; tests of hypotheses on the location parameter (median) were performed using rank statistics (Mann-Whitney, Kruskal-Wallis, and adjusted Kruskal-Wallis for ordered groups) with clinicopathological details stratified according to tumor stage (≤ 7 cm and > 7 cm; Ref. 31), Fuhrman's nuclear grade low (1 and 2) and high (3 and 4; Ref. 31), and patient sex. Kaplan and Meier survival curves were constructed, and the Log Rank test was performed to examine for differences in survival in a Phase I prognostic study. This is defined by Altman and Lyman (23) as a prognostic study that seeks to evaluate the association between a new marker and disease characteristics. All of the tests were performed using Prism 3 (GraphPad, San Diego, CA).

RESULTS

Mcm2 and Ki-67 Labeling in Normal and Neoplastic Renal Tissues. A total of 54 and 49 from 56 cases were evaluable for Mcm2 and Ki-67, respectively, the loss because of unsatisfactory staining. In normal kidney both Mcm2 and Ki-67 immunoreactivity was observed in nuclei of the glomerular and tubular compartments with positivity present in both distal and proximal tubules (Fig. 1). In areas where non-neoplastic kidney was infiltrated by chronic inflammatory cells (usually at the tumor periphery) an increased Mcm2 LI was observed. This was present both in regenerating tubules and glomeruli. Cycling cells mostly in the latter were observed in Bowman's capsule (Fig. 1). Mcm2 detected more proliferating cells than Ki-67 in normal kidney ($P = 0.02$; Table 2).

Table 2 Mcm2 and Ki-67 labeling indices (%) for normal kidney and combined tumors

	Mcm2		Ki-67	
	Median (range)	n	Median (range)	n
Tubules	0.3 (0.1–2.9)	10	0.1 (0–0.4)	10
Glomeruli	0.35 (0–1.7)	10	0.1 (0–0.9)	10
All tumors	35.7 (0.2–91.5)	54	3.7 (0.2–40.5)	49

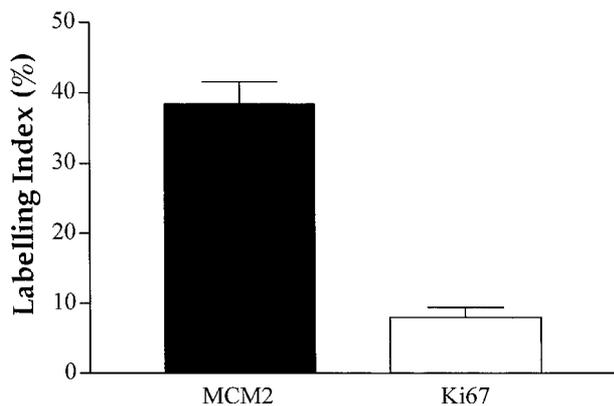


Fig. 2 Bar graph of Mcm2 and Ki-67 labeling indices in tumors of all histological types ($P < 0.001$; bars, \pm SE).

Unlike normal tissues where the proliferating compartment could not be readily characterized, in carcinomas most cells in cycle were present at the tumor periphery. This was notable only for carcinomas, with oncocytomas showing an apparent random distribution of cells in cycle. In tumors, although there was a significant correlation between Mcm2 and Ki-67 LIs ($P = 0.008$), Mcm2 identified significantly more malignant cells in cycle than Ki-67 in all of the tumor types ($P < 0.001$; Fig. 2; Table 2) and in clear cell ($P < 0.0001$), chromophil ($P = 0.0047$), and TCCs ($P = 0.003$; Fig. 3; Table 3). In oncocytomas the median Mcm2 LI was 21.1% compared with Ki-67 LI of 2.2%, although this difference was not significant ($P = 0.29$; Fig. 3; Table 3).

There was a significantly higher Mcm2 LI in malignant tumors derived from the renal pelvis (TCCs) compared with the renal parenchyma (RCCs; $P = 0.013$; Table 3), but no significant difference was observed between the renal parenchymal tumors of different histology ($P = 0.21$; Fig. 4).

Relationship between Mcm2, Clinicopathological Variables, Angiogenic Factors, and Survival. There was a significant correlation in RCCs between Mcm2 LI and grade ($P = 0.0006$; Fig. 4) but no association was observed with patient age ($P = 0.84$), patient sex ($P = 0.25$), tumor size ($P = 0.74$), or stage ($P = 0.33$). To determine whether the Mcm2 LI was associated with an angiogenic phenotype we correlated expression of the Tie2 ligands in clear cell carcinomas with Mcm2 LI. Both Ang-1 and Ang-2 were variably expressed in tumor samples (Fig. 5), and there was a significant association between Mcm2 LI and Ang-2 ($P = 0.03$) but not Ang-1 ($P = 0.37$). Survival curves were constructed using a Mcm2 LI of 20% as a

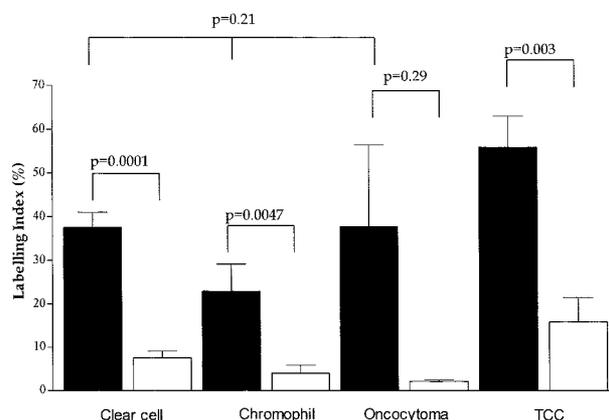


Fig. 3 Bar graph of Mcm2 (black) and Ki-67 (white) labeling indices in clear cell carcinomas, chromophil carcinomas, oncocytomas, and TCCs; bars, \pm SE.

cutoff (derived from cut-point analysis). Although not significant, this demonstrated that there were 0 of 9 deaths in patients with tumors having a low Mcm2 LI, whereas there were 5 of 30 deaths in patients with tumors having a high Mcm2 LI ($P = 0.14$; data unavailable for 4 patients; Fig. 6).

DISCUSSION

This is the first study examining the frequency and pattern of Mcm2 protein expression in a series of characterized normal and neoplastic renal tissues. We observed that Ki-67 was more fixation-dependent, with inadequate staining of 6 cases compared with 2 cases for Mcm2. This study has shown that a small percentage of cells in normal kidney have entered the cell cycle in both glomerular and tubular compartments. Our data shows that the number of cycling cells in normal kidney and in renal tumors has been significantly underestimated in prior studies that have used Ki-67 (5–10, 13, 14). Moreover, we also describe increased cell cycle entry in sites of inflammation. In view of the reported genetic changes in normal breast next to neoplastic breast tissues (32) it would be interesting to examine the non-neoplastic epithelium immediately adjacent to renal tumor to assess for differences in cell cycle.

Renal parenchymal tumors and renal pelvic tumors are derived from different cell populations (*i.e.*, stable and labile epithelium, respectively), and tumors derived from these origins showed differences in their proliferation status. No such difference was observed comparing tumors derived from the renal parenchyma. Our observation that TCCs have a significantly higher Mcm2 LI than RCCs parallels the earlier finding that normal urothelium contains a greater frequency of cells in cycle than normal kidney parenchyma (20). It is interesting that such differences are retained after neoplastic transformation of these epithelia, because this may exert significant effects on growth rate of the tumors that are formed. Expression of Mcms does not necessarily signify that a cell is capable of progressing through cell cycle, and it would be instructive to measure the expression of cell cycle phase-specific markers (33) in normal and neoplastic renal and transitional cell epithelial cells defined as being in cycle by expression of Mcms.

Table 3 Mcm2 and Ki-67 labeling indices for tumors of different histological types

	Clear cell		Chromophil		Oncocytoma		TCC	
	Median (range)	n	Median (range)	n	Median (range)	n	Median (range)	n
Mcm2	36.4 (0.2–73.8)	34	21.1 (9.8–57.7)	7	21.1 (1.5–91.5)	5	56.5 (30.1–79.6)	8
Ki-67	4.1 (0.2–35.8)	31	2.8 (0.8–12.6)	6	2.2 (1.2–2.8)	4	7.9 (2.2–40.5)	8
P	<0.0001		0.0047		0.29		0.003	

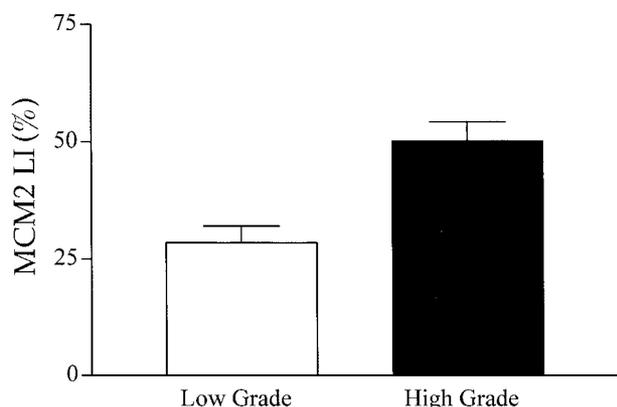


Fig. 4 Bar graph of Mcm2 LI in kidney tumors of low (Fuhrman 1 and 2; white) and high (Fuhrman 3 and 4; black) grade ($P < 0.0006$; bars \pm SE).

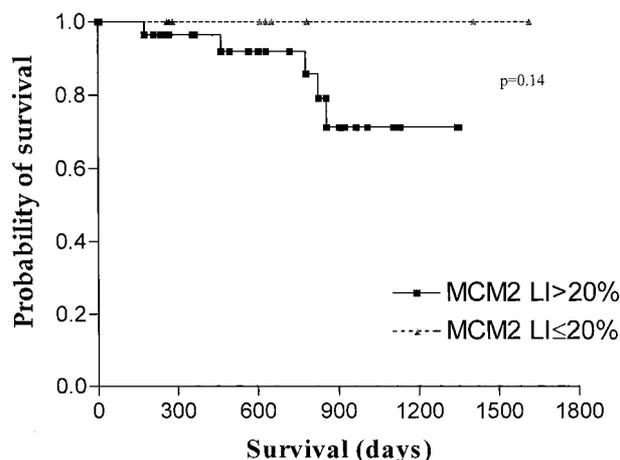


Fig. 6 : Kaplan and Meier survival curves stratifying patients with RCCs by high and low Mcm2 LI.

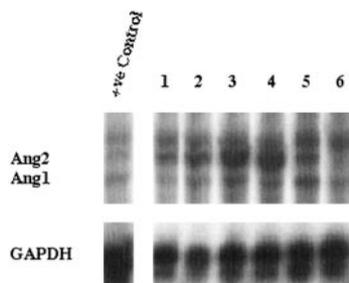


Fig. 5 RNase protection assay showing variable expression of Ang-1, Ang-2, with GAPDH spike signal in a representative series of six clear cell carcinomas (1–6); placenta was used as a positive control.

In association studies we confirmed a significant relationship between Mcm2 and grade as a measure of tumor differentiation (20) but did not observe a relationship to other clinicopathological parameters. However, we did demonstrate that Mcm2 identifies significantly more cells in cycle than Ki-67 in all histological types of renal tumors apart from oncocytomas, the latter result being because of the large variation in LIs evident in this type of neoplasm. The constitutive high frequency of cell cycle entry in clear cell tumors may be mediated by the *von Hippel-Lindau* gene that is mutated in sporadic clear cell renal cancers and may drive the cell cycle through several mechanisms, including altering transforming growth factor- α levels (34, 35), and modulating insulin growth factor signaling (36) and angiogenic factor levels (37). In support of the latter hypothesis is the highly significant correlation that we observed

between Mcm2 LI in clear cell RCCs and an angiogenic phenotype, indicated by its association with Ang-2 but not Ang-1 expression. We have observed previously that clear cell RCCs are characterized by elevated Ang-2, but not Ang-1, an angiogenic factor that regulates vessel stability and promotes angiogenesis directly (data not shown; Refs. 38–41). Indeed, the poor prognostic outcome that has been reported in some studies in RCCs with an angiogenic phenotype (31, 42, 43) may be because of enhanced nutrient and oxygen delivery allowing continued cell proliferation and tumor growth. The findings in this study suggest that Mcm2 has considerable potential as a prognostic marker in RCCs, as shown in the survival differences when stratified by Mcm2 LI in the Phase I study.

In summary, these findings confirm previous evidence that Mcm2 protein is a superior marker to Ki-67 in the assessment of cell cycle entry in archival histological material and suggest that normal kidney has a small percentage of cells within the glomerular and tubular compartments that have entered cell cycle. We also demonstrate that tumor cell cycle state is limited by the angiogenic phenotype, supporting animal models showing angiogenesis determines the likelihood of tumor dormancy. Mcm2 may also be useful for improving the accuracy of tumor grading (44) in addition to providing information on the probability of patient survival. A study examining its role as a prognostic marker in a large series using a multivariate analysis is indicated.

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