Carbonic Anhydrase IX Is an Independent Predictor of Survival in Advanced Renal Clear Cell Carcinoma: Implications for Prognosis and Therapy

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ABSTRACT

Purpose: **Metastatic renal cell carcinoma (RCC) has a poor prognosis and an unpredictable course. To date, there are no molecular markers which can reliably predict RCC outcome. We investigated whether a novel kidney cancer marker, carbonic anhydrase IX (CAIX), is associated with progression and survival.**

Experimental Design: **Immunohistochemical analysis using a CAIX monoclonal antibody was performed on tissue microarrays constructed from paraffin-embedded specimens from patients ($N = 321$) treated by nephrectomy for clear cell RCC. CAIX staining was correlated with response to treatment, clinical factors, pathologic features, and survival.**

Results: **CAIX staining was present in 94% of clear cell RCCs. Survival tree analysis determined that a cutoff of 85% CAIX staining provided the most accurate prediction of survival. Low CAIX (≤85%) staining was an independent poor prognostic factor for survival for patients with metastatic RCC, with a hazard ratio of 3.10 ($P < 0.001$). CAIX significantly substratified patients with metastatic disease when analyzed by T stage, Fuhrman grade, nodal involvement, and performance status ($P < 0.001$, $= 0.001$, $= 0.009$, $= 0.005$, respectively). For patients with nonmetastatic RCC and at high risk for progression, low CAIX predicted a worse outcome similar to patients with metastatic disease ($P = 0.058$). Overall expression of CAIX decreased with development of metastasis; as demonstrated by the lower CAIX staining levels in metastatic lesions relative to matched primary tumor specimens ($P = 0.036$).**

Conclusions: **On the basis of our data, CAIX is the most significant molecular marker described in kidney cancer to date. Decreased CAIX levels are independently associated with poor survival in advanced RCC. CAIX reflects significant changes in tumor biology, which should be used to predict clinical outcome and identify high-risk patients in need for adjuvant immunotherapy and CAIX-targeted therapies.**

INTRODUCTION

RCC[3] accounted for >31,000 new cases of cancer and contributed to ~12,000 deaths in the United States in 2001 (1). Clear cell carcinoma was the predominant subtype comprising up to 85% of RCCs. One-third of patients diagnosed with kidney cancer have evidence of metastatic disease at the time of diagnosis and up to half of those treated for localized disease eventually relapse (2). The natural history of RCC is complex and is influenced by factors other than stage (3). Patient and tumor-related factors have been proposed as prognostic factors (4–7). Therefore, understanding how the complex interactions between multiple prognostic factors contribute to the clinical behavior of RCC is essential for patient assessment, outcome prediction, and therapy planning.

CAIX protein, a member of the carbonic anhydrase family, is thought to play a role in the regulation of cell proliferation in response to hypoxic conditions and may be involved in oncogenesis and tumor progression (8–10). Previous studies using a monoclonal antibody against CAIX have shown that CAIX is induced constitutively in certain tumor types but is absent in most normal tissues with the exception of epithelial cells of the gastric mucosa (10–13). Furthermore, previous immunobiochemical studies of malignant and benign renal tissues revealed that CAIX was also highly expressed in RCC, suggesting that CAIX expression may be a useful diagnostic biomarker (14, 15).

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[3] The abbreviations used are: RCC, renal cell carcinoma; CAIX, carbonic anhydrase IX; UCLA, University of California Los Angeles; IL-2, interleukin 2; ECOG PS, Eastern Cooperative Oncology Group performance status; DSS, disease-specific survival; ROC, receiver-operator curve; 95% CI, 95% confidence interval.

Clinical tumor targeting studies by i.v. injection with a monoclonal antibody to CAIX have shown localization to RCC tumors in a mouse tumor model (16) and are being applied in clinical trials to treat metastatic RCC (17–19). However, the relationship between CAIX expression and RCC survivorship is unknown.

To investigate the importance and clinical significance of CAIX expression, we used tissue microarrays (20) for high-throughput molecular profiling of RCC tumor specimens based on their CAIX expression. Furthermore, UCLA has a RCC database with 1200 patients containing >263 clinical variables for each patient. We examined the clinical information from this data resource to evaluate the association of CAIX expression with clinical outcome.

PATIENTS AND METHODS

Patients. Our study cohort consisted of 321 patients who underwent a radical or partial nephrectomy for clear cell RCC at UCLA between 1989 and 2000. Following study protocol (KCP 99–233) approval by the UCLA Institutional Review Board, a retrospective study was performed with outcome assessment based on chart review of clinical and pathologic data. The median age was 62 years (27–89 years), and the male to female ratio was 2:1. The median follow-up for patients who died ($N = 170$) from an RCC-related cause was 14 months (1.3–101 months) and for all survivors was 45 months (0.3–117 months). The diagnosis of metastatic disease was determined at initial presentation. IL-2-based immunotherapy was administered to 86 patients, 70 of whom presented with metastatic disease, with the remaining 16 developing recurrent disease after nephrectomy for initially localized RCC.

Stage was determined according to the 1997 Union Internationale Contre le Cancer tumor-node-metastasis classification of malignant tumors (21). T, N, and M stages were determined by clinical and/or pathologic data. ECOG PS and metastatic status was determined at initial presentation (22).

Fifteen patients had concurrent resection of a metastatic lesion at the time of nephrectomy for the primary tumor. Histopathological evaluation confirmed that the metastatic lesion arose from the same histological type as the primary kidney tumor.

Tissue Array Construction. Archival tumor specimens from our cohort of 321 patients and 15 metastatic lesions were obtained from the Department of Pathology at the UCLA Medical Center. All tumors were of the clear cell subtype according to Union Internationale Contre le Cancer guidelines and were staged according to the 1997 tumor-node-metastasis classification and graded according to the Fuhrman grading scheme by a single pathologist (D. S.; Refs. 21, 23). Three core tissue biopsies, 0.6 mm in diameter, were taken from selected morphologically representative regions of each paraffin-embedded renal or metastatic tumor and precisely arrayed using a custom-built instrument as described previously (20). An additional core tissue biopsy was taken from a morphologically normal-appearing region of each tumor. Sections of 4-μm thickness of each tissue array block were transferred to glass slides using the paraffin sectioning aid system (adhesive coated slides PSA-CS4x, adhesive tape, UV lamp; Instrumedics Inc., Hackensack, NJ) to support the cohesion of 0.6-mm array elements. Quality control was assessed on each block by H&E staining after every five consecutive sections to confirm the grade and histological type of each tissue core spot.

Immunohistochemistry. The mouse monoclonal antibody (M75) used to detect the CAIX protein has been described previously (11, 24). Immunohistochemical staining of tissue sections with anti-CAIX antibody was done using a peroxidase technique with antigen retrieval using heat treatment, as previously described using the Dako staining systems (Dako Corporation, Carpenteria, CA; Ref. 24). The CAIX primary antibody was used at a 1:10,000 dilution. Semiquantitative assessment of the antibody staining was performed by a single pathologist (D. S.) blinded to the clinicopathological variables. Staining intensity was based on a 4-point scale from 0 to 3. The extent of staining was recorded as a percentage of the target tissue sample that had positive CAIX expression. Each spot was scored based on the staining intensity, the percentage of positive cells, and the percentage staining at maximal staining intensity. There were three spots/patient specimen. The overall score used for subsequent statistical analysis was the pooled mean from three spots of the same tumor. A score of zero was given to tissue spots that had no evidence of specific immunostaining.

Statistical Analysis. The primary outcome of interest was DSS from the time of nephrectomy to demise or to last follow-up. We used the default settings of the recursive partitioning function in S-Plus (Insightful Corp.) statistical analysis software for survival tree analysis to find appropriate cut-offs for classifying patients according to amount of CAIX expression.[4] The Kaplan-Meier method was used to estimate DSS (25); log-rank analysis was applied to test the difference between stratified survival functions. The Cox proportional hazards model (26) was used to test the statistical independence and significance of CAIX expression in predicting the risk of patient death based on a variety of potential prognostic factors (27). Logistic regression was used to quantify the area under the ROC. The binomial test was used to determine statistical significance of CAIX expression in the primary tumor compared with the metastasis. All *P*s were two sided, and $P < 0.05$ was considered significant. Statistical analyses were performed, and graphs were constructed using Stata statistical analysis software version 7.0 (Stata Corp, College Station, TX).

RESULTS

The clinical characteristics of the 321 patients (216 men and 105 women) at the time of nephrectomy for clear cell RCC are summarized in Table 1. Among these patients, the median age was 61 years, the median tumor size was 7 cm and 46% had metastatic disease at the time of presentation. The median follow-up time of all patients who died of a cancer-related death was 14 months (range, 1.3–101 months) and for all survivors was 45 months (range, 0.3–117 months).

[4] Y. Huang and S. Horvath. Analyzing tissue microarray data with classification and survival trees. UCLA Biostatistics, 2003, in preparation.

Table 1 Patient clinical characteristics and CAIX expression in clear cell carcinoma

			CAIX (No. of patients)			
		Overall (N = 321)	Negative	Positive	Low ≤ 85%	High > 85%
Gender						
Male		216				
Female		105				
Age, yr						
Mean	60.4 ± 11.7					
Median	61.6					
Range	27–89					
Tumor size, cm						
Median	7		5	7	7.5	6.5
Mean	7.3 ± 3.8		6.5 ± 4.6	7.3 ± 3.7	8.1 ± 3.4	7.1 ± 3.6
Range	0.8–18.0					
Metastasis						
No		172	15	157	32	140
Yes		149	6	143	34	115
T[a] stage						
1		114	13	101	22	92
2		39	2	37	8	31
3		150	6	144	29	121
4		18	0	18	7	11
Grade						
1		38	3	35	6	32
2		151	10	141	32	119
3		110	5	105	20	90
4		22	3	19	8	14
Nodes						
0		272	17	261	50	228
1		14	1	13	5	9
2		23	3	20	9	14
ECOG PS						
0		115	5	110	15	100
1		190	15	175	46	144
2		13	1	12	5	8
3		1	0	1	0	1
IL-2-based immunotherapy		86	1	85	14	72
Overall response		22			2	20
Complete response		7			0	7
No response		27			7	20
Stable disease		27	1		4	23
NE		3			1	2
Duration median follow-up, months						
Cancer-related deaths	14 (1.3–101)					
All survivors	44 (0.3–117)					
Relapses		38				
Deaths		170				

[a] T, tumor; NE, not evaluable.

CAIX Expression in Relation to Clinicopathological Variables. CAIX expression was seen in 94% (301 of 321) of tumor specimens. CAIX staining was predominantly found on the plasma membrane and varied according to the proportion of the target tissue in the core that stained positively (Fig. 1*A*). The staining intensity staining was strong with minimal variation. Tissue core biopsies taken from a morphologically normal-appearing region of each tumor specimen were uniformly negative for CAIX whereas tumor regions predominantly stained intensely.

Survival tree analysis of CAIX scoring information from the tissue arrays identified that a staining percentage of 85% was an ideal cutoff for stratification for patient survival. Staining percentages > 85%, irrespective of intensity, were considered high CAIX staining, whereas those ≤85% were considered low CAIX staining (Fig. 1*A*). Only 4.7% (N = 15) of patients in our cohort had staining percentages within the range of 80–90% (Fig. 1*B*). Most of the patients (N = 255, 79%) had >85% CAIX staining, whereas 21% (N = 66) had ≤85% CAIX staining. Survival of patients with CAIX-negative staining (0%) did not differ statistically from patients with low (≤85%) CAIX staining (data not shown). For patients with metastatic RCC, Kaplan-Meier estimated DSS showed that high CAIX was associated with a median survival of 24.8 months, whereas low CAIX had a median survival of only 5.5 months ($P < 0.001$; Fig. 1*C*).

For patients diagnosed with metastatic disease at the time of initial presentation, CAIX expression provided important

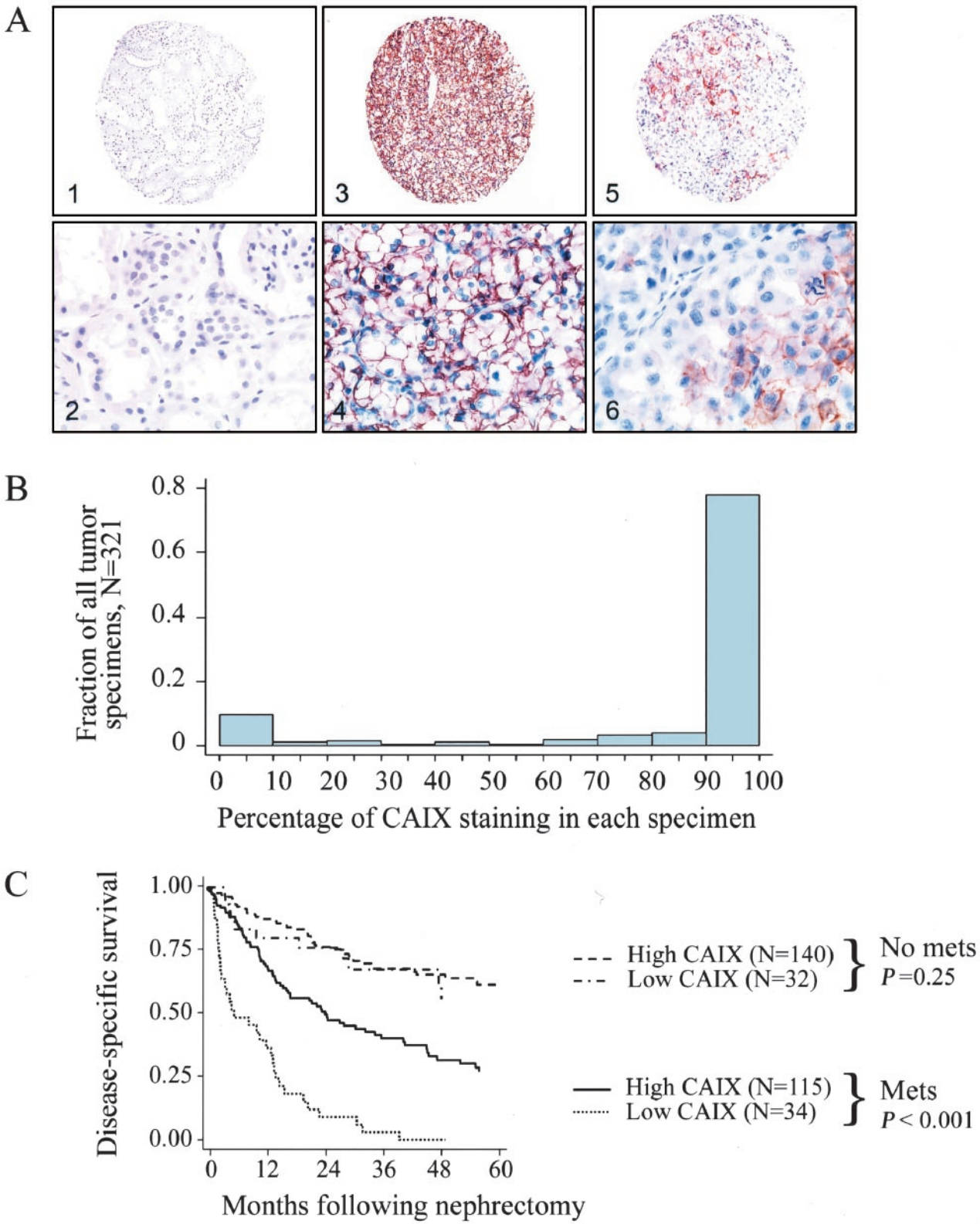

Fig. 1 Immunohistochemical analysis of CAIX and DSS according to the levels of CAIX expression for patients with clear RCC. *A*, immunohistochemical analysis shows the pattern of CAIX staining in the tissue cores (at ×100 and ×400 magnification). Normal kidney tissues (*A1–A2*) were ubiquitously negative for CAIX. Clear cell RCC had intense membrane staining for high levels (>85% positive staining, *A3–A4*) or low levels (≤85%, *A5–A6*) of CAIX. *B* shows distribution of CAIX staining for all tumor specimens. *C* shows the Kaplan-Meier curves of DSS for patients according to CAIX expression and metastatic status.

Table 2 Median DSS (months) according to CAIX expression for patients with metastatic clear cell carcinoma

	Low CAIX	High CAIX	*P*
Metastasis	5.5	24.8	<0.001
Low T stage (1 or 2)	12.2	54.4	<0.001
High T stage (3 or 4)	4.0	16.7	<0.001
Low grade (1 or 2)	15.2	32.7	0.001
High grade (3 or 4)	3.9	16.3	<0.001
No nodes	12.8	27.3	<0.001
Nodes > 0	2.9	12.5	0.009
ECOG PS = 0	9.8	44.2	0.005
ECOG PS > 0	4.8	21.2	<0.001

additional prognostic information when DSS was analyzed according to T stage and Fuhrman's grade. Table 2 lists the median survivals for patients with metastatic disease when stratified according to high or low CAIX staining. High CAIX expression predicted a better prognosis for both low and high T stage as shown by Kaplan-Meier estimates of DSS (Fig. 2, *A* and *B*). Interestingly, patients with both low T stage and low CAIX staining had a significantly worse median survival time (12.2 months) than patients with both high T stage and high CAIX staining (16.7 months; $P = 0.032$). Similarly, high CAIX staining demonstrated more favorable survival for both low- and high-grade tumors (Fig. 2, *C* and *D*). The survival time for low-grade tumors with low CAIX expression was not statistically different from the survival time for high-grade tumors with high CAIX (median, 15.2 *versus* 16.3 months; $P = 0.119$). No statistically significant associations were found between the level of CAIX expression and either T stage or grade for patients with metastatic disease.

Prediction of DSS according to nodal status and ECOG PS could be additionally substratified by CAIX expression in patients with metastatic disease (Fig. 3). For patients with no nodal disease, high CAIX staining predicted better survival than low CAIX staining, median 27.3 months *versus* 12.8 months, respectively ($P < 0.001$; Fig. 3*A*). Similarly, in patients with nodal disease ($N = 1$ or $N = 2$), high CAIX predicted a median survival of 12.5 *versus* 2.9 months for low CAIX ($P = 0.009$; Fig. 3*B*). Interestingly, median survival for patients with both node-negative disease and low CAIX expression was not statistically different from the survival of patients with both node-positive disease and high CAIX expression, 12.8 *versus* 12.5 months, respectively ($P = 0.103$; Table 2). Higher CAIX expression also predicted a more favorable survival for patients with ECOG PS = 0 with median survival of 44.2 months for high CAIX *versus* 9.8 months for low CAIX ($P = 0.005$; Fig. 3*C*). For patients with ECOG PS > 0, high CAIX staining predicted a median survival of 21.2 *versus* 4.8 months with low CAIX ($P < 0.001$; Fig. 3*D*).

When patients with localized RCC were analyzed altogether, CAIX staining initially did not appear to stratify survival ($P = 0.25$; Fig. 1*C*). However, using the Cox proportional hazards model, we identified a subset of patients with localized RCC and no nodal or metastatic involvement that could be additionally stratified by CAIX. Patients with T stage ≥ 3 and grade ≥ 2 were categorized as high risk for progression with a median survival of 28.5 months compared with low-risk patients (T stage ≤ 2 and grade = 1) who had a median survival of over 106 months ($P < 0.001$; Fig. 4*A*). There were no missing data for T stage or grade for patients with localized RCC. The high-risk patients were statistically distinct from the patients with metastatic disease who had a median survival of 16.7 months ($P = 0.021$; Fig. 4*A*). Stratification of high-risk patients according to high or low CAIX staining approached statistical significance ($P = 0.058$; Fig. 4*B*) and was limited by small sample size ($N = 47$). High-risk patients with high CAIX ($N = 41$) had a median survival of 30.3 months. However, high-risk patients with low CAIX staining ($N = 6$) had a worse prognosis with a median survival time of only 10 months and had a similar clinical outcome as patients with metastatic disease (16 months median survival). For low-risk nonmetastatic patients, CAIX status did not provide prognostic information. CAIX status also did not predict relapses ($N = 38$) in patients with nonmetastatic disease, perhaps because of small sample size.

Relationship of CAIX to DSS. Univariate analysis of established prognostic factors and their relationship to DSS in metastatic disease confirmed that CAIX status, T stage, grade, nodal status, and ECOG PS were all statistically significant prognosticators (Table 3*A*). There were no apparent relationships between CAIX and other prognostic variables such as age, gender, and tumor size. In univariate analysis, CAIX status for high-risk nonmetastatic patients approached statistical significance ($P = 0.068$) with hazard ratio of 2.53.

In multivariate Cox proportional hazards analysis, CAIX status was analyzed with T stage, grade, nodal status, and ECOG PS for their impact on DSS. For nonmetastatic disease, CAIX status was not an independent predictor of survival. However, for patients with metastatic disease, all of these covariates, except for nodal status, were significant independent predictors of DSS (Table 3*B*). Low CAIX staining in metastatic RCC was found to be independently associated with death from RCC, with a hazard ratio of 3.10 ($P < 0.001$; 95% CI, 1.99–4.83). The addition of CAIX status to a logistic regression model consisting of T stage, grade, nodal status, and ECOG PS for metastatic disease increased the area under an ROC curve from 0.66 to 0.76 indicating improved prediction of survival. The time period used to calculate the ROC curve was 36 months. Furthermore, we censored data for patients with >36 months follow-up and for patients who were alive but lost to follow-up. For the entire cohort of patients, 62% of patients with metastatic disease died from RCC by 36 months. The area under the ROC curve did not change when CAIX status was included for nonmetastatic disease.

Relationship between CAIX Expression in Primary Tumor and Metastatic Lesion. To determine the effect on CAIX expression when a tumor metastasizes, the level of CAIX expression in the metastatic lesion and the primary tumor were compared. Fifteen patients had resection of a metastatic lesion (9 lymph nodes, 2 liver, 1 lung, 1 adrenal, 1 colon, and 1 chest wall) at the time of nephrectomy for RCC. The tumor specimens were compared based on the percentage staining at maximal intensity. CAIX expression appeared to be less in the metastatic lesion (3 of 13) compared with the primary tumor (Fig. 5). CAIX expression in both the primary tumor and metastasis was absent in 1 patient and equal in another. Overall, CAIX staining

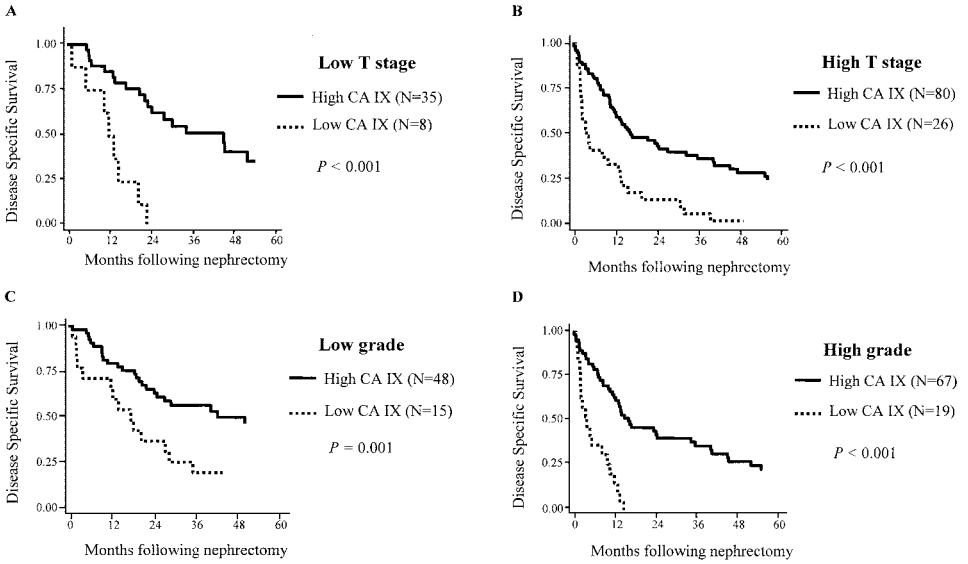

Fig. 2 DSS in patients with metastatic clear carcinoma. Kaplan-Meier estimates according to CAIX expression (*A*) low T stage (stages 1 and 2), (*B*) high T stage (stages 3 and 4), (*C*) low grade (grades 1 and 2), and (*D*) high grade (grades 3 and 4). N = number of patients.

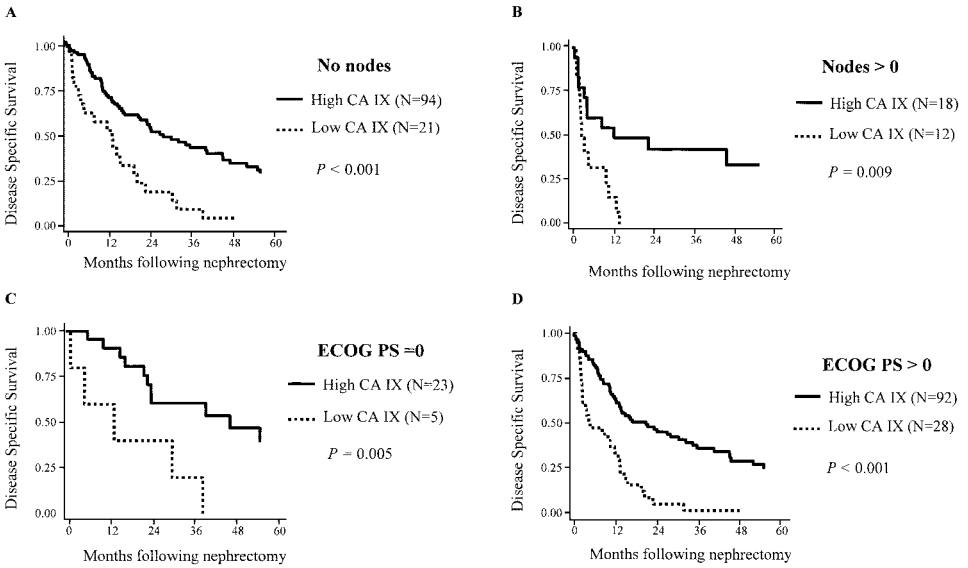

Fig. 3 DSS in patients with metastatic clear carcinoma. Kaplan-Meier estimates according to CAIX expression for (*A*) no nodes, (*B*) nodes > 0, (*C*) ECOG PS = 0, and (*D*) ECOG PS > 0. ECOG PS, Eastern Cooperative Oncology Group performance status. N = number of patients.

appeared greater in the primary tumor than in the metastatic lesion ($P = 0.036$; binomial test).

DISCUSSION

Traditionally, stage, grade, and performance status have been the most useful predictors of outcome for RCC (28). However, molecular markers have the potential to make a significant impact on the diagnosis and treatment of RCC. Tumor markers provide not only prognostic information to aid in the identification of patients at risk for recurrence or metastasis but could also facilitate the rational use of targeted therapeutic interventions as well. This concept has been elegantly demonstrated for the molecular marker, Her2/neu, and its use in the prognosis and treatment of breast cancer (29). The recent development of microarray technology will permit the rapid identification and validation of diagnostic and molecular markers. We report on one such marker, CAIX, which has been studied with tissue microarrays and correlated with clinical data to provide prognostic information.

Previous immunohistochemical investigations (24) have suggested that CAIX is a potential diagnostic biomarker for cervical neoplasms. An immunohistochemical study of RCC (15) also reported that CAIX was expressed in all examined RCCs, including granular, spindle, and papillary carcinomas, but not in those consisting of chromophobe histology or in benign renal lesions, including oncocytomas. Yet another study of 187 RCC demonstrated CAIX expression in 87% of tumors by immunohistochemistry (14). Our study of 321 primary clear

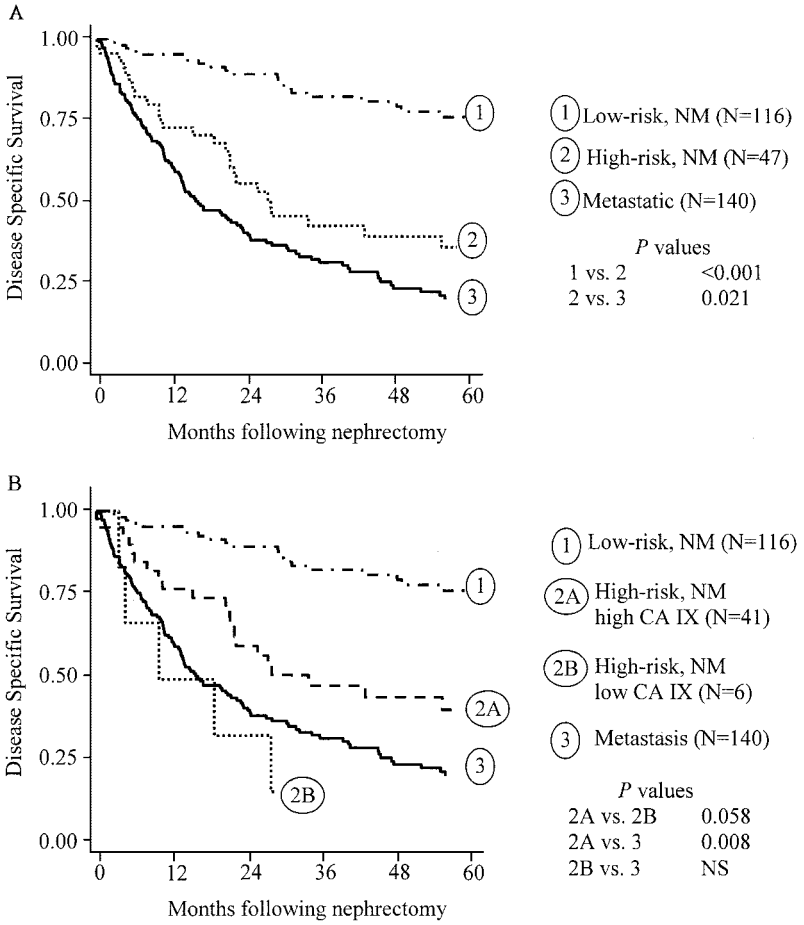

Fig. 4 DSS in patients with localized and metastatic clear cell RCC. Kaplan-Meier estimates according to (*2A*) high-risk (T stage ≥ 3 and grade ≥ 2) and low-risk (T stages ≤ 2 and grade = 1) patients with localized RCC and (*2B*) high-risk patients stratified by high and low CAIX expression. NM, nonmetastatic; N = number of patients; NS, not significant.

cell RCC tumors confirmed the high specificity of CAIX staining with 94% positive staining in clear cell carcinomas in the kidney. Other studies have shown that CAIX detection by reverse transcription-PCR assays in tumor specimens have a high correlation with immunohistochemistry (13, 14).

Targeted therapies directed at CAIX are being developed to exploit the exclusivity of CAIX expression in RCC for the treatment of metastatic disease. Early Phase I and II clinical trials are addressing the feasibility of radioimmunotherapy using a monoclonal antibody against CAIX coupled to a radioisotope and have shown only minor therapeutic responses for patients with metastatic RCC (16–19). Other therapy modalities target the immunogenicity of CAIX as a RCC tumor antigen (30) by developing tumor-cell vaccines and dendritic cell vaccines (31, 32). Yet another targeted approach would be to inhibit CAIX activity with chemical inhibitors. A recent study showed that a carbonic anhydrase inhibitor, acetazolamide, was able to inhibit the invasive capacity of renal cancer cells *in vitro* (33). Because CAIX is a cell surface protein unlike intracellular carbonic anhydrase isoenzymes, the design of specific chemical inhibitors of CAIX that are cell impermeable may demonstrate higher selectivity and less toxicity for suppressing renal cancer invasion.

The molecular role of CAIX in tumorigenesis is currently being elucidated, and RCC provides a unique model to study the role of hypoxia in solid tumor oncogenesis and progression. Constitutive expression of CAIX as a result of von Hippel-Lindau protein mutations (34) has been described for RCC. However, recent studies now indicate that expression of CAIX is regulated by the hypoxia-inducible factor 1 transcriptional complex that mediates expression of a number of genes in response to hypoxic conditions (35). Furthermore, higher CAIX expression has been seen in perinecrotic regions of several tumor types (10, 36). It has been postulated that cell surface carbonic anhydrases regulate acid-base balance to optimize conditions in the tumor invasiveness (10). Acidification of the extracellular matrix is known to induce expression of angiogenic factors (37) and may inhibit cellular immunity (38), which additionally promotes tumor aggressiveness. In addition, there is some evidence for the association of CAIX with loss of contact inhibition and anchorage dependence of cancer cells (33).

We found that low CAIX expression was a predictor of worse survival in patients with advanced RCC. The relationship between low CAIX expression and poor prognosis has been shown in studies with cervical carcinoma (39), colorectal carcinoma (40), and esophageal cancer (41). In contrast, other studies have found that increased CAIX expression correlated with worse survival in cervical carcinoma (42), lung cancer (38), and breast cancer (38). The reasons for these differences

Table 3 Cox analyses for DSS for metastatic clear cell carcinoma

	A. Cox univariate analysis		
	Hazard ratio	95% CI	Significance
Variable			
CAIX, low expression	3.17	2.07–4.86	<0.001
ECOG PS	1.62	1.18–2.24	0.003
Grade	1.52	1.15–2.01	0.004
Tumor stage	1.44	1.12–1.87	0.005
Nodal status	1.32	1.02–1.71	0.033
	B. Cox multivariate analysis		
	Hazard ratio	95% CI	Significance
Variable			
CAIX, low expression	3.10	1.99–4.83	<0.001
ECOG PS	1.67	1.20–2.36	0.003
Tumor stage	1.37	1.04–1.79	0.023
Grade	1.37	1.03–1.83	0.032
Nodal status	1.15	0.88–1.52	NS

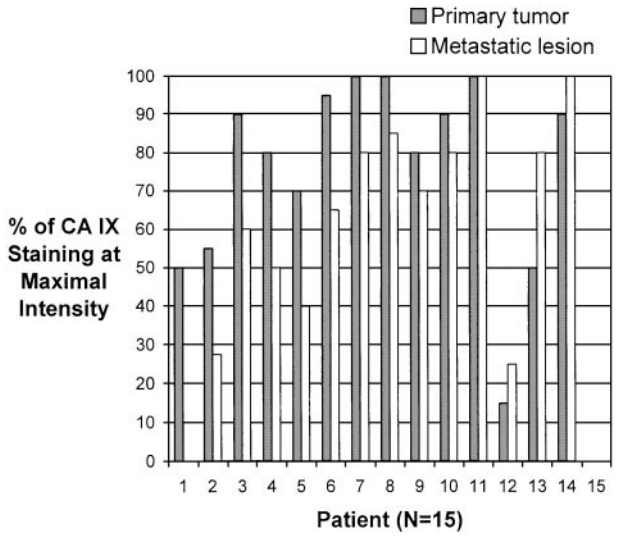

Fig. 5 CAIX expression in the primary tumor and metastatic lesion. *Bar graph* showing the percentage of CAIX staining at the maximal intensity in the primary tumor and corresponding metastatic lesion in 15 patients ($P = 0.036$).

are unclear but may be related to whether CAIX expression reflects tumor progression or directly influences tumor behavior. Furthermore, in our study, low CAIX status identified a subset of patients with high-risk localized RCC who had a clinical outcome similar to patients with metastatic disease. This group of patients would be excellent candidates for adjuvant immunotherapy trials.

Survival tree analysis used a method of recursive partitioning to define the 85% threshold for high and low CAIX staining that maximized the survival distributions between potential groups (43). The subsequent covariates were used in a Cox regression to obtain *P*s. Similar statistical results were found when the CAIX threshold was lowered to 0% (*e.g.*, positive or negative CAIX expression, $P = 0.008$; data not shown), but this did not maximize the survival distributions. Furthermore, the range of 0–85% for the low CAIX group reflected a homogeneous population that was not able to be additionally stratified by smaller ranges (data not shown). The 15 cases within the range of 80–90%, which were not reliably separated into by the 85% cutoff, constituted only 4.7% of our cohort of 321 patients, whereas the remaining 95.3% of our population could be discerned by the 85% cutoff. External validation of CAIX staining levels by other investigators will be important to arrive at a standardized cutoff value for application to patient care.

Our findings show that decreased CAIX expression occurs in tumors with the highest malignant potential. This is unlikely to be explained by the loss of differentiation because there was no correlation with Fuhrman grade. Furthermore, the overall expression of CAIX appears to decrease with development of metastases; the level of CAIX is less in the metastatic lesion than in the parental primary tumor (Fig. 5). This suggests that CAIX may play a functional role in tumor progression. We hypothesize that in the earlier stages of tumor progression, noxious conditions such as hypoxia or ischemia induce CAIX expression as an adaptation to confer proliferation advantage for tumor growth and spread; however, when this malignant potential is attained in the later stages of tumor growth, continued CAIX expression is no longer a requirement. Our analysis does not preclude the alternative hypothesis that the cumulative effects of genetic lesions involved in cancer progression could alter the pathways of hypoxia response (44) and therefore affect CAIX expression. Additional studies will be needed to determine whether genetic changes underlie differences in CAIX expression in the primary tumors and in metastatic lesions.

Using a Cox proportional multivariate analysis with established prognostic variables, CAIX status demonstrated independent prognostic significance for metastatic RCC. Including CAIX status with prognostic factors such as T stage, grade, and ECOG PS for patients with metastatic RCC demonstrated an increase of the area under the ROC curve from 0.66 to 0.76, indicating improved prediction of survival. For patients with localized RCC, the addition of CAIX status to the regression model did not change the area under the ROC curve, perhaps because of small sample size. Additional clinical validation and standardization will be necessary for CAIX status to be used directly for patient management.

RCC belongs to a small group of tumor types that have been shown to respond to biological immune therapy. Although the responses to systemic cytokine therapy for metastatic RCC have been promising, the overall results have been inadequate perhaps because limited activity in some patients and substantial toxicity in others. Therefore, careful patient selection and stratification to various types of adjuvant immunotherapies may delineate those patients most likely to respond to treatment. Preliminary data from our cohort of patients suggested a relationship between CAIX and immunotherapy response that could have implications for clinical-trial assignment and targeted therapies. IL-2-based immunotherapy was administered to 86 patients for metastatic disease. When stratified according to CAIX status, 73 (84%) patients had high CAIX staining and 14 (16%) had low CAIX (Table 1). All complete responses to IL-2 immunotherapy (8%) included patients within the high CAIX staining group. Furthermore, overall response rate to IL-2 was

greater in the group with high CAIX (27%) than in the group with low CAIX (14%). This finding could be exploited in the design of clinical trials of IL-2-based therapy and other biological response modifiers for RCC by stratifying patients based on CAIX status. Similarly, patient recruitment for targeted therapies based on monoclonal antibodies to CAIX (18) or immunotherapy with CAIX-based RCC vaccines (32) should also consider stratification based on CAIX staining. We also found a subset of patients with high-risk localized RCC and low CAIX status that behave clinically similar to patients with metastatic disease. This may indicate the presence of micrometastasis and that adjuvant immunotherapy may be of benefit for this group of patients. Overall, given that nephrectomy for known metastatic disease has been demonstrated to be helpful in prospective trials (45), determining a patient's CAIX status by immunohistochemistry could easily be incorporated into the selection and design of treatment regimens.

In conclusion, our investigation of CAIX expression in a large number of RCC demonstrates that CAIX expression is highly associated with survivorship for kidney cancer. Low CAIX expression predicts a worse outcome for patients with locally advanced RCC and is an independent predictor of poor survival in patients with metastatic RCC. CAIX status may potentially aid in the selection of patients who might benefit from IL-2 or CAIX-targeted therapies. Furthermore, patients with high-risk localized RCC and low CAIX may be potential candidates for adjuvant immunotherapy. Our observations with CAIX demonstrate that the integration of molecular markers with established prognostic factors will result in more accurate prognosis and will direct novel therapies to improve the survival of patients with metastatic RCC.

REFERENCES

1. Jemal, A., Thomas, A., Murray, T., and Thun, M. Cancer statistics, 2002. CA - Cancer J. Clin., *52:* 23–47, 2002.

2. Figlin, R. A. Renal cell carcinoma: management of advanced disease. J. Urol., *161:* 381–386; discussion 386–387, 1999.

3. Pantuck, A. J., Zisman, A., and Belldegrun, A. S. The changing natural history of renal cell carcinoma. J. Urol., *166:* 1611–1623, 2001.

4. Bretheau, D., Lechevallier, E., de Fromont, M., Sault, M. C., Rampal, M., and Coulange, C. Prognostic value of nuclear grade of renal cell carcinoma. Cancer (Phila.), *76:* 2543–2549, 1995.

5. Elson, P. J., Witte, R. S., and Trump, D. L. Prognostic factors for survival in patients with recurrent or metastatic renal cell carcinoma. Cancer Res., *48:* 7310–7313, 1988.

6. Motzer, R. J., Mazumdar, M., Bacik, J., Berg, W., Amsterdam, A., and Ferrara, J. Survival and prognostic stratification of 670 patients with advanced renal cell carcinoma. J. Clin. Oncol., *17:* 2530–2540, 1999.

7. Bui, M. H., Zisman, A., Pantuck, A., Han, K., Wieder, J., and Belldegrun, A. Prognostic factors and molecular markers for renal cell carcinoma. Exp. Rev. Anticancer Ther., *1:* 565–575, 2001.

8. Pastorek, J., Pastorekova, S., Callebaut, I., Mornon, J. P., Zelnik, V., Opavsky, R., Zat'ovicova, M., Liao, S., Portetelle, D., Stanbridge, E. J., *et al*. Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment. Oncogene, *9:* 2877–2888, 1994.

9. Wykoff, C. C., Pugh, C. W., Maxwell, P. H., Harris, A. L., and Ratcliffe, P. J. Identification of novel hypoxia dependent and independent target genes of the von Hippel-Lindau (VHL) tumour suppressor by mRNA differential expression profiling. Oncogene, *19:* 6297–6305, 2000.

10. Ivanov, S., Liao, S. Y., Ivanova, A., Danilkovitch-Miagkova, A., Tarasova, N., Weirich, G., Merrill, M. J., Proescholdt, M. A., Oldfield, E. H., Lee, J., Zavada, J., Waheed, A., Sly, W., Lerman, M. I., and Stanbridge, E. J. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am. J. Pathol., *158:* 905–919, 2001.

11. Zavada, J., Zavadova, Z., Pastorekova, S., Ciampor, F., Pastorek, J., and Zelnik, V. Expression of MaTu-MN protein in human tumor cultures and in clinical specimens. Int. J. Cancer, *54:* 268–274, 1993.

12. Oosterwijk, E., Ruiter, D., and Joedemaeker, P. Monoclonal antibody G250 recognizes a determinant present in renal cell carcinoma and absent from normal kidney. Int. J. Cancer, *38:* 489–494, 1986.

13. Murakami, Y., Kanda, K., Tsuji, M., Kanayama, H., and Kagawa, S. MN/CA9 gene expression as a potential biomarker in renal cell carcinoma. BJU Int., *83:* 743–747, 1999.

14. Uemura, H., Nakagawa, Y., Yoshida, K., Saga, S., Yoshikawa, K., Hirao, Y., and Oosterwijk, E. MN/CA IX/G250 as a potential target for immunotherapy of renal cell carcinomas. Br. J. Cancer, *81:* 741–746, 1999.

15. Liao, S. Y., Aurelio, O. N., Jan, K., Zavada, J., and Stanbridge, E. J. Identification of the MN/CA9 protein as a reliable diagnostic biomarker of clear cell carcinoma of the kidney. Cancer Res., *57:* 2827–2831, 1997.

16. Steffens, M. G., Oosterwijk, W. J., Zegwaart, H. N., Boerman, O. C., Debruyne, F. M., Corstens, F. H., and Oosterwijk, E. Immunohistochemical analysis of tumor antigen saturation following injection of monoclonal antibody G250. Anticancer Res., 1197–1200, 1999.

17. Steffens, M. G., Boerman, O. C., Oosterwijk, W. J., Oosterhof, G. O., Witjes, J. A., Koenders, E. B., Oyen, W. J., Buijs, W. C., Debruyne, F. M., Corstens, F. H., and Oosterwijk, E. Targeting of renal cell carcinoma with iodine-131-labeled chimeric monoclonal antibody G250. J. Clin. Oncol., *15:* 1529–1537, 1997.

18. Divgi, C. R., Bander, N. H., Scott, A. M., O'Donoghue, J. A., Sgouros, G., Welt, S., Finn, R. D., Morrissey, F., Capitelli, P., Williams, J. M., Deland, D., Nakhre, A., Oosterwijk, E., Gulec, S., Graham, M. C., Larson, S. M., and Old, L. J. Phase I/II radioimmunotherapy trial with iodine-131-labeled monoclonal antibody G250 in metastatic renal cell carcinoma. Clin. Cancer Res., *4:* 2729–2739, 1998.

19. Steffens, M. G., Boerman, O. C., de, M. P., Oyen, W. J., Buijs, W. C., Witjes, J. A., van den, Broek, W. J., Oosterwijk, W. J., Debruyne, F. M., Corstens, F. H., and Oosterwijk, E. Phase I radioimmunotherapy of metastatic renal cell carcinoma with 131I-labeled chimeric monoclonal antibody G250. Clin. Cancer Res., 3268s–3274s, 1999.

20. Kononen, J., Bubendorf, L., Kallioniemi, A., Barlund, M., Schraml, P., Leighton, S., Torhorst, J., Mihatsch, M. J., Sauter, G., and Kallioniemi, O. P. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat. Med., *4:* 844–847, 1998.

21. Sobin, L. H., and Fleming, I. D. TNM Classification of Malignant Tumors, 5 Ed. (1997). Union Internationale Contre le Cancer and the American Joint Committee on Cancer. Cancer (Phila.), *80:* 1803–1804, 1997.

22. Roila, F., Lupattelli, M., Sassi, M., Basurto, C., Bracarda, S., Picciafuoco, M., Boschetti, E., Milella, G., Ballatori, E., Tonato, M., *et al*. Intra and interobserver variability in cancer patients' performance status assessed according to Karnofsky and ECOG scales. Ann. Oncol., *2:* 437–439, 1991.

23. Fuhrman, S. A., Lasky, L. C., and Limas, C. Prognostic significance of morphologic parameters in renal cell carcinoma. Am. J. Surg. Pathol., *6:* 655–663, 1982.

24. Liao, S. Y., Brewer, C., Zavada, J., Pastorek, J., Pastorekova, S., Manetta, A., Berman, M. L., DiSaia, P. J., and Stanbridge, E. J. Identification of the MN antigen as a diagnostic biomarker of cervical intraepithelial squamous and glandular neoplasia and cervical carcinomas. Am. J. Pathol., *145:* 598–609, 1994.

25. Kaplan, E., and Meier, P. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc., *53:* 457–481, 1958.

26. Cox, D. R., and Oakes, D. Analysis of survival data. New York: Chapman and Hall, 1990.

27. Kalbfleisch, J. D., and Prentice, R. L. The statistical analysis of failure time data. New York: John Wiley, 1980.

28. Zisman, A., Pantuck, A. J., Dorey, F., Said, J. W., Shvarts, O., Quintana, D., Gitlitz, B. J., deKernion, J. B., Figlin, R. A., and Belldegrun, A. S. Improved prognostication of renal cell carcinoma using an integrated staging system. J. Clin. Oncol., *19:* 1649–1657, 2001.

29. Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., and Norton, L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N. Engl. J. Med., *344:* 783–792, 2001.

30. Vissers, J. L., De, V. I., Schreurs, M. W., Engelen, L. P., Oosterwijk, E., Figdor, C. G., and Adema, G. J. The renal cell carcinoma-associated antigen G250 encodes a human leukocyte antigen (HLA)-A2.1-restricted epitope recognized by cytotoxic T lymphocytes. Cancer Res., *59:* 5554–5559, 1999.

31. Uemura, H., Beniers, A. J., Okajima, E., Debruyne, F. M., and Oosterwijk, E. Vaccination with anti-idiotype antibodies mimicking a renal cell carcinoma-associated antigen induces tumor immunity. Int. J. Cancer, *58:* 555–561, 1994.

32. Tso, C. L., Zisman, A., Pantuck, A., Calilliw, R., Hernandez, J. M., Paik, S., Nguyen, D., Gitlitz, B., Shintaku, P. I., de Kernion, J., Figlin, R., and Belldegrun, A. Induction of G250-targeted and T-cell-mediated antitumor activity against renal cell carcinoma using a chimeric fusion protein consisting of G250 and granulocyte/monocyte-colony stimulating factor. Cancer Res., *61:* 7925–7933, 2001.

33. Parkkila, S., Rajaniemi, H., Parkkila, A. K., Kivela, J., Waheed, A., Pastorekova, S., Pastorek, J., and Sly, W. S. Carbonic anhydrase inhibitor suppresses invasion of renal cancer cells *in vitro*. Proc. Natl. Acad. Sci. USA, *97:* 2220–2224, 2000.

34. Ivanov, S. V., Kuzmin, I., Wei, M. H., Pack, S., Geil, L., Johnson, B. E., Stanbridge, E. J., and Lerman, M. I. Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel-Lindau transgenes. Proc. Natl. Acad. Sci. USA, *95:* 12596–12601, 1998.

35. Wykoff, C. C., Beasley, N. J., Watson, P. H., Turner, K. J., Pastorek, J., Sibtain, A., Wilson, G. D., Turley, H., Talks, K. L., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J., and Harris, A. L. Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res., *60:* 7075–7083, 2000.

36. Olive, P. L., Aquino-Parsons, C., MacPhail, S. H., Liao, S. Y., Raleigh, J. A., Lerman, M. I., and Stanbridge, E. J. Carbonic anhydrase 9 as an endogenous marker for hypoxic cells in cervical cancer. Cancer Res., *61:* 8924–8929, 2001.

37. Shi, Q., Abbruzzese, J. L., Huang, S., Fidler, I. J., Xiong, Q., and Xie, K. Constitutive and inducible interleukin 8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorigenic and metastatic. Clin. Cancer Res., *5:* 3711–3721, 1999.

38. Giatromanolaki, A., Koukourakis, M. I., Sivridis, E., Pastorek, J., Wykoff, C. C., Gatter, K. C., and Harris, A. L. Expression of hypoxia-inducible carbonic anhydrase-9 relates to angiogenic pathways and independently to poor outcome in non-small cell lung cancer. Cancer Res., *61:* 7992–7998, 2001.

39. Brewer, C. A., Liao, S. Y., Wilczynski, S. P., Pastorekova, S., Pastorek, J., Zavada, J., Kurosaki, T., Manetta, A., Berman, M. L., DiSaia, P. J., and Stanbridge, E. J. A study of biomarkers in cervical carcinoma and clinical correlation of the novel biomarker MN. Gynecol. Oncol., *63:* 337–344, 1996.

40. Saarnio, J., Parkkila, S., Parkkila, A. K., Haukipuro, K., Pastorekova, S., Pastorek, J., Kairaluoma, M. I., and Karttunen, T. J. Immunohistochemical study of colorectal tumors for expression of a novel transmembrane carbonic anhydrase. MN/CA IX, with potential value as a marker of cell proliferation. Am. J. Pathol., *153:* 279–285, 1998.

41. Turner, J. R., Odze, R. D., Crum, C. P., and Resnick, M. B. MN antigen expression in normal, preneoplastic, and neoplastic esophagus: a clinicopathological study of a new cancer-associated biomarker. Hum. Pathol., *28:* 740–744, 1997.

42. Loncaster, J. A., Harris, A. L., Davidson, S. E., Logue, J. P., Hunter, R. D., Wycoff, C. C., Pastorek, J., Ratcliffe, P. J., Stratford, I. J., and West, C. M. Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. Cancer Res., *61:* 6394–6399, 2001.

43. Zhang, H., and Singer, B. Recursive partitioning in the health sciences, p. 238. New York: Springer-Verlag, 1999.

44. Rak, J., Yu, J. L., Kerbel, R. S., and Coomber, B. L. What do oncogenic mutations have to do with angiogenesis/vascular dependence of tumors? Cancer Res., *62:* 1931–1934, 2002.

45. Flanigan, R. C., Salmon, S. E., Blumenstein, B. A., Bearman, S. I., Roy, V., McGrath, P. C., Caton, J. R., Jr., Munshi, N., and Crawford, E. D. Nephrectomy followed by interferon α-2b compared with interferon α-2b alone for metastatic renal-cell cancer. N. Engl. J. Med., *345:* 1655–1659, 2001.

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