Published in final edited form as:

Urology. 2014 January ; 83(1): . doi:10.1016/j.urology.2013.09.026.

Urinary Concentrations of Aquaporin-1 and Perilipin-2 in Patients with Renal Cell Carcinoma Correlate with Tumor Size and Stage but Not Grade

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Abstract

OBJECTIVE—To evaluate trends in urine aquaporin-1 (AQP1) and perilipin 2 (PLIN2) concentrations in patients with clear cell and papillary renal cell carcinoma (RCC) this investigation determined the relationship between the urine concentration of these biomarkers and tumor size, grade and stage.

MATERIALS and METHODS—Biomarker concentrations were determined by sensitive and specific Western blot procedures normalized to urine creatinine excretion. Analysis included 61 patients undergoing a partial or a radical nephrectomy for clear cell or papillary renal cancer and 43 age- and sex-matched control patients. Relationships between urine biomarker concentrations and tumor size, stage, and grade were assessed.

RESULTS—Patients with renal cell carcinoma had 35-fold and 9-fold higher median urinary AQP1 and PLIN2 concentrations, respectively, compared to controls (p values). Both tumor markers decreased following tumor resection, to concentrations equivalent to those of controls. The sensitivity and specificity were both 100% for AQP1, and 92% and 100% for PLIN2. There was a significant linear correlation between tumor size and pre-nephrectomy AQP1 (Spearman coefficient 0.78, P<0.001) and PLIN2 (Spearman coefficient 0.69, P<0.001) concentrations. There was a correlation of both markers with tumor stage (overall P=0.030), when stage was dependent primarily on tumor size (stages T1 and T2), but not with stage T3 which reflected extra-renal spread. Neither marker showed a significant correlation with tumor grade.

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CONCLUSIONS—AQP1 and PLIN2 are significantly increased in patients with clear cell and papillary renal cell carcinoma compared to controls. Preoperative urinary concentrations of these markers reflect tumor size and stage.

Keywords

Kidney Cancer; Urine Biomarkers; Aquaporin-1; Perilipin 2

INTRODUCTION

Renal cell carcinoma (RCC) is the most lethal urologic malignancy¹ and there has been a steady rise in its incidence^{1–5}. For example, the overall age adjusted renal cancer incidence has increased from 7.4/100,000 in 1975 to 17.6/100,000 in 2006.⁵ The National Cancer Institute had projected that over 64,500 patients would be newly diagnosed with kidney cancer, while over 13,500 would die from this disease in 2012.⁶

Due to increased diagnostic use of abdominal imaging, there has been a consequent increase in incidental discovery of occult renal masses, and also a stage migration towards smaller tumors, and thus with a higher potential for cure.^{1,3,4,7} From 1989 to 1997, Memorial Sloan-Kettering Cancer Center found a decrease in the mean size of resected tumors from 7.8 to 5.3 cm along with an increased percentage of pT1 tumors from 4 to 22%.⁸ Based on Surveillance, Epidemiology and End Results (SEER) cancer registry data, the diagnosis of tumors localized to the kidney has increased from 46% during the time period from 1975 to 1990, to 62% during 1991 to 2006.⁵ Despite an increase in the percentage of lower stage disease, the incidence of locally advanced and metastatic disease continues to increase as does the overall mortality rate.^{2,3,5,7} It has been suggested that further improvement in mortality rate would likely requiring further screening of larger volumes of patients.¹ Unfortunately, the cost effectiveness of CT or MRI imaging to screen for RCC and differentiate benign from potentially malignant masses has yet to be proven.^{9,10}

An alternative to radiological screening would involve the use of a sensitive and specific tumor marker. Currently there is no readily available screening biomarker for RCC.¹¹ An initial investigation found elevated urine concentrations of aquaporin 1 (AQP1) and adipophilin (ADFP, since renamed as perilipin 2, PLIN2) in patients with clear cell and papillary RCC, compared to controls.¹² A second validation study also found significantly elevated concentrations of the two urine markers in the RCC group compared to a second control group.¹³ Moreover, biomarker concentrations were not significantly influenced by non-cancerous kidney diseases like diabetic nephropathy, urinary tract infection, and glomerulonephritis.¹³ Both studies found a positive relationship between the size of the renal cancer and urine biomarker concentrations, but were limited in the number of RCC patients and lacked the ability to assess the significance of stage or grade on biomarker concentrations.^{12,13} The goal of this investigation was to combine and evaluate the data sets of the two previous studies to determine if urinary AQP1 and PLIN2 concentrations have any relation to tumor size, stage or grade.

MATERIALS AND METHODS

PATIENTS

Approval was obtained from the Washington University Institutional Review Board, and written informed consent was obtained from all patients. From July 8, 2008 to April 22, 2010, urine samples were obtained prior or during surgery (pre-nephrectomy) from patients undergoing either a partial or radical nephrectomy for renal masses suspicious for RCC on radiological imaging. Inclusion in this analysis required a postoperative pathological

diagnosis of either clear cell RCC (52 patients) or papillary RCC (9 patients). A post-nephrectomy urine sample was obtained from 47 of these patients during the first post-operative clinic visit 2 to 4 weeks post-surgery, however, one patient did not return for 6 months.

Preoperative urine samples were obtained from 43 control patients who underwent non-urological surgery. Any patient with a previous urological malignancy was excluded from the control group. The control group did not undergo postoperative urine samples.

CLINICAL AND PATHOLOGICAL DATA

Demographic and medical history was recorded including age, sex, weight, and smoking history. Serum creatinine concentrations were obtained and the eGFR was calculated using the Modification of Diet in Renal Disease equation.¹⁴ Postoperative pathology reports provided RCC cell type, tumor size based on the largest dimension, TNM stage, and Fuhrman grade. All renal stages were converted to the American Joint Committee on Cancer 2010 TNM staging system.¹⁵

AQP1 AND PLIN2 MEASUREMENT

Urine biomarker concentrations were determined by sensitive and specific Western blot procedures and normalized to the urinary creatinine concentration as described previously.^{12,13} Arbitrary absorbance units of the Western blot bands for both AQP1 and PLIN2 were normalized to the urinary creatinine excretion, measured by the Jaffe reaction¹², and corrected for the absorbance of AQP1 and PLIN2 in several patient samples common to each study and run on each Western blot in order to compare blot-to-blot results. Each gel and blot consisted of one lane of molecular weight standards, 14 individual patient/control samples and samples from 2 common patients. Results are reported as Absorbance Units per mg Urine Creatinine for each cancer and control patient. The inter-assay variation from gel to gel for AQP1 was 10% while that for PLIN2 was 9%.^{12, 13}

STATISTICAL ANALYSIS

Comparisons between controls and RCC patients were performed for AQP1 and PLIN2, and for age, sex, smoking history, weight, and the estimated glomerular filtration rate (eGFR). The receiver operating characteristic (ROC) analysis was utilized to determine the area under the ROC for both pre-nephrectomy AQP1 and PLIN2. The optimal cut-off values were obtained as the one corresponding to the coordinate closest to the perfect classification coordinate (specificity=sensitivity=1) in the ROC curve and the resulting sensitivities and specificities were calculated. Pre- and post-nephrectomy AQP1 and PLIN2 concentrations were compared for the RCC patients. Correlations of urine AQP1 and PLIN2 concentrations for the RCC patients were further assessed based on pathological features of size, stage, and grade.¹⁵

An initial multivariate linear regression analysis evaluated the role of the patient's age, sex, smoking history, eGFR, BMI, tumor size, stage, grade, and histological subtype on urine AQP1 and PLIN2 concentrations (results not shown), respectively. Due to co-linearity between size and stage, a second multivariate analysis was performed removing stage from the above model. This second analysis is reported in the results section. During the analysis, size and eGFR were analyzed as continuous variables, while age, sex, smoking status, BMI, grade and tumor subtype were evaluated as categorical variables. For the category of age, the patients were dichotomized into those younger or older than age sixty. Smoking status was dichotomized into two categories based on a smoking history of either greater than or less than 20 pack years since smoking is a risk factor for RCC.¹⁵ Since obesity is a risk factor for RCC, the BMI was divided into three categories: normal (BMI < 25), overweight

(BMI 25.1 to 30), and obese (BMI >30).¹⁶ Due to a limited number of patients with grade 4 RCC, grade was analyzed as either low grade (Fuhrman grade 1 and 2) or high grade (Fuhrman grade 3 and 4). Finally, tumor subtype was divided into clear cell and papillary RCC.

In evaluating clinical and laboratory features between patients, comparison of a continuous variable between control and RCC patients was conducted using the two sample t-test assuming non-equal variance. Distributions of a categorical variable between patients were compared using the Chi-square test of independence. Paired pre-nephrectomy and post-nephrectomy urine markers in RCC patients were compared using the Wilcoxon signed rank test. The Spearman correlation coefficient was calculated to assess relationships between urine markers and tumor size. Comparison of urine markers between/among groups of a categorical variable were conducted using the Mann-Whitney U test. Tests performed were all two-sided with statistical significance set at the 0.05 level. Analysis was performed using R statistical software (R Development Core Team, version 2.15.1) and Analyse-it for Excel 2010.

Sample size calculations were based on results of the control group using the mean and standard deviation of the urinary AQP1 and PLIN2 concentrations of our recent study.¹³ To detect a 2-fold (conservative assumption) increase in biomarker concentrations in the RCC patients in the present study, based on a two-sided t-test and 90% power, would require 12 patients for AQP1 and 9 for PLIN2 (0.01 significance).

RESULTS

Descriptive Characteristics

The RCC and control groups did not differ significantly based on age, sex, smoking history, eGFR, or weight. Clinical characteristics of the patients are shown in Table 1. Fifty-two (85%) of the RCC patients had clear cell carcinoma, while 9 (15%) possessed papillary RCC. The mean pathological tumor size and standard deviation for the RCC group was 4.4 and 3.9 cm. The distribution of the pathological stages was as follows: pT1a 41 (67%), pT1b 7 (12%), pT2 5 (8%), pT3 8 (13%), and zero pT4 patients. The classification based on Fuhrman grade was 6 (10%) grade 1, 37 (61%) grade 2, 15 (25%) grade 3, and 2 (3%) grade 4 tumors. One patient with papillary RCC did not receive a Fuhrman grade. None of the RCC patients had positive lymph nodes, while three patients (5%) had known preoperative metastasis.

Pre-nephrectomy AQP1 and PLIN2 values for the RCC and control groups are provided in Table 1. The median pre-nephrectomy urine AQP1 and PLIN2 concentrations were both significantly higher in the RCC groups compared to preoperative concentrations in controls. Receiver operating characteristic analysis of the pre-nephrectomy urine concentrations determined the optimal cut-off concentrations for both AQP1 and PLIN2. A cut-off value of 3 relative absorption units per milligram urine creatinine gives AQP1 both a sensitivity and specificity of 100%. For PLIN2, a value of 13 relative absorption units per milligram urine creatinine provides a sensitivity and specificity of 92% and 100%.

Forty-seven (77%) of the RCC patients provided a postoperative urine sample, a median of 31 (range 11–187) days following surgery. Post-nephrectomy urine biomarker concentrations decreased significantly compared to pre-nephrectomy concentrations for both AQP1 (Wilcoxon Signed Ranked Test, $P < 0.001$) and PLIN2 (Wilcoxon Signed Rank Test, $P < 0.001$). Figure 1 shows the matched pre-nephrectomy and post-nephrectomy urine biomarker concentrations for the RCC group. Both post-nephrectomy urine AQP1 (Wilcoxon Signed Ranked Test, $P = 0.076$) and PLIN2 (Wilcoxon Signed Rank Test,

$P=0.064$) concentrations in the RCC group were statistically indistinguishable from the preoperative urine marker concentrations in the controls.

Association of Markers with Clinical and Pathological Characteristics

The relationship between pre-nephrectomy AQP1 and PLIN2 concentrations and tumor size is illustrated in Figure 2. Pre-nephrectomy AQP1 concentrations were positively correlated with tumor size (Fig 2A, Spearman's Rank Correlation Coefficient 0.780, $P<0.001$). Using various regression models, for all tumor sizes, a linear fit of urine AQP1 concentrations to tumor size ($R^2=0.47$) was not as good as a polynomial fit ($R^2=0.59$). The relationship between urine AQP1 concentrations and pathological stage is shown in Figure 2B. There was a significant increase in urine AQP1 concentrations between control and pT1a and again from pT1a to pT1b (Mann-Whitney U, $P<0.001$ and $P=0.003$, respectively) and then from pT1b to pT2 (Mann-Whitney U, $P=0.030$). Between pT2 and pT3 the urine AQP1 level was not different (Mann-Whitney U, $P=0.127$).

Pre-nephrectomy PLIN2 concentrations were positively correlated with tumor size (Figure 2C, Spearman's Rank Correlation Coefficient 0.688, $P<0.001$). Using various regression models, a linear fit of urine PLIN2 concentrations to tumor size $R^2=0.17$ was not as good as a polynomial fit ($R^2=0.31$) for all tumor sizes. Overall correlations for PLIN2 were not as robust as for AQP1. Of the six patients with the largest tumors, five were stage pT3 and had high Fuhrman grades. These patient's PLIN2 concentrations influenced the polynomial in a negative direction. For PLIN2, there was a significant increase in concentrations from that of the control, and between pT1a and pT1b (Mann-Whitney U, $P<0.001$ and $P=0.008$, respectively) (Figure 2D). The increase was not significant between pT1b and pT2 or between pT2 and pT3 (Mann-Whitney U, $P=0.515$ and $P=0.608$, respectively). While there was a significant increase in urinary AQP1 and PLIN2 concentrations between control patients and all patients with RCC (Table 1, $P<0.001$) no significant difference was found between low (Grades 1 and 2) and high grade (Grades 3 and 4) tumors for either AQP1 (Kruskal-Wallis, $P=0.110$) or PLIN2 (Kruskal-Wallis, $P=0.205$) concentrations.

Results of the multivariate analysis for predictors of AQP1 and PLIN2 concentrations are shown in Table 2. Tumor size was the only independent association for AQP1 or PLIN2 urine concentration. Any differences in either biomarker concentration in the urine did not depend on patient age, smoking history, BMI or kidney function (eGFR) (Table 2).

COMMENT

This is the first study to show that the urine biomarkers of clear cell and papillary renal cell carcinoma, AQP1 and PLIN2, reflect tumor stage. Results are consistent with previous histological studies on resected tumors.¹⁸⁻²⁰ Those histological studies measured mRNA and protein levels in excised tumors by in situ hybridization and immunohistochemistry, while our studies measured urine protein concentrations. Thus, urine AQP1 and PLIN2 concentrations reflect tumor AQP1 and PLIN2 expression, and also reflect tumor size. Thus urine AQP1 and PLIN2 concentrations provide a valuable non-invasive method for identification of an imaged renal mass as a clear cell or papillary renal cancer, and may also indicate tumor existence and tumor size in the absence of radiologic findings (such as in an application for screening).

Our two previous investigations demonstrated increased concentrations in urine AQP1 and PLIN2 in patients with documented RCC.^{12,13} There were significantly elevated urine concentrations of both markers when compared to healthy controls and to patients with common non-cancerous kidney diseases. Following resection of the renal tumor by either partial or radical nephrectomy, both markers normalized to concentrations essentially

equivalent to the controls. This phenomenon further strengthens the argument for the specificity of these urine proteins in RCC screening.^{12,13} Cancer biomarker specificities are invariably assessed in the relevant biological fluid prior to surgical removal of the tumor. If solely based on the pre-surgical concentration in the urine, AQP1 and PLIN2 are 100% specific by ROC analysis. Curiously, 4 patients had post-surgical biomarker concentrations essentially equivalent to their pre-surgical levels (Figure 1). If based upon the pre-versus post-surgical biomarker concentrations, the specificity would not be 100% but about 85%; still an impressive specificity. The concentration of both AQP1 and PLIN2 decreased in the urine following nephrectomy in the 3 patients with noted metastatic disease at the time the nephrectomy. We do not think that exosomal AQP1 and PLIN2 shed by metastases would be filtered at the glomerulus and show in the urine, however, this needs to be determined in a larger sampling of patients with metastatic disease at the time of nephrectomy.

Tumor stage is based on size and whether the tumor has spread beyond the kidney, while tumor grade describes microscopic tumor cell appearance. While urine concentrations of AQP1 and PLIN2 reflected tumor size and hence stage; they did not reflect histologic tumor grade. We did not find a difference in urine AQP1 or PLIN2 concentrations between patients with clear cell versus papillary tumors. Similarly, there was no significant difference in tumor AQP1 protein expression between clear cell and papillary RCC, based on immunohistochemical analysis.¹⁸ Urine AQP1 significantly increased from control patients through pT1a and pT1b and further to pT2. When investigating PLIN2, the marker concentrations were significantly increased when comparing control patients to pT1a and then to pT1b, but the trend did not significantly continue on to pT2 or pT3 (Figure 2). These trends in AQP1 and PLIN2 seen with stage further illustrate the concept that concentrations are more related to size than stage. It should also be noted that the association is largely driven by the data from the clear cell carcinomas since 52 of the 61 patients were diagnosed with this tumor subtype. Once tumors are pT3, where the classification is based on either extra-renal extension and protrusion into circulation, there is no correlation with either of the two urinary marker concentrations. Lastly, in regards to size, our multivariate analysis showed that size was the only independent predictor for tumor marker concentrations. This analysis of AQP1 and PLIN2 based on stage, grade, and tumor subtype (clear cell vs. papillary) was enabled by combining the results from two separate studies,^{12,13} due to an increased number and diversity of tumors in this analysis.

Expression of AQP1 and PLIN2 in tumor cells has also been evaluated based on other pathological parameters. In pathology specimens, RCC tumors show decreased expression of both AQP1 and PLIN2 as tumors progress from low to high Fuhrman grades.^{18,19,21–23} In the present group of patients, urine AQP1 and PLIN2 concentrations mirrored previous histological findings as no association was found between tumor grade or histological subtype (clear cell vs. papillary). Furthermore, when evaluating urinary PLIN2 concentrations in relation to tumor size, we found that the best polynomial fit to the data was negatively influenced by lower PLIN2 concentrations in the six patients with the largest tumors (Figure 2C). Five of these six patients had Fuhrman grade 3 or 4 tumors resulting in a non-linear increase in PLIN2 despite increased tumor size. This suggests that unspecified molecular factors contributing to increased Fuhrman grade blunt PLIN2 expression.

CONCLUSION

The results of our investigation show that urine AQP1 concentrations distinguished clear cell and papillary RCC from controls with a sensitivity and specificity of 100% and 100% while urine PLIN2 displayed sensitivity and specificity of 92% and 100%. Multivariate analysis showed that only tumor size was an independent predictor of urine AQP1 and PLIN2 concentrations. Urine concentrations of both markers were positively correlated with

tumor stages T1a, T1b and T2 since these stage classifications are driven by tumor size. Long-term follow up of these patients will also be required to determine if either marker has the potential to predict local recurrences, distant metastasis, or survival differences.

Acknowledgments

The authors would like to thank Anesthesiology Clinical Coordinators Megan Kalin, B.A. and Karen Frey, C.C.P.R. and Urology Clinical Coordinators Alethia Paradis, M.S. and Janise Webb, C.T.R. for their help in the study.

Support for this study was provided by the Department of Anesthesiology and the Division of Urology, Washington University in St. Louis School of Medicine, a grant from the Longer Life Foundation, a grant from the Bear Cub Fund of Washington University, a grant from the Barnes-Jewish Hospital Foundation of Washington University School of Medicine CTSA UL1 TR000448, and a grant from the National Institutes of Health R01CA141521 each to JJM.

Washington University has a patent pending for the use of urinary AQP1 and PLIN2 to diagnose kidney cancer.

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Figure 1.
Pre- and post-nephrectomy urine concentrations of AQP1 and PLIN2 in 47 patients with clear cell or papillary renal cell carcinoma.

Figure 2. Pre-nephrectomy urine concentrations of AQP1 (panels A and B) and PLIN2 (panels C and D) in 61 patients with clear cell or papillary renal cell carcinoma compared with tumor size (panels A and C) or tumor stage (panels B and D). Closed diamonds (panels A and C) indicate biomarker concentrations in patients with stage pT3 tumors. The dashed lines in panels A and C depict the linear correlation of biomarker concentration to tumor size while the polynomial correlation is depicted by the solid lines. The tumor stage analysis (B and D) includes data from 43 control patients with no known renal tumors.

Table 1

Clinical and laboratory features of all patients.

Parameters	Renal Cell Carcinoma (N=61)	Controls (N=43)	P-Values
Age: Mean (SD)	58 (13)	59 (13)	0.527
Sex: No. (%)			
Male	40 (66)	26 (60)	0.594
Female	21 (34)	17 (40)	
Smoker: No. (%)			
Yes	31 (51)	21 (49)	0.842
No	30 (49)	22 (51)	
eGFR: Mean (SD)	86 (30)	85 (20)	0.737
Weight (kg): Mean (SD)	92 (26)	92 (20)	0.931
Pre-nephrectomy or preoperative urine AQP1: Median (Q1,Q3)	53 (4, 86)	1.5 (1,2)	<0.001
Pre-nephrectomy or preoperative urine PLIN2: Median (Q1, Q3)	59 (22, 96)	6 (4,7.5)	<0.001
Post-operative urine AQP1: Median (Q1, Q3)	3 (1,7.5)	NA	NA
Post-operative urine PLIN2: Median (Q1, Q3)	4 (2,7.5)	NA	NA

Table 2

Multivariate analysis of factors predicting AQP1 and PLIN2 concentrations.

Variable	AQP1			PLIN2		
	Estimate	Standard Error	P-Value	Estimate	Standard Error	P-Value
Age (>60)	-4.25	8.07	0.600	1.82	16.82	0.915
Sex (Male)	15.56	8.69	0.080	29.96	18.14	0.105
Smoker (Yes)	9.87	8.99	0.278	7.36	18.75	0.696
eGFR	-0.07	0.14	0.637	0.21	0.30	0.483
BMI (Overweight)	-5.55	12.06	0.647	-11.16	25.15	0.659
BMI (Obese)	-1.23	12.29	0.921	-24.84	25.63	0.337
Size	7.29	1.16	<0.001	7.47	2.43	0.003
Grade (3&4)	-5.06	5.20	0.335	-7.92	21.68	0.717
Subtype (Papillary)	10.33	11.94	0.391	-21.70	24.91	0.388