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Carbonic Anhydrase IX Expression Predicts Outcome of Interleukin 2 Therapy for Renal Cancer

Michael Atkins,^{1,4} Meredith Regan,^{2,4} David McDermott,^{1,4} James Mier,^{1,4} Eric Stanbridge,^{5,6}
Amanda Youmans,¹ Philip Febbo,^{2,4} Melissa Upton,¹ Mirna Lechpammer,²
and Sabina Signoretti,^{2,4}

Abstract Purpose: Renal cancer response to interleukin 2 (IL-2) therapy and patient survival has been correlated with tumor histology and carbonic anhydrase IX (CAIX) expression. In an effort to confirm and expand these observations, we examined CAIX expression in pathology specimens from renal cancer patients who had previously received IL-2 therapy.

Experimental Design: Paraffin-embedded tissue sections of renal cancer were immunostained with the MN-75 monoclonal antibody to CAIX and expression levels were correlated with histologic findings and clinical outcome.

Results: Tissue specimens were obtained from 66 patients; 27 of whom (41%) had responded to IL-2–based therapy. Fifty-eight specimens were assessed as clear cell, with 56, 33, and 4 having alveolar, granular, and papillary features, respectively. Twenty-four (36%), 31 (47%), and 11 (17%) were classified into good, intermediate, and poor prognosis groups according to the Upton pathology model. Forty-one specimens (62%) had high CAIX expression. Twenty-one of 27 (78%) responding patients had high CAIX expressing tumors compared with 20 of 39 (51%) nonresponders (odds ratio, 3.3; $P = 0.04$). Median survival was prolonged ($P = 0.04$) and survival >5 years was only seen in high CAIX expressers. In patients with intermediate pathologic prognosis, all nine responders had high CAIX expression versus 11 of 22 nonresponders. A resultant group with good pathologic prognosis alone or with intermediate pathologic prognosis and high CAIX contained 26 of 27 (96%) responders compared with 18 of 39 (46%) nonresponders (odds ratio, 30; $P < 0.01$) and exhibited longer median survival ($P < 0.01$).

Conclusions: CAIX expression seems to be an important predictor of outcome in renal cell carcinoma patients receiving IL-2–based therapy and may enhance prognostic information obtained from pathology specimens.

Metastatic renal cell carcinoma remains a therapeutic challenge. High-dose interleukin 2 (IL-2), the only U.S. Food and Drug Administration–approved therapy, produces tumor regression in 15% to 20% of patients (1), with only 7% of patients showing durable long-term freedom from recurrence (2). Two recently completed phase III trials supported

the superiority of the high-dose IL-2 regimen in terms of response rate, complete response, and durable response relative to lower dose regimens containing either IL-2 alone or IL-2 in combination with IFN α (3, 4). Unfortunately, high-dose IL-2 treatment is associated with considerable toxicity and expense, making it an impractical standard therapy. Improved treatment strategies and/or better methods of identifying those patients likely to benefit from IL-2–based therapy are needed.

Considerable data is now available to help predict the outcome for patients with advanced renal cancer receiving cytokine-based immunotherapy. Factors that have been variably associated with response include performance status, number of organs with metastases (one versus two or more), absence of bone metastases, prior nephrectomy, degree of treatment-related thrombocytopenia, absence of prior IFN therapy, thyroid dysfunction, rebound lymphocytosis, erythropoietin production, and posttreatment elevations of tumor necrosis factor- α and IL-1 blood levels (5).

Figlin et al. identified prior nephrectomy and time from nephrectomy to relapse as important predictors of survival in patients receiving IL-2–based therapy (6). In their series, patients who received systemic immunotherapy for metastatic disease >6 months after nephrectomy had the best

Authors' Affiliations: ¹Division of Hematology/Oncology, Beth Israel Deaconess Medical Center; ²Dana-Farber/Harvard Cancer Institute; ³Brigham and Women's Hospital, Harvard Medical School; ⁴Dana-Farber/Harvard Cancer Central Renal Cancer Program, Boston, MA; and Departments of ⁵Microbiology and ⁶Molecular Genetics, Irvine College of Medicine, University of California, Irvine, California. Received 9/30/04; revised 2/4/05; accepted 2/7/05.

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Note: M. Upton is currently at the Anatomic Pathology Division, Department of Pathology, University of Washington Medical Center, Seattle, WA.

Requests for reprints: Michael B. Atkins, Division of Hematology/Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215. Phone: 617-667-1930; Fax: 617-975-8030; E-mail: Matkins@bidmc.harvard.edu.

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median survival and had a 3-year survival rate of 46%. A recent multivariate analysis confined to patients who received IL-2 after nephrectomy revealed survival to be inversely associated with lymph node involvement, constitutional symptoms, sarcomatoid histology, metastases involving multiple sites or sites other than bone or lung, and a thyroid-stimulating hormone level of >2.0 mIU/L (7). They proposed a scoring algorithm based on these features in which survival at 1-year was predicted to vary from 10% to 92%. Recent data from the Cytokine Working Group phase III trial suggested that disease site factors, such as primary in place or hepatic or bone metastases, may be more predictive of a poor response to low-dose IL-2 and IFN treatment than to high-dose IL-2 (4, 5, 8). Furthermore, this study suggested the greatest benefit from high-dose IL-2, relative to lower dose regimens, might be seen in patients with primaries in place and/or liver and bone metastases. These data call into question some of the prior studies and suggest that additional predictors of response and survival in patients receiving cytokine-based immunotherapy are necessary for optimal patient selection.

We recently reported the pathologic characteristics of renal cancer specimens that seem to correlate with response to IL-2 therapy (9). In this analysis, we determined that response to IL-2 was significantly associated with clear cell histology with alveolar features and the absence of papillary or significant granular features. Patients with these features in their kidney tumor specimens had a 25% response rate (29 of 113) compared with 4% response rate (2 of 50) for patients with non-clear cell tumors or clear cell tumors containing papillary or $>50\%$ granular features. The results in the kidney tumor specimens were confirmed in a separate analysis of metastatic lesions. In the metastatic setting, responses were limited to patients with clear cell tumors with the favorable histologic patterns described in the primary tumor specimens. This data encouraged further analysis of renal cancer specimens in an effort to determine other features that might predict for response.

Carbonic anhydrase IX (CAIX) has recently been identified as a molecular marker that is potentially predictive of renal cancer prognosis (10). CAIX expression is mediated by the HIF-1 transcriptional complex and induced in many tumor types but is absent in most normal tissues. Bui et al. used a monoclonal antibody to detect immunohistochemical evidence of CAIX expression on paraffin-embedded renal cell carcinoma specimens. They showed that $>90\%$ of renal cell carcinomas express CAIX and that its expression decreases with advancing stage (10). In their analysis, high CAIX expression in primary tumors was seen in 79% of patients and was associated with improved overall survival in patients with advanced disease. In addition, Bui et al. suggested that CAIX expression might be associated with response to IL-2-based therapy. Responses were observed in 20 of 72 (27%) patients with high CAIX expression compared with only 2 of 14 (14%) patients with low CAIX expression. In an effort to confirm and expand upon this initial observation, we evaluated renal cancer specimens collected from patients who had received IL-2 as part of Cytokine Working Group trials for CAIX expression and correlated the results with response and survival as well as other clinical and pathologic predictive models of response.

Materials and Methods

Patients. We had previously evaluated pathologic features that would predict for response to IL-2 from pathology specimens collected from a representative cohort of 231 patients who had participated in Cytokine Working Group or Beth Israel Deaconess Medical Center IL-2-based renal cancer trials from 1990 to 2001 (9). Tissue blocks were then collected from 66 of these patients (27 responding and 39 non-responding patients) for a nested case-control study that enriched for patients who had exhibited a tumor response to IL-2-based therapy. All patients had previously provided informed consent to participate in their Cytokine Working Group or Beth Israel Deaconess Medical Center clinical trial, which included the possibility of central review of their pathology material. The current investigation received a waiver from the Institutional Review Board by the Beth Israel Deaconess Medical Center.

Immunohistochemistry. Specimens were stained for CAIX expression using the mouse monoclonal antibody, MN-75 (11). Immunohistochemical staining of tissue sections with anti-CAIX antibody was done in an automated stainer (Optimax Plus 2.0 bc, BioGenex, San Ramon, CA) using the MultiLink-HRP kit (BioGenex) in a 750-W oven for 15 minutes (12). The CAIX primary antibody was used at a 1:10,000 dilution. Semiquantitative assessment of the antibody staining was done by a single pathologist blinded to the clinicopathologic variables. The extent of staining was recorded as a percentage of the tumor tissue sample that had positive CAIX expression. Each specimen was scored based on the staining intensity, the percentage of positive cells, and the percentage staining at maximal staining intensity. As described by Bui et al., specimens in which $>85\%$ of cells stained for CAIX were labeled as high CAIX expressing, whereas those in which $\leq 85\%$ of cells expressed CAIX were labeled as low CAIX expressing tumors.

Statistical analysis. The association of covariates of interest with CAIX expression, dichotomized as high ($>85\%$) versus low ($\leq 85\%$) expression, or with IL-2 response was evaluated using Fisher's exact test or Cochran-Armitage trend test for ordered categorical data with exact *P*s; odds ratios (OR) and exact 95% confidence intervals were reported. Whether the association of CAIX expression with IL-2 response was homogeneous across the different values of the pathologic prognostic model was evaluated using the Breslow-Day Test for homogeneity of ORs. Survival and follow-up times are calculated from the time of initiation of IL-2 treatment until death from any cause, or censored at time last known alive. Log-rank tests were used to evaluate the association of covariates with overall survival; because of the case-control design, we do not report response rates, median survival, or hazard ratios, which would likely have been influenced by the oversampling of patients who responded to IL-2.

Table 1. Characteristics of 66 patients who had received IL-2-based therapy

Characteristic	Nonresponder (%) (n = 39)	Responder (%) (n = 27)	All patients (%) (n = 66)
Male gender	33 (85)	18 (67)	51 (77)
IL-2 dose			
Low	23 (59)	10 (37)	33 (50)
High	16 (41)	17 (63)	33 (50)
Response			
CR/PR	—	10/17 (41)	10/17 (41)
MR/None	3/36 (59)	—	3/36 (59)
Survival*			
Alive	5 (13)	15 (56)	20 (30)
Dead	34 (87)	12 (44)	46 (70)

* At 3.9 years median follow-up from initiation of IL-2 therapy.

Table 2. Pathologic evaluation of 66 patients' specimens

Characteristic	Nonresponder (%) (n = 39)	Responder (%) (n = 27)	All patients (%) (n = 66)
Specimen			
Primary	34 (87)	22 (81)	56 (85)
Metastatic lesion	5 (13)	5 (19)	10 (15)
Specimen source			
Resection	33 (85)	23 (85)	56 (85)
Biopsy	6 (15)	3 (11)	9 (14)
FNA	0 (0)	1 (4)	1 (1)
Clear cell tumor	32 (82)	26 (96)	58 (88)
Alveolar features	30 (86)	26 (96)	56 (90)
Granular features	25 (66)	8 (30)	33 (51)
Papillary features	3 (8)	1 (4)	4 (6)
Pathology risk group (Upton)			
Good	7 (18)	17 (63)	24 (36)
Intermediate	22 (56)	9 (33)	31 (47)
Poor	10 (26)	1 (4)	11 (17)
CAIX expression			
Low ($\leq 85\%$)	19 (49)	6 (22)	25 (38)
High ($>85\%$)	20 (51)	21 (78)	41 (62)

Results

Demographics. Tumor blocks were obtained from 66 patients who had received IL-2–based therapy. The demographics, treatment, and response characteristics of the 66 patients from whom these tissue samples were obtained are described in Table 1. Fifty-one of the 66 patients were male and 27 (41%) had responded to IL-2–based therapy. Responses included 10 complete and 17 partial responses. Exactly half of the patients had received high-dose IL-2– and half low-dose IL-2–containing regimens. Median survival and follow-up were 2.6 and 3.9 years, respectively.

Pathology specimen characteristics. The characteristics of the pathology specimens examined are displayed in Table 2. Of

note, the majority of specimens (85%) were obtained from the renal primary tumor. Fifty-eight specimens were assessed as clear cell with 56, 33, and 4 of these being classified as having alveolar, granular, or papillary features, respectively. Twenty-four (36%), 31 (47%), and 11 (17%) were classified as good, intermediate, or poor prognosis based on the previously described pathologic model (9). High CAIX expression was seen in 41 (62%) specimens. Examples of high and low CAIX staining specimens are shown in Fig. 1.

Association of carbonic anhydrase IX expression with response to interleukin 2. Figure 2 depicts the relationship between CAIX expression and response to IL-2. Twenty-one of 27 (78%) responding patients exhibited high CAIX staining compared with only 20 of 39 (51%) nonresponding patients (OR, 3.3; $P = 0.04$). Survival was significantly longer in patients whose tumor specimens stained highly for CAIX relative to those whose specimens expressed low levels of CAIX ($P = 0.03$; Fig. 3). Survival of >5 years was only seen in patients whose tumor specimens highly expressed CAIX.

Association of carbonic anhydrase IX staining with pathologic classification and response to interleukin 2. Table 3 displays the relationship between CAIX staining, pathologic category, and response to IL-2 therapy. A trend exists for the association of better pathologic predictive group and high CAIX staining ($P = 0.09$). Specifically, 71%, 65%, and 36% of specimens classified as good, intermediate, or poor, respectively, expressed high levels of CAIX. This trend is completely lost if the eight non-clear cell patients are removed from the analysis as two of the remaining three specimens with poor pathologic features stained highly for CAIX (data not shown). Of note, of the 31 specimens classified as having intermediate pathology, all nine from patients responding to IL-2 therapy stained highly for CAIX as compared with only 11 of 22 (50%) of those obtained from patients who did not respond to IL-2 therapy.

Refinement of pathology prediction model. The finding of a strong association with CAIX staining and response to IL-2 in the intermediate pathology group, suggested that the three compartment pathologic model proposed by Upton et al. could be compressed into two compartments with the addition of CAIX staining characteristics. In this refined model (Fig. 4), the good risk group contained specimens with either good risk

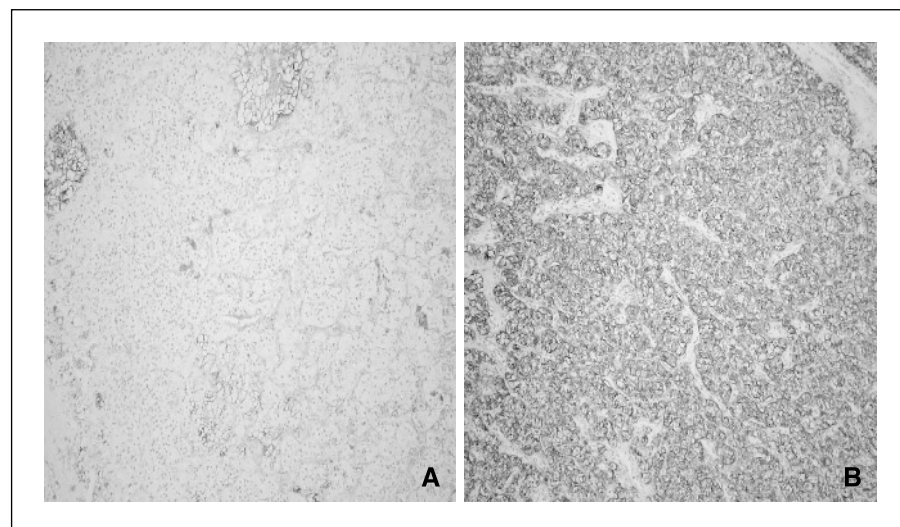
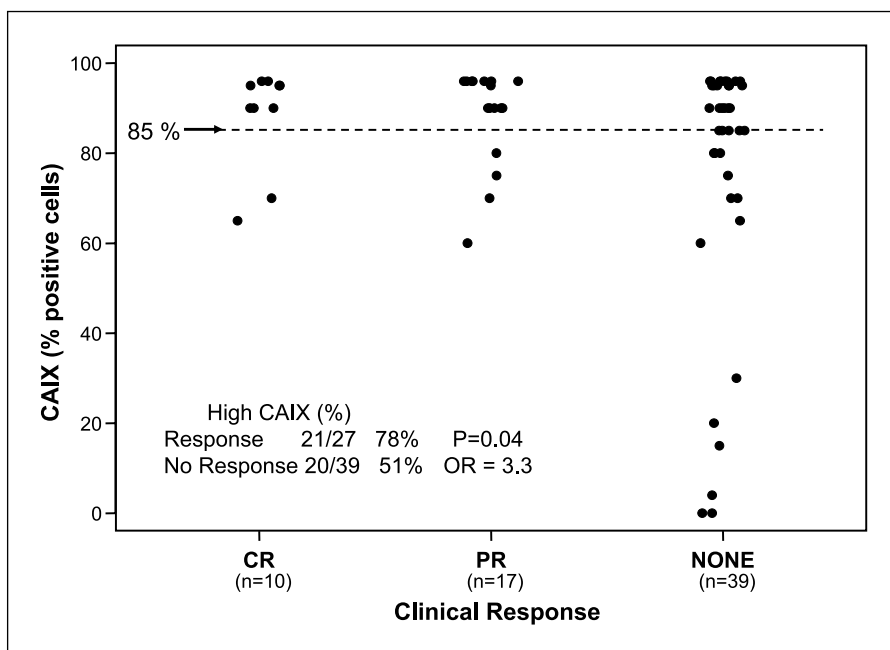


Fig. 1. Immunohistochemical stains of renal cancer tissue using the MN-75 anti-CAIX antibody. A, representative of a low CAIX staining. B, representative of high CAIX staining.

Fig. 2. Relationship between CAIX staining and response. Dotted line, 85% of cells staining cutoff developed by Bui et al. Separate columns show complete (CR), partial (PR), and nonresponders; 78% of the responders (CR or PR) were high CAIX expressers compared with only 51% of the nonresponders.



pathology alone or intermediate risk pathology and high CAIX staining, whereas the poor risk group contained specimens with poor risk pathologic features alone or intermediate risk pathology and low CAIX staining. The good risk group contained 44 patients including 26 of 27 responding patients (96%) compared with only 18 of 39 (46%) of nonresponding patients (OR, 30; $P < 0.01$; Table 4). A corresponding survival benefit was also seen (Fig. 5). If the eight non-clear cell patients are excluded from the model (only two of whose specimens stained highly for CAIX), then all 26 responding patients are in the good risk group compared with 18 of 32 (56%) of the nonresponding patients.

Relationship to other predictors of cytokine responsiveness or prognosis. An effort was made to associate CAIX staining

with other variables previously reported predictive of response to cytokine treatment or overall survival. Factors examined included the dose of IL-2 therapy, sites of disease (primary in place, bone, or liver metastases), the Memorial Sloan Kettering Cancer Center clinical classification, and Fuhrman histologic grade. The results are displayed in Table 5. Of note, the ability of CAIX staining to predict for response was unaffected by the IL-2 dose given (OR for high-dose IL-2, 3.6; OR for low-dose IL-2, 2.5). In addition, patients with tumor with predominant Fuhrman grade 3 or 4 or without bone or liver metastases were still over six times more likely to respond to IL-2 if their tumors stained highly for CAIX than if their tumors stained weakly. High CAIX staining also predicted for prolonged survival in patients with low or

Fig. 3. Kaplan Meier survival curves for patients with high and low CAIX staining tumors. Survival of >5 years was only seen in patients with high CAIX expressing tumors.

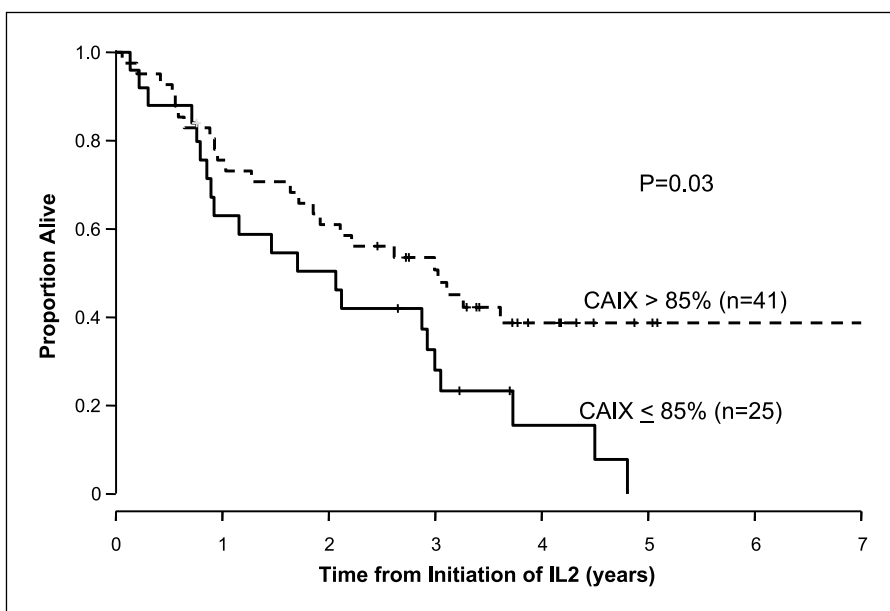


Table 3. Association of CAIX staining and pathologic predictive group and response to IL-2 therapy

Pathology risk group	With high CAIX (%)		
	Nonresponder (n = 39)	Responder (n = 27)	All patients (n = 66)
Good	5/7 (71)	12/17 (71)	17/24 (71)
Intermediate	11/22 (50)	9/9 (100)	20/31 (65)
Poor	4/10 (40)	0/1 (0)	4/11 (36)

intermediate risk by the Memorial Sloan Kettering Cancer Center classification ($P < 0.01$). Insufficient specimens were available to create a multivariate model that included all of these features with CAIX staining.

Discussion

IL-2 leads to clinical response in 15% to 20% of patients with renal cell carcinoma but is associated with considerable toxicity. To reduce risk and to maximize potential benefits of IL-2 therapy, it is desirable to identify those patients most likely to respond to treatment. Previous clinical trials have identified clinical and laboratory features predicting poor survival following IL-2 therapy (6, 7, 13). Others have identified clinical and laboratory factors that might be associated with response to IL-2-based therapy (14, 15). Several of these factors including thyroid dysfunction, thrombocytopenia or IL-1 production are treatment related and thus not useful in selection of patients for IL-2-based therapies. Other factors such as absence of nephrectomy, presence of bone or liver metastases, or poor performance status seem inadequate predictors of response in patients receiving high-dose IL-2 therapy (4). Although these clinical and laboratory factors may help to identify patients with renal cell carcinoma whose life expectancy is short even with systemic immunotherapy, they do not help to predict which patients are likely to respond to treatment. Given that response to treatment remains a strong surrogate marker for clinical outcome and was the principal

justification for Food and Drug Administration approval of IL-2 therapy in this disease, identification of factors that can predict for response remains a useful goal.

Prior analyses have suggested that histologic features might predict for response to IL-2-based therapy (4). This prompted a more thorough analysis of the proteins expressed on the tumor for possible molecular correlates of response to IL-2 therapy. Carbonic anhydrase IX seemed a logical place to start as high expression of this molecule in primary tumor specimens had previously been associated with prolonged survival in patients with stage IV disease (10). In addition, there was a suggestion from this data that high expression of CAIX correlated with response to IL-2 (27% versus 13% response rates).

The data reported here largely confirms the results of the University of California at Los Angeles group (10). Whereas the nested case-control design of our study makes assessment of response rates based on CAIX staining characteristics impossible, we too observed a 2-fold higher response rate in patients with high CAIX relative to low CAIX expressing tumors. Given that the response rate to IL-2 for patients selected for this analysis was 41%, (nearly double that typically seen for the treatment of unselected patients), we would estimate that the response to IL-2 for patients with high CAIX staining tumors would be in the 25% to 30% range compared with 10% to 15% for patients whose tumors stain low for CAIX. Whereas these differences in response rates may not be sufficient to justify exclusion of patients with low CAIX expressing tumors from IL-2-based therapy, the fact that all patients surviving >5 years following IL-2 therapy had high CAIX expressing tumors suggests this feature may be a better determinant of efficacy than is indicated solely by response rate.

The value of CAIX staining is best seen when combined with other predictors of response. For example, combining CAIX staining with our previously reported pathologic predictors of response defined a "good prognostic" group that was 30-fold more likely to respond to IL-2 and exhibited significantly longer median survival relative to the "poor prognostic" group. Adjusting for the patient selection bias in our analysis, we estimate that about 50% of patients would

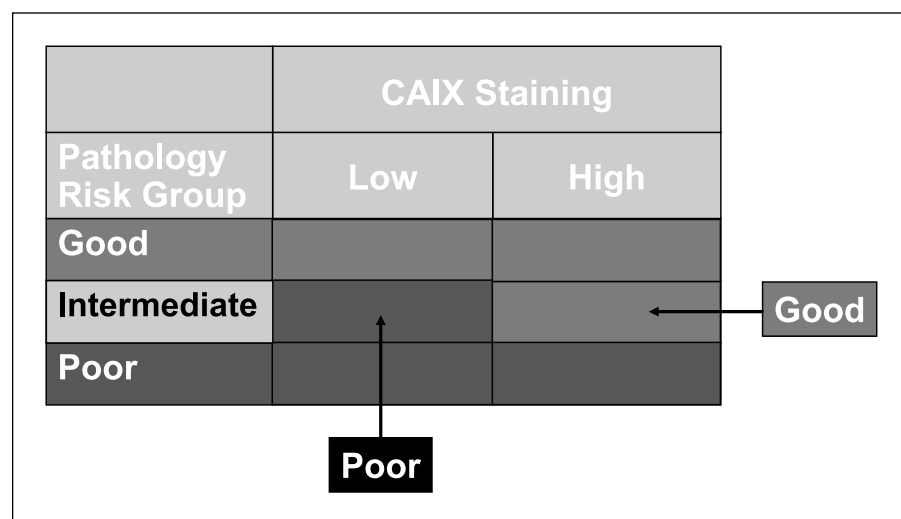


Fig. 4. The proposed new model for combining pathology predictive group with CAIX staining. Three pathologic risk groups previously reported by Upton et al. (9) can theoretically be collapsed into two groups having distinct response rates to IL-2 therapy and survivals as shown in Table 4 and Fig. 5, respectively.

Table 4. Association of the refined model incorporating CAIX staining and pathology risk groups with response to IL-2 therapy

Refined pathology risk group	Nonresponder (%) (n = 39)	Responder (%) (n = 27)
Good (Good risk path or intermediate risk path with high CAIX)	18 (46)	26 (96)
Poor (Poor risk path or intermediate path with low CAIX)	21 (54)	1 (4)

fall into each of these refined predictive groups and that the response rate in the good predictive group would be in the 30% to 40% range, whereas that in the poor predictive group would be <5%. This refined model requires independent and/or prospective validation possibly including a "matched pair" data set analysis; however, if confirmed, it would suggest that patients in the poor predictive group should not be considered for IL-2 containing therapy. Inclusion of data from more patients would be necessary to incorporate other predictive or prognostic features, such as MSKCC classification (16), Fuhrman grade, dose of IL-2 (3, 4), or the University of California at Los Angeles classification (7) into the selection model.

The mechanism by which CAIX may predict for prognosis in IL-2 recipients remains unclear. Whereas, it is conceivable that G250 may serve as a tumor regression antigen that is being recognized by IL-2 activated T cells, other hypotheses are more appealing. CAIX is a hypoxia-inducible gene whose transcription is regulated by HIF1 (17). Its principal function is to maintain extracellular pH in the face of hypoxic and acidotic stress. Its expression is induced in renal cancer cell lines lacking von Hippel-Lindau (VHL) protein even under

normoxic conditions and acidic pH can further enhance its expression in these cells (18). Little is known about protein importance that pH-regulatory mechanisms play in tumor cell survival, proliferation, invasion, and metastasis. Whereas it is conceivable that pH at the tumor site may be critical to immune mediated tumorlysis, little or no evidence exists to support this hypothesis. Alternatively, as CAIX expression is mediated by the HIF1 and the lability of the HIF1 subunit is dependent on VHL function, it is possible that high CAIX expression is a marker for a predominantly VHL-defective HIF-1 driven tumor. In this context, CAIX expression may serve as a surrogate for some other critical HIF-mediated protein that is more directly associated with immune responsiveness. Under this model, VHL wild-type tumors would be considered less likely to express CAIX and also less likely to respond to IL-2. Efforts to correlate CAIX staining with VHL expression and identify HIF inducible factors that are coexpressed with CAIX are under way and could shed considerable light on the merits of this proposed mechanism.

The fact that CAIX expression in metastatic lesions is less than seen in a patient's corresponding primary tumor suggests another model might be at play (10). Under this model, one could hypothesize that as tumors progress they become less dependent on HIF related factors and possibly driven more by mutations controlling other pathways (particularly PTEN/AKT and mammalian target of rapamycin pathway). It is possible that these secondary genetic changes are associated with immune suppression (19) making the tumors less responsive to immunotherapy and, possibly more aggressive (20). Whatever the mechanism, the fact that pathologic and molecular features of the tumor can predict outcome of IL-2 therapy suggests that more detailed analysis of genetic and gene expression factors related to response to IL-2-based therapy is warranted.

In the past 2 years, several novel treatments have been reported to have antitumor activity in patients with renal cancer. These include inhibitors of vascular endothelial growth factor function (21), inhibitors of receptor tyrosine kinases

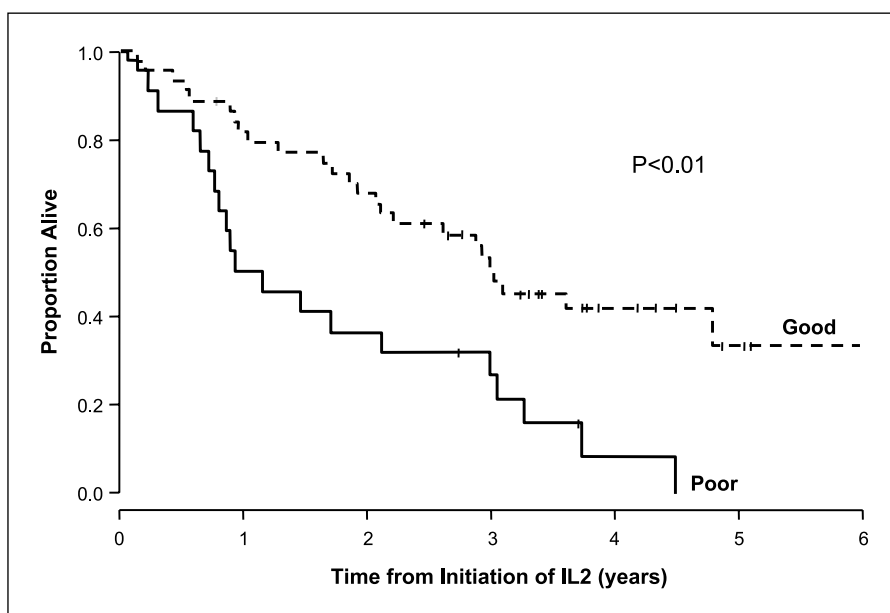
Fig. 5. Kaplan Meier survival curves for patients in good and poor predictive groups based on the refined model combining pathologic predictive group and CAIX staining.

Table 5. Association of CAIX expression with response to IL-2, stratified by various factors; percents sum across row, within the nonresponder or responder group

Characteristic	Nonresponder (n = 39)		Responder (n = 27)		Odds Ratio
	Low (%)	High (%)	Low (%)	High (%)	
All patients	19 (49)	20 (51)	6 (22)	21 (78)	3.3 (0.99-12.1)
IL-2 Dose					
Low	12 (52)	11 (48)	3 (30)	7 (70)	2.5 (0.4-18.6)
High	7 (44)	9 (56)	3 (18)	14 (82)	3.6 (0.6-26.5)
Primary in place					
No	19 (53)	17 (47)	6 (25)	18 (75)	3.4 (1.0-12.6)
Yes	0 (0)	3 (100)	0 (0)	3 (100)	—
Bone or liver metastasis*					
No	16 (59)	11 (41)	3 (18)	14 (82)	6.8 (1.4-43.6)
Yes	3 (25)	9 (75)	2 (29)	5 (71)	0.8 (0.10-13.3)
MSKCC					
Unknown	1 (100)	0 (0)	0 (0)	1 (100)	—
Low (1)	2 (33)	4 (67)	1 (33)	2 (67)	—
Intermediate (2)	16 (52)	15 (48)	4 (19)	17 (81)	4.5 (1.1-22.2)
Intermediate/poor*	0 (0)	1 (100)	1 (50)	1 (50)	—
Fuhrman overall					
2	0 (0)	2 (100)	1 (25)	3 (75)	—
3 or 4	19 (51)	18 (49)	5 (22)	18 (78)	3.8 (1.0-15.6)
Fuhrman predominant					
1 or 2	0 (0)	7 (100)	3 (27)	8 (73)	—
3 or 4	19 (59)	13 (41)	3 (19)	13 (81)	6.3 (1.3-40.0)

*As a result of missing data, three patients could not be fully classified.

with promiscuous targets including VEGFR1 and VEGFR2, PDGFR, C-Kit, Raf kinase, and mammalian target of rapamycin (22–25). In addition, IFN has been shown in several phase III studies to exhibit a modest survival advantage (26, 27). It would be of interest to determine the role of CAIX staining in predicting benefit to each of these agents. The results of such analyses could shed light on the mechanism underlying the predictive power of CAIX as well as contribute additional data useful to the establishment of rational treatment selection criteria for patients with this disease.

The potential value of this information suggests that it would be beneficial to require central tissue collection in future studies involving novel agents in patients with renal cancer.

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