Mcm2, Geminin, and KI67 Define Proliferative State and Are Prognostic Markers in Renal Cell Carcinoma

Tim J. Dudderidge,[1,2] Kai Stoeber,[1,2] Marco Loddo,[2] Geraldine Atkinson,[3] Thomas Fanshawe,[4] David F. Griffiths,[5] and Gareth H. Williams,[1,2]

Abstract **Purpose:** The origin licensing factors minichromosome maintenance 2 (Mcm2) and Geminin have recently been identified as critical regulators of growth and differentiation. Here we have investigated the regulation of these licensing factors together with Ki67 to further elucidate the cell cycle kinetics of renal cell carcinoma (RCC). Furthermore, we have examined the role of Ki67, Mcm2, and Geminin in disease-free survival after nephrectomy in patients with localized RCC.
Experimental Design: Tissue sections from 176 radical nephrectomy specimens were immunohistochemically stained with Mcm2, Geminin, and Ki67 antibodies. Labeling indices (LI) for these markers were compared with clinicopathologic parameters (median follow-up 44 months).
Results: In RCC, Mcm2 is expressed at much higher levels than Ki-67 and Geminin, respectively [medians 41.6%, 7.3%, and 3.5% ($P < 0.001$)] and was most closely linked to tumor grade ($P < 0.001$). For each marker, Kaplan-Meier survival curves provided strong evidence that increased expression is associated with reduced disease-free survival time ($P < 0.001$). Additionally, an Mcm2 − Ki67 LI identified a unique licensed but nonproliferating population of tumor cells that increased significantly with tumor grade ($P = 0.004$) and was also of prognostic value ($P = 0.01$). On multivariate analysis, grade, vascular invasion, capsular invasion, Ki67 LI >12%, and age were found to be independent prognostic markers.
Conclusions: Although Ki67 is identified as an independent prognostic marker, semiquantitative assessment is difficult due to the very low proliferative fraction identified by this marker. In contrast, Mcm2 identifies an increased growth fraction that is closely linked to grade, provides prognostic information, and is amenable to semiquantitative analysis in routine pathologic assessment.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/11/7/2510/1961499/2510-2517.pdf by guest on 14 December 2024

There are 6,200 new cases of renal cancer in the United Kingdom each year, accounting for 2% of all adult cancers. Around 90% of these are renal cell carcinomas (RCC) with an expected 5-year survival of 45%. In 2002, there were 3,360 deaths in the United Kingdom from renal cancer (1). Successful treatment of RCC can only be achieved through surgical excision at an early stage. This traditionally involves a radical nephrectomy, although partial nephrectomy (2) and tumor ablative approaches (3) are increasingly being used. There are concerns regarding the oncologic results of ablative procedures as there is no histologic confirmation of complete tumor removal. Furthermore, up to 10% of patients undergoing partial nephrectomy develop local tumor recurrence (4), which suggests that some small tumors, normally considered suitable for partial nephrectomy, should be managed with more radical surgery. Thus, a tissue marker that identified the biological potential of RCC could guide clinicians when selecting surgical treatment options for these patients. Where limited growth potential is identified, such a marker could support the use of a less invasive, tumor ablative technique.

Metastatic RCC is resistant to conventional chemotherapy and radiotherapy and has a poor prognosis with a median survival of 8 months (5). Adjuvant immunotherapy with IFN-α and interleukin-2 can achieve responses in 10% to 20% of patients (6, 7) and improved results are seen when treatment is preceded by cytoreductive nephrectomy (8, 9). However, in general, the results of adjuvant therapy have been disappointing and new agents that may improve the outlook for these patients are awaited (10). To effectively target the individuals most likely to benefit from adjuvant therapy, markers that assess the biological potential of tumors are needed. Such markers should allow clinicians to withhold potentially toxic therapies from patients with highly aggressive tumors, who cannot be helped by further treatment, as well as from those patients whose tumors are indolent and unlikely to recur.

The current preferred method of assessing the growth potential of RCC is the Fuhrman nuclear grade (11). This relies on a subjective assessment of the nuclear characteristics of tumor cells and is recognized as being highly subjective

Authors' Affiliations: [1]Wolfson Institute for Biomedical Research, [2]Department of Histopathology, and [3]Royal Free and University College Medical School, University College London, London, United Kingdom; [4]Center for Applied Medical Statistics, Department of Public Health and Primary Care, University of Cambridge, Cambridge, United Kingdom; and [5]Department of Pathology, University of Wales College of Medicine, Heath Park, Cardiff, United Kingdom
Received 9/1/04; revised 12/6/04; accepted 1/6/05.
Grant support: Cancer Research UK scientific program grant SP2360/0103.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Requests for reprints: David F. Griffiths, Department of Pathology, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, United Kingdom. Phone: 44-29-2074-5316; Fax: 44-29-2074-2701; E-mail: griffithsdfr@cf.ac.uk.
©2005 American Association for Cancer Research.

(12). Even in the hands of expert genitourinary pathologists, there is only fair agreement (interobserver agreement test κ = 0.29-0.33; refs. 13, 14). Thus, there is a need to identify reproducible, objective prognostic markers to complement Fuhrman nuclear grade (15), and many targets have been studied with this aim.

Ki67 has been suggested as having prognostic value in RCC (16). This 345 kDa protein is present in proliferating cells in all cell-cycle phases (G_1, S, G_2, and M) and absent in quiescent cells (G_0); thus, it can be used as a marker of growth fraction (17). In RCC, it has been shown to be a prognostic factor in both univariate analysis (16) and multivariate analysis (18–20), although conflicting evidence has challenged these findings (21, 22). Ki67 is not routinely used in clinical practice as semiquantitative analysis is difficult to undertake and reproduce because of the low-growth fraction in RCC. More intensive "counting" methods are time-consuming and not suitable for routine pathologic assessment. A prognostic tumor biomarker with a broader range of expression would enable histopathologists to objectively and quickly evaluate individual cases in a clinical setting. The initiation of DNA synthesis is a final and critical step in growth control and, therefore, of importance in carcinogenesis. Initiation of eukaryotic DNA replication is dependent on the assembly onto chromatin of prereplicative complexes containing origin recognition complex, Cdc6, Cdt1, and minichromosome maintenance (Mcm) 2 to 7 proteins, thus rendering origins licensed for one round of DNA replication during S phase (23–25). The prereplicative complexes are activated by cyclin-dependent kinases and Cdc7/ASK kinase leading to recruitment of elongation factors, Cdc45, DNA polymerases and RPA, to origins (26, 27). Recruitment of these factors to replication forks results in unwinding of the DNA helix and initiation of DNA synthesis. Replication initiation is tightly coupled to removal of the license, preventing relicensing after origin firing. This step is critical as origins must fire once, and once only each cell cycle, to ensure genomic stability. To avoid relicensing, mammalian cells have adopted a number of mechanisms. These include the inactivation of licensing factors during S, G_2, and M phases (a process controlled by cyclin-dependent kinase activity) regulated proteolysis, and changes in gene expression. The other significant mechanism is the expression of a repressor of origin licensing known as Geminin (27). This protein acts by binding Cdt1, thereby blocking MCM loading onto chromatin (28–30). In human cells, we have shown by membrane elution that Geminin expression is restricted to the S-G_2-M phases of the cell cycle, in keeping with its function of preventing rereplication (31). Importantly, the constituents of the prereplicative complex can be regarded as relay stations coupling growth regulatory pathways with DNA replication, thereby serving as novel biomarkers of growth (32).

We and others have shown in a range of different tumor types that dysregulation of the MCM proteins is an early event in tumorigenesis and have exploited these novel markers of growth in primary diagnosis, surveillance, and prognosis (33–38). Importantly, these studies show that the superior sensitivity of the MCM proteins over Ki67 resides in the fact that these novel markers of growth identify not only cycling cells but also noncycling cells with proliferative potential (39). Interestingly, this replication phenotype, Ki67 negative but MCM positive, is exhibited by premenopausal breast and primary oocytes, resting tissues that retain proliferative capacity and can rapidly respond to growth stimuli (39).[6]

Here we have investigated the regulation of selected origin licensing proteins in RCC to more precisely define the cell cycle kinetics of this tumor using a combined analysis of Ki67, Mcm2, and Geminin. This expression analysis has been combined with clinical outcome measures and clinicopathologic parameters to evaluate the prognostic value of these novel markers in RCC.

Materials and Methods

Patients and archival case material. Patients who had surgery for a primary RCC were identified by a computer search of the histopathology records of two hospitals, University of Wales College of Medicine, Cardiff, and Royal Glamorgan Hospital, Llantrisant, between January 1, 1991, and December 31, 1996. Urothelial carcinomas, childhood tumors, and cases with metastatic spread (including lymph node metastases) at presentation or operation were excluded, leaving 176 patients in the study group. Ethical approval for the study was received from the Bro Taf Local Research Ethics Committee.

A full description of the collection of tissue and the collation of patient data used in this study has been previously published (40). All patients in the study had undergone a radical nephrectomy but with no formal sampling of lymph nodes. Histology reports and slides were available for all these cases. For each tumor, the slides were reviewed by a pathologist with no knowledge of the clinical outcome and assessed for histologic type by the Heidelberg classification (41), Fuhrman nuclear grade (11), presence of capsular penetration with cellular invasion of perinephric fat (42), and the extent of any vascular invasion seen by microscopy (40).

Most patients had been reviewed annually as outpatients for 4 to 8 years; the following information was recorded from the patients' notes: date of birth, sex, date of surgery, date last seen, date of death, cause of death, and the date on which recurrent or metastatic disease was first identified. Other clinical data including the patient performance status was not consistently available at the time of review and, thus, were not available for analysis. The impact of performance status on disease-free survival in localized RCC is in any case controversial (43–45).

Two patients were lost to follow-up at 25 and 29 months. In all other survivors, the last recorded clinical contact was after January 1, 1998, giving a median (range) follow-up of 44 months (25-99 months). The date at which locally recurrent or metastatic disease was first identified was used in the analysis of disease-free survival. Disease-free survival was used in preference to actual survival as this was felt to be a more sensitive indicator of aggressive disease. Furthermore, patients who develop metastases often require adjuvant immunotherapy making this end point of clinical relevance. In 14 patients for whom the cause of death was recorded as RCC, the date of first recurrence was unavailable. In these cases, the date of death was considered the end point for disease-free survival. For disease-free survival, deaths from causes other than RCC were considered to be censored at the date of death.

Antibodies. Mouse anti-human monoclonal Mcm2 antibody (clone 46) was obtained from BD Transduction Laboratories (Lexington, KY). Mouse anti-human Ki67 antibody (clone MIB-1) was obtained from DAKO (Glostrup, Denmark). Affinity-purified rabbit polyclonal antibody against full-length human Geminin protein was generated as previously described (31). In summary, rabbits were injected with recombinant Geminin following a standard immunization protocol

[6] K.L. Eward, et al. DNA replication licensing in somatic and germ cells, submitted for publication.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/11/7/2510/1961499/2510-2517.pdf by guest on 14 December 2024

(Eurogentech, Seraing, Belgium). Rabbits were subsequently bled and the sera were affinity purified. Specificity of the anti-Geminin antibody was shown by immunoblotting, by quenching all immunohistochemical staining after preincubating antibodies with recombinant Geminin, and by flow cytometric analyses of asynchronous MOLT-4 cells after preincubation with both anti-Geminin antibody and recombinant Geminin or anti-Geminin antibody alone (31).

Immunohistochemistry. Paraffin sections were available for all patients in the series and for each specimen a block was selected that contained a sample of peripheral tumor. Sections of formalin-fixed, paraffin-embedded tissue were cut onto charged slides [either Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) or DAKO ChemMate 75 μm capillary gap slides S2024 (DAKO, Ely, United Kingdom)] at 4 μm thickness. The slides were dewaxed in xylene and rehydrated through graded alcohols. For antigen retrieval, slides were pressure cooked in 0.1 mol/L citrate buffer (pH 6) at 15 p.s.i. (103.4 kPa) for 1 to 4 minutes.

Tissue sections cut on to Superfrost Plus slides were stained manually using a standard protocol. Following antigen retrieval, the slides were washed thrice (using TBS with 0.1% Tween 20 for this and subsequent washes). Endogenous peroxidase activity was quenched with peroxidase-blocking solution (DAKO, Ely, United Kingdom) for 15 minutes. After washing, the primary antibody was applied for 1 hour at room temperature using antibody concentrations optimized in previous experiments on surplus tissue from the series (data not shown). The antibody concentrations found to be effective were 1/400 (Mcm2), 1/500 (G95), and 1/50 (Ki67). After washing, primary antibodies were detected using the ChemMate DAKO EnVision Detection kit (DAKO K5001, DAKO, Ely, United Kingdom). The slides were incubated with

Table 1. Relationship between Mcm2, Geminin, Ki67, and Mcm2 − Ki67 with other factors

Covariate	*n*	Mcm2	Geminin	Ki67	Mcm2 − Ki67
Grade					
Grade 1	20	36.14 (7.02)*	4.05 (1.07)	5.32 (1.48)	28.80 (5.63)
Grade 2	104	39.98 (2.54)	4.90 (0.54)	9.97 (1.00)	30.39 (2.05)
Grade 3	38	49.80 (4.09)	8.74 (1.42)	11.27 (1.56)	38.54 (3.80)
Grade 4	14	65.02 (6.00)	8.97 (2.52)	19.33 (4.92)	45.70 (5.88)
$P^{\dagger}$		<0.001	<0.001	0.002	0.004
Gender					
Male	120	44.19 (2.45)	5.82 (0.58)	11.22 (1.05)	33.29 (2.01)
Female	56	42.99 (3.74)	6.40 (1.07)	9.27 (1.38)	33.58 (3.15)
$P^{\ddagger}$		0.73	0.59	0.18	0.98
Age (mean, 63.7 y; SD, 11.3 y)					
<60 y	61	47.73 (3.43)	6.25 (0.86)	12.51 (1.39)	35.71 (2.82)
>60 y	115	41.78 (2.53)	5.88 (0.66)	9.55 (1.05)	32.16 (2.12)
$P^{\ddagger}$		0.15	0.36	0.01	0.22
Size (mean, 71.3 mm; SD, 30.3 mm)					
15-45 mm	43	31.57 (3.54)	4.11 (0.93)	7.96 (1.58)	24.92 (3.17)
50-65 mm	44	44.94 (3.71)	5.44 (0.77)	11.48 (1.53)	33.21 (2.92)
70-90 mm	46	47.89 (4.52)	7.87 (1.27)	11.18 (1.89)	35.54 (3.58)
95-150 mm	43	50.65 (3.95)	6.46 (1.07)	11.46 (1.67)	39.19 (3.54)
$P^{\dagger}$		0.001	0.009	0.08	0.003
Vascular invasion					
None	108	36.86 (2.36)	4.81 (0.51)	7.97 (0.82)	28.67 (1.99)
MVI	24	50.76 (5.46)	9.30 (2.00)	11.86 (2.26)	40.48 (5.23)
RVI	30	57.22 (5.26)	8.00 (1.66)	16.92 (2.83)	40.30 (3.96)
IVCI	14	56.20 (6.97)	5.39 (1.06)	14.18 (2.87)	42.02 (6.66)
$P^{\S}$		<0.001	0.03	0.001	0.006
Capsular invasion (one missing)					
Not seen	144	41.51 (2.24)	5.10 (0.47)	9.28 (0.83)	32.17 (1.91)
Present	31	54.51 (4.69)	9.85 (1.85)	16.62 (2.57)	39.22 (3.60)
$P^{\ddagger}$		0.01	0.01	0.005	0.07
Histologic type (2 collecting duct, 28 uncertain)					
Conventional	117	43.07 (2.42)	5.47 (0.60)	11.12 (1.12)	32.51 (1.92)
Papillary	23	36.94 (4.74)	5.89 (1.03)	8.01 (1.45)	26.86 (3.86)
Chromophobe	5	28.15 (11.34)	9.63 (7.28)	5.54 (2.04)	61 (9.60)
$P^{\ddagger,\parallel}$		0.34	0.27	0.59	0.25

Abbreviations: VI, microvascular invasion; RVI, renal vein invasion; IVCI, inferior vena cava invasion.

*Mean (SE), expressed as percentages.

†Jonckheere-Terpstra test.

‡Mann-Whitney *U* test.

§Kruskal-Wallis test.

$^{\parallel}$Compares conventional with papillary histologic type (n = 140).

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/11/7/2510/1961499/2510-2517.pdf by guest on 14 December 2024

the secondary antibody for 2 hours and developed with 3,3-diaminobenzidine for 10 minutes. Slides were then counterstained with Mayer's hematoxylin, differentiated in 1% acid alcohol, dehydrated, and cleared in xylene. Coverslips were applied with Leica CV Mount (Leica, Nussloch, Germany). Incubation without the primary antibody was used as a negative control and colonic epithelial sections were used as positive controls. Those sections mounted on DAKO ChemMate capillary gap slides were stained using the DAKO TechMate 500 immunostainer (DAKO, Cambridge, United Kingdom).

Protein expression profile analysis. To determine the labeling index (LI) in each tumor, slides were evaluated at low power (×100) to identify regions with the most intense degree of staining. Of note was the distribution of high-intensity staining at the advancing edge, with central areas showing consistently lower fractions of all markers. From the selected areas, three to five fields at ×400 magnification were captured with a charged coupled device camera and analySIS software (SIS, Münster, Germany). Images were subsequently printed for analysis. Quantitative analysis was undertaken with the assessor unaware of the clinicopathologic variables. Cells were identified as positive if there was any nuclear staining present and any stromal or inflammatory cells on the field were excluded. A mean of 681 nuclei was counted for each case. The LI was calculated by dividing the number of positive cells by the total number of cells counted. Reassessment of 10 randomly selected cases by an independent assessor showed high levels of agreement.

Statistical analysis. The expression profiles of each marker were compared using a Student's paired t test. Associations between marker expression and other factors were assessed using the Mann-Whitney U, Kruskal-Wallis, and Jonckheere-Terpstra tests. Because of multiple testing, two-sided $P < 0.01$ were judged to be statistically significant. Kaplan-Meier cumulative survival curves were constructed for Mcm2, Geminin, Ki67, and Mcm2 minus Ki67 (Mcm2 − Ki67). A log-rank test assessed the statistical significance of these curves. Multivariate analysis of the data was carried out using Cox's regression model for survival data. Analysis was carried out using SPSS 11.5 for Windows (SPSS, Inc., Chicago, IL) and S-PLUS 6 (MathSoft, Inc., Seattle, WA).

Results

Patient characteristics and classic prognostic markers. Details of the tumor series and relationship of clinicopathologic parameters to survival has been previously presented (40). Demographic and pathologic details of the patient cohort are included in Table 1. From the 176 patients in the series, high-quality immunohistochemical staining was available in 170 cases for Mcm2, 168 cases for Geminin, and 167 cases for Ki67. Data for the complete panel of markers were available for 164 patients and the multivariate analysis undertaken was based on these cases only.

Origin licensing in normal kidney and renal cell carcinoma. The pattern of origin licensing protein expression was first assessed in morphologically normal renal tissue adjacent to the stained tumor sections. The expression of Mcm2, Geminin, and Ki67 was extremely low (<1%) in keeping with previous observations that lowered proliferative capacity in stable tissues (e.g., liver and thyroid) is coupled to repression of origin licensing through down-regulation of MCM helicase subunits (24, 39, 46). In contrast, RCC shows high levels of Mcm2 and Geminin expression indicative of cell cycle reentry (31, 39, 46).

Relationship between Mcm2, Geminin, and Ki67 expression and clinicopathologic characteristics. The distributions of LIs for each marker as well as the median and interquartile ranges are shown in Fig. 1. Mcm2 expression in RCC was much higher

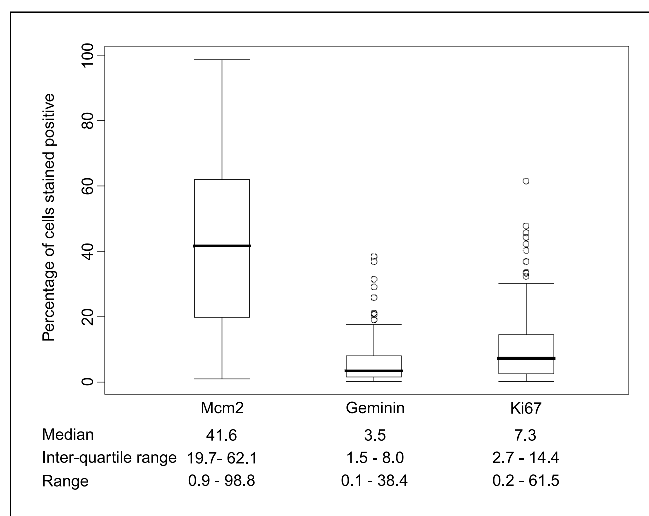

Fig. 1. The median (*solid black line*), interquartile range (*boxed*), and range (*enclosed by lines*) of Mcm2, Geminin, and Ki67 expression are shown (outlying cases are shown by isolated points). The broader distribution of Mcm2 compared with Ki67 reflects the additional licensed nonproliferating growth fraction, identified by Mcm2 but not Ki67. The low-growth fraction identified by Geminin compared with Ki67 is in keeping with its restricted expression to the S-G_2-M phases of the cell cycle. Overall, Mcm2 is expressed at significantly greater levels compared with Ki67, which itself is expressed more greatly than Geminin (paired t test, $P < 0.001$ in each case).

than the Ki67 expression, which was itself higher than the Geminin expression in these tumors [median: Mcm2, 41.6%; Ki67, 7.6%; Geminin, 3.5% (paired t test, $P < 0.001$ in each case)]. Furthermore, Mcm2 expression was distributed over a far broader range than the other markers (0.9-98.8%). The low-growth fraction identified by Geminin compared with Ki67 is in keeping with its restricted expression to the S-G_2-M phases of the cell cycle (31). There was a clear association of increasing Mcm2 LI with each increment of increasing grade ($P < 0.001$; Fig. 2; Table 1) and this trend was also seen to a lesser degree with Geminin and Ki67 (Fig. 3). To assess the influence of licensed nonproliferating cells, an Mcm2 − Ki67 index was calculated. Interestingly, this also increased significantly with increasing grade ($P = 0.004$) with the majority of grade 4 patients falling into this licensed, nonproliferating cell category. There were also trends of increasing Mcm2 expression with increasing tumor size ($P = 0.001$), vascular invasion ($P < 0.001$), and capsular invasion ($P = 0.001$; Table 1).

Mcm2, Geminin, and Ki67 expression and disease-free survival: Univariate and multivariate analyses. Kaplan-Meier survival curves for Mcm2, Geminin, Ki67, and Mcm2 − Ki67 are shown in Fig. 4. For each individual protein, the result of the associated log-rank test indicates there is strong evidence that increased levels of expression are associated with reduced disease-free survival time ($P < 0.001$). Interestingly, an Mcm2 − Ki67 index of >30% is also a significant prognostic factor ($P = 0.01$).

A multivariate (Cox regression) survival analysis was undertaken to determine if the markers studied offered prognostic information significantly greater than that gained from the classic markers, grade, vascular invasion, age, tumor size, capsular invasion, and histologic type (Table 2). Significant and independent prognostic factors in this analysis included tumor grade, which had the greatest prognostic power, followed by degree of vascular invasion, capsular

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/11/7/2510/1961499/2510-2517.pdf by guest on 14 December 2024

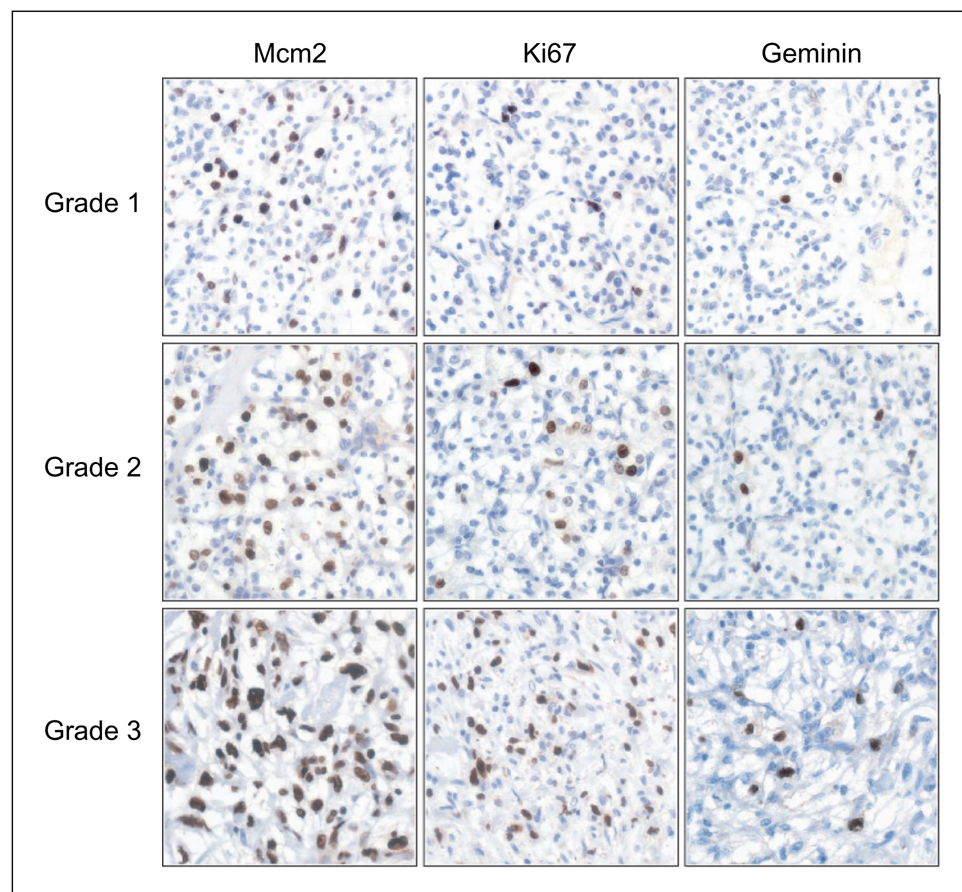

Fig. 2. Photomicrographs of paraffin-embedded tissue sections of RCC grades 1 to 3 immunohistochemically stained with antibodies to Mcm2, Ki67, and Geminin protein (positive cells stain brown). Mcm2 expression increases dramatically with increasing grade in these typical cases, in contrast to Geminin and Ki67, which only show modest increases. Original magnification, ×400.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/11/7/2510/1961499/2510-2517.pdf by guest on 14 December 2024

invasion, Ki67 (LI >12%), and, to a lesser extent, age. Tumor size and histologic type were omitted from the final Cox analysis as they had no influence when other variables were included. Although Mcm2, Geminin, and Mcm2 − Ki67 were all significant prognostic factors, there was no significant improvement in prognostic value when these were included separately in the multivariate analysis.

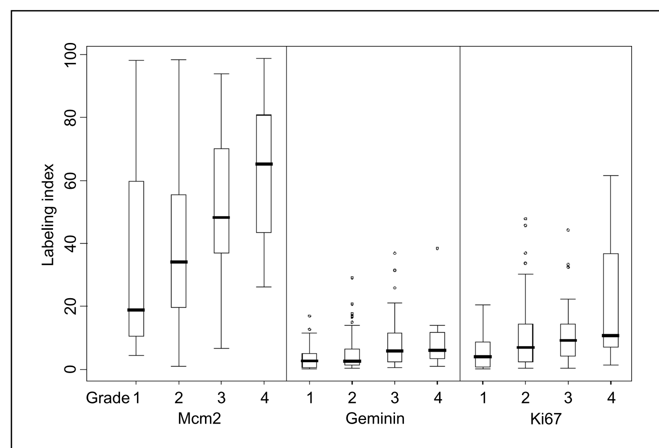

Fig. 3. The median (*solid black line*), interquartile range (*boxed*), and range (*enclosed by lines*) of Mcm2, Geminin, and Ki67 expression are shown according to tumor grade (outlying cases are shown by isolated points). The mean and interquartile range of Mcm2 increase dramatically with increasing grade compared with Geminin and Ki67.

Discussion

The initiation of DNA replication is a final and critical step in growth control lying downstream of the mitogenic signaling pathways (32). The initiation of DNA replication is regulated by a macromolecular complex of proteins called the prereplicative complex. A core component of the prereplicative complex consists of the Mcm2-7 complex of proteins, which unwinds the DNA helix, a prerequisite for the initiation of DNA synthesis. Geminin is a negative regulator of DNA replication and functions by inhibiting the recruitment of Mcm2-7 proteins onto chromosomal origins of replication. The prereplicative complex can, therefore, be regarded as a molecular switch or relay station connecting the growth signaling pathway to the process of genome duplication and is important for understanding the deregulated growth that characterizes neoplasia (32, 39). Using antibodies raised against the MCM family of proteins, we and others have shown that these represent novel biomarkers of growth with powerful diagnostic and prognostic potential in a wide range of tumors including cervical, prostate, bladder, colon, and esophageal cancer (33 – 38, 47 – 49).

We and others have previously shown that repression of origin licensing is a powerful downstream mechanism by which metazoan cells lower proliferative capacity (24, 39, 46). The withdrawal of cells into reversible arrest (G_0) or terminally differentiated states is coupled to down-regulation of Mcm2-7 and the origin licensing inhibitor Geminin

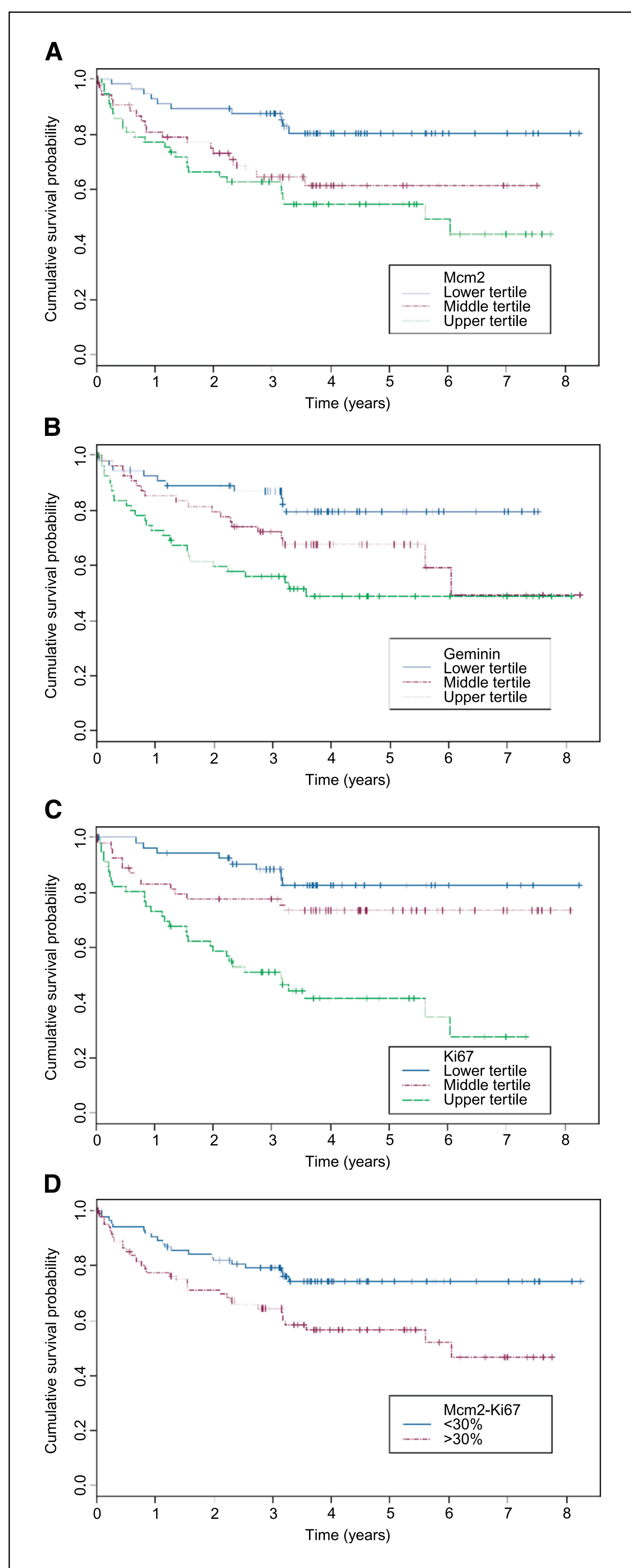

Fig. 4. Kaplan-Meier curves showing cumulative disease-free survival. *A*, Mcm2 tertiles (lower tertile, <27.87%; middle tertile, 27.87-56.21%; upper tertile, >56.21%), log rank test $P < 0.001$. *B*, Geminin tertiles (lower tertile, <2.04%; middle tertile, 2.04-5.98%; upper tertile, >5.98%), log rank test $P < 0.001$. *C*, Ki67 tertiles (lower tertile, <3.90%; middle tertile, 3.90-12.53%; upper tertile, >12.53%), log rank test $P < 0.001$. *D*, Mcm2 − Ki67 (low, <30%; high, >30%), log rank test $P = 0.01$.

Table 2. Cox regression analysis for predictors of survival

Covariate	Hazard ratio (95% confidence interval)	*P*
Grade		
Grade 1	1	
Grade 2	2.71 (0.36, 20.7)	0.34
Grade 3	4.99 (0.62, 40.2)	0.13
Grade 4	30.2 (3.57, 256.0)	0.002
Vascular invasion		
None	1	
MVI	1.57 (0.68, 3.62)	0.29
RVI	1.64 (0.74, 3.65)	0.22
IVCI	3.10 (1.32, 7.29)	0.01
Age (y)	0.98 (0.95-1.00)	0.07
Capsular invasion		
Not seen	1	
Present	2.26 (1.18-4.33)	0.01
Ki67		
Low (<12%)	1	
High (>12%)	2.22 (1.22-4.03)	0.009

(39).[7] However, MCM labeling also identifies noncycling cells with proliferative potential (39).[6] These are MCM-expressing cells that do not express Ki67 and whose failure to initiate DNA synthesis is reflected in the lack of Geminin expression in the S-G_2-M phases. Expression analysis of normal kidney parenchyma shows only very occasional MCM, Ki67, and Geminin-positive cells in keeping with repression of origin licensing proteins in the quiescent (G_0) state. Analysis of RCC reveals that the largest growth fraction is identified by Mcm2 but only a small proportion of these cells are cycling as identified by Ki67. The small number of cycling cells is reflected in the low values of Geminin, indicative of a failure of the majority of tumor cells to progress through the S-G_2-M phases of the cell cycle. Thus, a large fraction of the tumor cells in RCC reside in a licensed nonproliferating state. Interestingly, we have observed this replication phenotype in premalignant lesions of cervix and bladder (33–35).

Previous studies exploiting *in vitro* and *in vivo* model systems have shown that activation of the differentiation program in metazoan cells is coupled to down-regulation of the origin licensing factors Mcm2-7 and Geminin as cells exit the proliferative cell cycle and enter a terminally differentiated state (24).[8] In this study, the block to the differentiation program in RCC, indicated by increasing grade, was associated with increased expression of Ki67, Mcm2, and Geminin. Notably, there was also an increase in the proportion of "licensed cells with proliferative potential", indicated by the increase in the Mcm2 − Ki67 LI, the most prevalent cell type in grade 4 tumors. These findings indicate severe dysregulation of the origin licensing pathway in high-grade RCC. We found that levels of Mcm2, Geminin, and

[7] S. Kingsbury, et al. Repression of DNA replication licensing in quiescence is independent of geminin to define the cell cycle state of progenita cells.
[8] L. Barkley, personal communication.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/11/7/2510/1961499/2510-2517.pdf by guest on 14 December 2024

Ki67, as determined by immunocytochemistry and quantification with a LI, provide prognostic information with respect to disease-free survival in RCC patients undergoing radical nephrectomy for localized disease. Moreover, an increasing Mcm2 − Ki67 LI was also associated with reduced disease-free survival, suggesting that the proportion of noncycling Mcm2 expressing tumor cells strongly influences its potential for growth.

It is interesting that whereas a clear relationship between Mcm2 and grade has been shown in this study, there is, nevertheless, a fairly heterogeneous expression of Mcm2 in grade 1 and 2 tumors (Fig. 3), indicating a broad spectrum of growth potential within each tumor grade. Up to 10% of patients treated with partial nephrectomy develop local recurrence (4). Although our data does not specifically address the prediction of local recurrence, we have shown that Mcm2 is a sensitive marker of tumor growth and future studies could assess the value of Mcm2 as a predictor of local recurrence after partial nephrectomy. Furthermore, the effect of immunotherapy upon origin licensing in metastatic RCC could provide a valuable predictor of disease response. This approach has already been taken in a study assessing proliferative response using Ki67 antibody (50). However, whereas Donskov et al. (50) found that Ki67 was a prognostic factor, Ki67 response to immunotherapy did not identify treatment responders. Here we have shown that Mcm2 is a much more sensitive growth marker than Ki67 in RCC; thus, the antiproliferative effect of immunotherapy may be more easily measured using Mcm2. Indeed, we have recently exploited this approach to identify responders of endocrine neoadjuvant therapy in breast cancer.[9]

There is controversy in the literature regarding the prognostic value of Ki67 in the assessment of RCC (15). Here, we clearly show that Ki67 is an independent prognostic factor in RCC. However, its use in routine clinical practice is constrained because of the very low-growth fraction identified by this marker leading to difficulties with semiquantitative assessment. In contrast, Mcm2 identifies an increased growth fraction that makes it amenable to semiquantitative analysis during routine pathologic assessment. Although not an independent marker of disease-free survival, because of its close link to grade, we have shown that Mcm2 provides prognostic information that could complement current prognostic factors when assessing these patients.

In summary, analysis of the origin licensing pathway in RCC has shown that dysregulation of the DNA replication licensing machinery is a major event during renal carcinogenesis. This study has shown that the MCM helicase proteins represent potentially useful prognostic molecular markers that could be exploited in routine clinical practice. Moreover, this study provides supporting evidence that Ki67 is an independent prognostic marker of disease-free survival in RCC.

[9] A. Shetty, personal communication.

References

1. Cancer Research UK [homepage on the Internet]. London: Cancer Research UK; c1999-2004 [updated 2004 Feb 2; cited 2004 Jun 15]. About cancer-statistics-Kidney Cancer. Available from: www.cancer-researchuk.org.
2. Novick AC. Nephron-sparing surgery for renal cell carcinoma. Annu Rev Med 2002;53:393−407.
3. Anderson CJ, Havranek EG. Minimally invasive ablative techniques in renal cancer. BJU Int 2004;93: 707−9.
4. Uzzo RG, Novick AC. Nephron sparing surgery for renal tumors: indications, techniques and outcomes. J Urol 2001;166:6−18.
5. Haas GP, Hillman GG, Redman BG, Pontes JE. Immunotherapy of renal cell carcinoma. CA Cancer J Clin 1993;43:177−87.
6. Wirth MP. Immunotherapy for metastatic renal cell carcinoma. Urol Clin North Am 1993;20:283−95.
7. Motzer RJ, Russo P, Nanus DM, Berg WJ. Renal cell carcinoma. Curr Probl Cancer 1997;21:185−232.
8. Mickisch GH, Garin A, van Poppel H, de Prijck L, Sylvester R. Radical nephrectomy plus interferon-α-based immunotherapy compared with interferon α alone in metastatic renal-cell carcinoma: a randomised trial. Lancet 2001;358:966−70.
9. Flanigan RC, Salmon SE, Blumenstein BA, et al. Nephrectomy followed by interferon α-2b compared with interferon α-2b alone for metastatic renal-cell cancer. N Engl J Med 2001;345:1655−9.
10. Fishman M, Antonia S. Novel therapies for renal cell carcinoma—an update. Expert Opin Investig Drugs 2003;12:593−609.
11. Fuhrman SA, Lasky LC, Limas C. Prognostic significance of morphologic parameters in renal cell carcinoma. Am J Surg Pathol 1982;6:655−63.
12. Medeiros LJ, Jones EC, Aizawa S, et al. Grading of renal cell carcinoma: Workgroup No. 2. Union Internationale Contre le Cancer and the American Joint Committee on Cancer (AJCC). Cancer 1997;80:990−1.
13. Lanigan D, Conroy R, Barry-Walsh C, Loftus B, Royston D, Leader M. A comparative analysis of grading systems in renal adenocarcinoma. Histopathology 1994;24:473−6.
14. Al Aynati M, Chen V, Salama S, Shuhaibar H, Treleaven D, Vincic L. Interobserver and intraobserver variability using the Fuhrman grading system for renal cell carcinoma. Arch Pathol Lab Med 2003;127:593−6.
15. Mejean A, Oudard S, Thiounn N. Prognostic factors of renal cell carcinoma. J Urol 2003;169:821−7.
16. Hofmockel G, Tsatalpas P, Muller H, et al. Significance of conventional and new prognostic factors for locally confined renal cell carcinoma. Cancer 1995;76: 296−306.
17. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984;133: 1710−5.
18. Visapaa H, Bui M, Huang Y, et al. Correlation of Ki-67 and gelsolin expression to clinical outcome in renal clear cell carcinoma. Urology 2003;61: 845−50.
19. Rioux-Leclercq N, Turlin B, Bansard J, et al. Value of immunohistochemical Ki-67 and p53 determinations as predictive factors of outcome in renal cell carcinoma. Urology 2000;55:501−5.
20. Aaltomaa S, Lipponen P, Ala-Opas M, Eskelinen M, Syrjanen K. Prognostic value of Ki-67 expression in renal cell carcinomas. Eur Urol 1997;31:350−5.
21. Gelb AB, Sudilovsky D, Wu CD, Weiss LM, Medeiros LJ. Appraisal of intratumoral microvessel density, MIB-1 score, DNA content, and p53 protein expression as prognostic indicators in patients with locally confined renal cell carcinoma. Cancer 1997;80: 1768−75.
22. Cheville JC, Zincke H, Lohse CM, et al. pT1 clear cell renal cell carcinoma: a study of the association between MIB-1 proliferative activity and pathologic features and cancer specific survival. Cancer 2002;94: 2180−4.
23. Dimitrova DS, Prokhorova TA, Blow JJ, Todorov IT, Gilbert DM. Mammalian nuclei become licensed for DNA replication during late telophase. J Cell Sci 2002; 115:51−9.
24. Blow JJ, Hodgson B. Replication licensing-defining the proliferative state? Trends Cell Biol 2002; 12:72−8.
25. Mendez J, Stillman B. Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. Mol Cell Biol 2000;20:8602−12.
26. Masai H, Arai K. Cdc7 kinase complex: a key regulator in the initiation of DNA replication. J Cell Physiol 2002;190:287−96.
27. Nishitani H, Lygerou Z. Control of DNA replication licensing in a cell cycle. Genes Cells 2002;7:523−34.
28. McGarry TJ, Kirschner MW. Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell 1998; 93:1043−53.
29. Wohlschlegel JA, Dwyer BT, Dhar SK, Cvetic C, Walter JC, Dutta A. Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science 2000;290: 2309−12.
30. Tada S, Li A, Maiorano D, Mechali M, Blow JJ. Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. Nat Cell Biol 2001; 3:107−13.
31. Wharton SB, Hibberd S, Eward KL, et al. DNA replication licensing and cell cycle kinetics of oligodendroglial tumours. Br J Cancer 2004;91:262−9.
32. Williams G, Stoeber K. Clinical applications of a novel mammalian cell-free DNA replication system. Br J Cancer 1999;80:20−4.
33. Stoeber K, Halsall I, Freeman A, et al. Immunoassay for urothelial cancers that detects DNA replication protein Mcm5 in urine [letter]. Lancet 1999;354:1524−5.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/11/7/2510/1961499/2510-2517.pdf by guest on 14 December 2024

34. Stoeber K, Swinn R, Prevost AT, et al. Diagnosis of genito-urinary tract cancer by detection of minichromosome maintenance 5 protein in urine sediments. J Natl Cancer Inst 2002;94:1071–9.

35. Williams GH, Romanowski P, Morris L, et al. Improved cervical smear assessment using antibodies against proteins that regulate DNA replication. Proc Natl Acad Sci U S A 1998;95:14932–7.

36. 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.

37. Wharton SB, Chan KK, Anderson JR, Stoeber K, Williams GH. Replicative Mcm2 protein as a novel proliferation marker in oligodendrogliomas and its relationship to Ki67 labelling index, histological grade and prognosis. Neuropathol Appl Neurobiol 2001;27: 305–13.

38. Kruger S, Thorns C, Stocker W, Muller-Kunert E, Bohle A, Feller AC. Prognostic value of MCM2 immunoreactivity in stage T_1 transitional cell carcinoma of the bladder. Eur Urol 2003;43:138–45.

39. Stoeber K, Tlsty TD, Happerfield L, et al. DNA replication licensing and human cell proliferation. J Cell Sci 2001;114:2027–41.

40. Griffiths DF, Verghese A, Golash A, et al. Contribution of grade, vascular invasion and age to outcome in clinically localized renal cell carcinoma. BJU Int 2002;90:26–31.

41. Kovacs G, Akhtar M, Beckwith BJ, et al. The Heidelberg classification of renal cell tumours. J Pathol 1997;183:131–3.

42. Thomas DH, Verghese A, Kynaston HG, Griffiths DF. Analysis of the prognostic implications of different tumour margin types in renal cell carcinoma. Histopathology 2003;43:374–80.

43. Palmer PA, Vinke J, Philip T, et al. Prognostic factors for survival in patients with advanced renal cell carcinoma treated with recombinant interleukin-2. Ann Oncol 1992;3:475–80.

44. Zisman A, Pantuck AJ, Dorey F, et al. Mathematical model to predict individual survival for patients with renal cell carcinoma. J Clin Oncol 2002;20: 1368–74.

45. Leibovich BC, Blute ML, Cheville JC, et al. Prediction of progression after radical nephrectomy for patients with clear cell renal cell carcinoma: a stratification tool for prospective clinical trials. Cancer 2003; 97:1663–71.

46. Stoeber K, Mills AD, Kubota Y, et al. Cdc6 protein causes premature entry into S phase in a mammalian cell-free system. EMBO J 1998;17:7219–29.

47. Davies RJ, Freeman A, Morris LS, et al. Analysis of minichromosome maintenance proteins as a novel method for detection of colorectal cancer in stool. Lancet 2002;359:1917–9.

48. Going JJ, Keith WN, Neilson L, Stoeber K, Stuart RC, Williams GH. Aberrant expression of minichromosome maintenance proteins 2, 5 and Ki-67 in dysplastic squamous oesophageal epithelium and Barrett's mucosa. Gut 2002;50:373–7.

49. Williams GH, Swinn R, Prevost AT, et al. Diagnosis of oesophageal cancer by detection of minichromosome maintenance 5 protein in gastric samples. Br J Cancer 2004;91:714–9.

50. Donskov F, Marcussen N, Hokland M, Fisker R, Madsen HH, von der MH. *In vivo* assessment of the antiproliferative properties of interferon-α during immunotherapy: Ki-67 (MIB-1) in patients with metastatic renal cell carcinoma. Br J Cancer 2004; 90:626–31.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/11/7/2510/1961499/2510-2517.pdf by guest on 14 December 2024