Longer Life Foundation Final Research Report

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Abstract: Based on microarray studies showing that several genes were highly expressed in cancerous compared to normal kidney cells, we hypothesized that novel proteins would be found in high concentrations in the urine of patients with kidney cancer. In an IRB-approved clinical investigation, we found (by Western blotting) markedly increased urine concentrations of aquaporin-1 (AQP-1) and adipophilin (ADRP) in patients with clear cell and papillary carcinomas which together account for over 80 percent of kidney cancers. A month or more after tumor removal the concentration of these two novel proteins decreased to barely or undetectable levels in the patients' urine. These proteins appear sensitive and specific and, if validated and matured into a clinically useful assay, they would be the first ever non-invasive biomarkers for detecting renal cancer and its recurrence. The specific aim of the proposal was to: **Identify candidate novel biomarker proteins in the urine of patients with renal cancer.**

Through this grant from the Longer Life Foundation we were able to validate the sensitivity and specificity of AQP-1 and ADFP to differentiate patients with clear cell and papillary subtypes of kidney cancer as small as 1 centimeter from control patients with ostensibly no cancer. These surgical control patients were matched by age, sex, estimated glomerular filtration rate, weight and smoking history to be statistically indistinguishable from the patients having surgery for kidney cancer. In addition to AQP-1 and ADFP, the urine of both the cancer and control cohorts was used to measure the concentrations of neutrophil-gelatinase associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1), two emerging biomarkers of general kidney injury, to compare their sensitivity and specificity to that of AQP-1 and ADFP. Based on our results, KIM-1 will detect patients with kidney cancer recognizing it as a kidney injury while NGAL is less sensitive. Neither KIM-1 nor NGAL are specific for kidney cancer. AQP-1 and ADFP appear to be sensitive and specific for clear cell and papillary subtypes that account for over 80 percent of kidney cancer.

By the SEER Stat Facts of the Kidney Cancer Homepage of the National Cancer Institute, one in 70 adults in the United States will develop kidney cancer during their lifetime. In men, renal cancer was seventh among newly diagnosed cancers in 2009 ahead of both leukemia and pancreatic cancer, and in women it was eighth in newly diagnosed cancers ahead of both ovarian and pancreatic cancer. Development of a cost effective and high through-put ELISA for either or both of these proteins could be used to annually screen the general population or at least at risk populations for kidney cancer eventually by convenient dip-stick technology. Systematic screening would identify this devastating disease at an early treatable stage, certainly earlier than occurs by haphazard discovery or by clinical presentation, thereby saving lives. Discovery of the kidney cancers at an early stage would also allow tumor resection by relatively non-invasive laparoscopic and nephron-sparing partial nephrectomy rather than open total uninephrectomy thereby decreasing patient morbidity, hospitalization costs and preserving future kidney function.

Summary and Description in Lay Language: Renal (kidney) cancer is a silent killer. By the time renal cancer causes symptoms which prompt patients to visit their physician, the tumor has usually advanced beyond a curative stage. In the US in 2009 over 54, 000 new cases of kidney cancer were diagnosed and almost 13,000 patients died from this disease. This represents about 3 percent of adult malignancies but kidney cancer is the sixth leading cause of cancer deaths. Over three billion dollars was spent on kidney cancer alone in the United States in 2009. There are known risk factors for kidney cancer such as smoking, obesity and hypertension. Unfortunately, there is no test that can be effectively and easily used to screen populations at high risk to develop kidney cancer. This is tragic, because when diagnosed early, surgery often cures the disease and prolongs life first by tumor removal and second by preserving viable kidney function. Previous studies by many groups have characterized gene and protein expression in tumor tissue once it was removed, but this has not translated into any clinically relevant tests to prospectively diagnose kidney cancer. The goal of our study was to identify novel biomarkers – molecules in urine that are either atypically present or absent that will signal the presence of otherwise undetectable cancers, and allow their early removal. Based on preliminary data, we have found two novel proteins, aquaporin-1 and adipophilin in the urine of patients with kidney cancer that are greatly decreased in their urine after surgery to remove the tumor. These biomarkers were characterized to determine their sensitivity and specificity to diagnose kidney cancer. The accuracy of the markers to specifically diagnose kidney cancer was evaluated by comparing the marker levels in age- and sex-matched normal individuals who had undergone surgery for non-kidney related issues and to compare these results to diagnoses based on two general biomarkers of kidney injury. Based on our study, one of the general kidney injury biomarkers did differentiate patients with kidney cancer from the surgical control patients. The other general biomarker was less discriminating in diagnosing kidney cancer. Unfortunately, both of these general biomarkers were not specific for kidney cancer suggesting that additional tests would need to be done to specifically diagnose kidney cancer. Our markers, aquaporin-1 and adipophilin, were found to be sensitive and specific at discriminating patients with the most prevalent forms of kidney cancer (accounting for over 80 percent of kidney cancers) from the surgical control patients. Patients with tumors as small as 1 centimeter (about 0.4 inch) could readily be discovered.

In the future, we will develop more inexpensive, high throughput, real-time assays for the biomarkers that can give same-day results. Overall, this investigation has the potential to improve the health of everyone in general and those in at-risk populations by developing a method to detect kidney cancer at a stage early enough to allow a significant chance for cure and preserve existing kidney function all of which will lead to a longer life.

Introduction: Kidney cancer is generally silent and frequently fatal (1-4). About 80 percent of kidney tumors are discovered incidentally during abdominal imaging performed for unrelated diagnostic reasons. When symptomatically diagnosed due to blood in the urine, abdominal pain or abdominal mass; renal cancer has already metastasized in 30-40 percent of patients. Renal cancer is notoriously resistant to chemotherapy, and metastatic disease means a poor prognosis, with a 5 year survival of 5 percent. Even with earlier diagnosis due to incidental radiologic detection, up to 30 percent of patients will ultimately have metastatic disease (1-4).

Renal cell carcinoma accounts for about 3 percent of adult malignancies but is the sixth leading cause of cancer deaths. Thus, it should not be considered a rare disease. More than 57,000 cases of kidney cancer were diagnosed, almost 13,000 deaths attributable to this disease occurred, and more than 3 billion dollars was spent on kidney cancer alone in the United States in 2009. By the SEER Stat Facts of the Kidney Cancer Homepage of the National Cancer Institute, one in 70 adults in the United States will develop kidney cancer during their lifetime. In men, renal cancer was seventh among newly diagnosed cancers in 2009 ahead of both leukemia and pancreatic cancer, and in women it was eighth in newly diagnosed cancers ahead of both ovarian and pancreatic cancer. Having suitable biomarkers of kidney cancer are important to disease diagnosis and documenting response to therapy. We have shown that aquaporin-1 (AQP1) and adipophilin (ADFP) are significantly increased in urine of patients with clear cell and papillary kidney cancer, which together account for more than 80 percent of all kidney cancers (5). The suitability of these markers entails specificity of the diagnostic procedure to differentiate patients with kidney cancer at an early stage (T1a tumors less than 4 cm.) from control patients. By the time kidney tumors grow large enough to be symptomatically diagnosed (hematuria, flank pain or a palpable flank mass), they are often (30-40percent) metastatic (1-4). Increasingly, kidney cancer is found incidentally when otherwise asymptomatic individuals undergo abdominal imaging (ultrasound, CT or MRI) for unrelated diagnostic purposes. Nonetheless, despite pre-symptomatic diagnosis, about one-third of these tumors have micro-metastasized to local lymph nodes or adjacent organs at the time of diagnosis. Metastatic kidney cancer is notoriously resistant to chemotherapy with a 2 year survival of less than 20 percent. Thus a diagnosis of renal cancer portends poor patient outcome. In contrast, early diagnosis has a more promising prognosis. Since renal cancer is largely unilateral, a small imaged renal mass confined in the kidney can be removed by nephrectomy with survival rates exceeding 70 percent. Furthermore, early detection enables partial rather than total nephrectomy, and relatively non-invasive laparoscopic surgery rather than an open flank incision total nephrectomy; reducing morbidity, cost, accelerates patient recovery and preserves future renal function.

Unfortunately, there is no valid biomarker for the early detection of kidney cancer. The established test for overall kidney function, serum creatinine, is not sensitive enough for early renal cancer detection. The emerging urinary biomarkers of kidney disease, KIM-1 and NGAL, although indicative of kidney disease per se, may not be sensitive or specific enough to validly diagnose kidney cancer. The unmet need and barrier to progress in the field is a sensitive and specific diagnostic test for the early detection of kidney cancer from other urologic cancers, particularly at a curative stage. As stated above, we have shown that AQP1 and ADFP are significantly increased in urine of patients with clear cell and papillary kidney cancer. These are the first ever sensitive and specific urine biomarkers for detecting kidney cancer. Having a low cost and reliable means of diagnosing patients with kidney cancer will improve clinical practice by identifying patients at an early non-metastatic stage when there are more options for tumor removal and the preservation of future kidney function. This study seeks to validate the usefulness of AQP-1 and ADFP to diagnose patients with kidney cancer in a second cohort of patients.

Methods:

AQP-1 and ADFP Western blots: AQP-1 and ADFP were quantitated as described (5). Briefly, urine was centrifuged (1,800g, 10 min) to remove debris and mixed with a protease inhibitor tablet (Roche) prior to processing for Western blot analysis or freezing at -80°C. Urinary creatinine concentration was quantified by the Jaffe reaction. Protein from 100 µl of fresh spun urine was precipitated with 1.5 ml ice cold acetone-methanol (1:1), centrifuged and washed with fresh acetone-methanol (1.5 ml). Precipitated proteins were dissolved in an amount of SDS-sample buffer such that 5µl of sample reflected the amount of urine containing 1µg of creatinine. Urine samples processed for Western blot were stored at 4^{o}C prior to analysis. The blocked membranes were incubated with 1/500 dilution of anti-AQP-1 (H-55) antibody or a 1/200 dilution of anti-ADFP (H-80) antibody (both from Santa Cruz Biotechnology, Inc. Santa Cruz, CA) in blocking buffer containing 0.1% Tween-20 overnight. After washing, the membranes were incubated with a 1/2000 dilution of donkey anti-rabbit IgG IRDye 680 (LI-COR Biosciences, Lincoln, NE) in blocking buffer with 0.1% Tween-20 for 1 hour. Both AQP-1 and ADFP were visualized and quantified using a LI-COR Odyssey Infrared Imager and proprietary software. AQP-1 and ADFP were quantified using arbitrary absorbance units. On each gel, the same 2 pre-excision urine samples were analyzed, and used to normalize the signal response across all gels run within the same or different days. Over the span of numerous gels for AQP-1, the variation in the signal of these common samples was 10% and of gels for ADFP the variation was 9%.

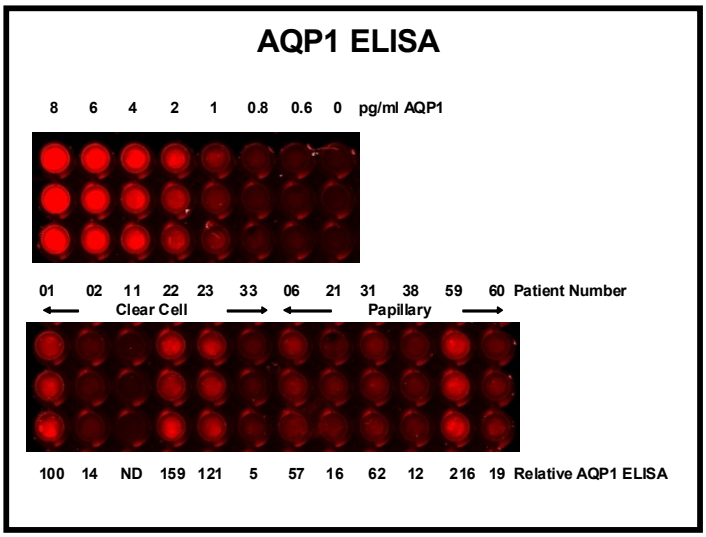
AQP-1 ELISA Development: Through the course of Western blot procedures, we have identified several polyclonal and monoclonal antibodies that have been used in preliminary studies to optimize the capture and detection of each protein. Recombinant His-tagged AQP-1 was expressed in HEK293 cells and the purified protein used as standard to develop sensitive and specific ELISAs for AQP-1. The results were normalized to the urinary creatinine concentration.

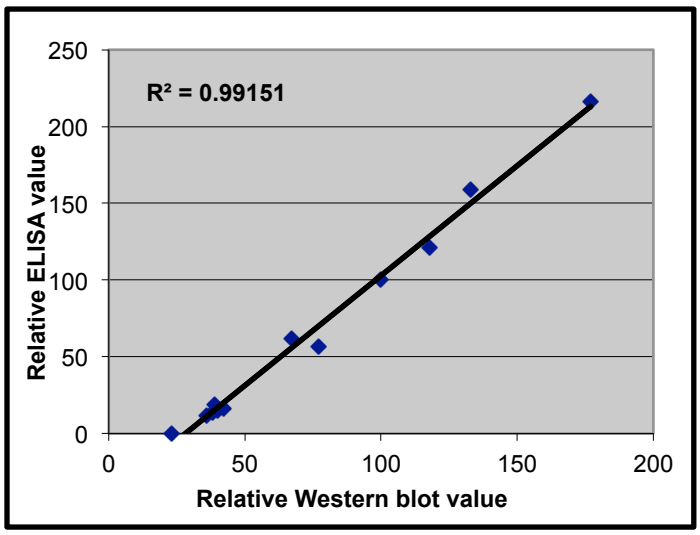
Results:

Patient Characteristics. Forty six patients with a presumptive diagnosis of kidney cancer were enrolled. Based on the final pathological results, 35 patients were found to have clear cell and papillary subtypes of kidney cancer with tumor sizes ranging from 0.6 to 18 centimeters. Eighteen patients had tumors at stage T1a; under 4 centimeters in greatest dimension. Overall, 12 patients were diagnosed with chromophobe, oncocytoma, angiomyolipoma and cysts. With over 30,000 surgeries performed each year at Barnes-Jewish Hospital, matching patients in a surgical control cohort by age, sex, weight (obesity is a risk factor for kidney cancer), smoking history (another risk factor) and overall kidney function to the kidney cancer patient cohort was achieved. This is illustrated by a histogram (Figure 1) which shows a bimodal distribution of patient age in the cancer cohort that was mimicked fairly well in the control patient group. This bimodal distribution may be due to an asymptomatic imaged renal mass discovered incidentally (younger age) and discovery due to symptoms (older age).The 35 patients with a final diagnosis of either clear cell (32) or papillary (3) subtypes of kidney cancer were statistically indistinguishable from the 44 surgical control patients by age (0.94 by t-test), sex (0.84 by Chi-square test), weight (0.76 by Wilcoxon rank sum test), smoking history (0.96 by Chi-square test) and estimated glomerular filtration rate as an overall measure of kidney function (0.71 by t-test).

Biomarker Characteristics. Regression analysis of the 35 patients with clear cell and papillary factored by tumor size gave Spearman rank correlations of 0.709 (r^2= 0.05) and 0.191 (r^2=0.24), respectively for the non-specific kidney injury biomarkers KIM-1 and NGAL (data not shown). Similar analysis for the kidney cancer markers gave Spearman rank correlations of 0.894 (r^2= 0.83) and 0.923 (r^2=0.81), respectively for ADFP and AQP-1 (Figures 2 and 3). By area under the receiver operating characteristic curve analysis, KIM-1 and NGAL were 0.976 and 0.845 respectively (data not shown). For AQP-1 and ADFP, the area under the curves was 0.99 and 0.94 for AQP-1 and ADFP respectively (Figures 4 and 5).

Preliminary ELISA.





In preliminary studies, we have developed an ELISA for AQP1 in urine (left panel) giving results fairly in accordance with the AQP1 relative quantitation by Western blot (right panel) when both were normalized to the urinary excretion of creatinine. The means of detection was IRDye-tagged antibody to the detection antibody of the sandwich ELISA for AQP-1. This ELISA will be improved to maximize sensitivity to be able to measure AQP-1 in both patient urine and serum and have at least the sensitivity of the Western blot for urine samples. We are testing effects of urine salt and urea on ELISA results and transitioning to horse radish peroxidase second antibody or luminescent detection to improve sensitivity.

Discussion:

This investigation shows that KIM-1 concentrations were increased in the urine of patients with clear cell and papillary kidney cancers and were significantly correlated with tumor size (Spearman rank correlation 0.709). These results might suggest that KIM-1 may have potential utility as biomarker to detect occult renal cell and papillary carcinomas. However, as discussed below, based on the literature, KIM-1 lacks the specificity to correctly diagnose kidney cancer per se. In contrast, urine NGAL concentrations were not significantly increased in renal cancer patients and were less related to tumor size (Spearman rank correlation 0.191). These results suggest that NGAL has little potential as a renal cancer biomarker.

Kidney injury molecule-1 (KIM-1) is a promising biomarker of renal injury undergoing clinical evaluation. By immunohistochemistry, KIM-1 expression was found to be significantly increased in clear cell and papillary carcinoma, and not present in adjacent normal kidney (6 and 7). Urinary KIM-1 excretion was increased in patients with clear cell or papillary tumors, and the urine KIM-1 excretion somewhat mirrored tumor size (6) Our study found a reasonable correlation with tumor size ranging from 0.6 to 3.8 cm With a Spearman rank correlation of 0.709. That KIM-1 is found in increased amounts in the urine of patients with these renal tumors is additionally important since the tumors are partially or completely surrounded by a fibrous capsule (8 and 9). This suggests communication of the renal tumors with the urine-forming tubular elements of the kidney or the ability of an encapsulated tumor to affect adjacent “normal” kidney, supports the use of urine as a source to discover and measure biomarkers specific for kidney cancer. From this study (especially the ROC analysis of 0.976) and studies cited above, one would outwardly conclude that urinary KIM-1 is a sensitive and specific biomarker of kidney injury. Nevertheless, based on the literature, KIM-1 is over-expressed and excreted in the urine of patients with numerous types of kidney injury such as that due to diabetes, glomerulosclerosis, IgA nephropathy, polycystic kidney disease, nephrotoxicants and ischemia (10-12). Therefore, the impressive ROC analysis is deceiving when true disease-specificity is considered. While it is non-specific to many kidney diseases, KIM-1 appears to be a sensitive biomarker of any kidney injury but here lacks the specificity needed for a biomarker of kidney cancer. Since clear cell and papillary carcinoma affects over 80 percent of those with kidney cancer, KIM-1 analysis would detect the vast majority of individuals with this devastating disease; however, as described while sensitive to detect kidney injury, it does not have the specificity to directly diagnose kidney cancer as the disease entity per se. Recently, we have identified increased concentrations of urinary AQP-1 and ADFP as having specificity to diagnose clear cell or papillary kidney cancer (5).

Here in this study sponsored by the Longer Life Foundation, we have validated the sensitivity and specificity of AQP-1 and ADFP to distinguish patients with imaged renal masses pathologically identified conclusively as clear cell and papillary subtypes of kidney cancer from control patients. This assessment is based on the Spearman rank correlations of 0.923 and 0.894 for AQP-1 and ADFP, respectively, and the AUROC values of 0.99 and 0.92 respectively. Individually, AQP-1 and ADFP would minimize the number of false positive or false negative results. What is needed to solidify the specificity issue for diagnosing kidney cancer is to measure urinary AQP-1 and ADFP in a variety of patients with common renal diseases such as diabetic nephropathy or various glomerulonephritites. This represents the first non-invasive assay to diagnose kidney cancer at a treatable stage of the disease and at a time to preserve post-surgical kidney function. Perhaps a combination of non-specific but sensitive marker to any kidney injury (such as KIM-1) along with sensitive and specific markers to kidney cancer, AQP-1 and ADFP, will be necessary to correctly diagnose this disease and the presence of co-morbid kidney diseases.

Future Plans: This validation data was included in a grant application to the NIH resulting in a fundable award with an impact score of 25 and a percentile of 9.

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URINARY BIOMARKERS OF RENAL CELL CARCINOMAS

An aim of this R01 grant was to develop and refine ELISAs for AQP-1, ADFP and other candidate biomarkers of renal carcinomas.

Additional plans involve measuring urinary AQP-1 and ADFP levels in patients with common kidney diseases such as diabetic nephropathy and glomerulonephritites.

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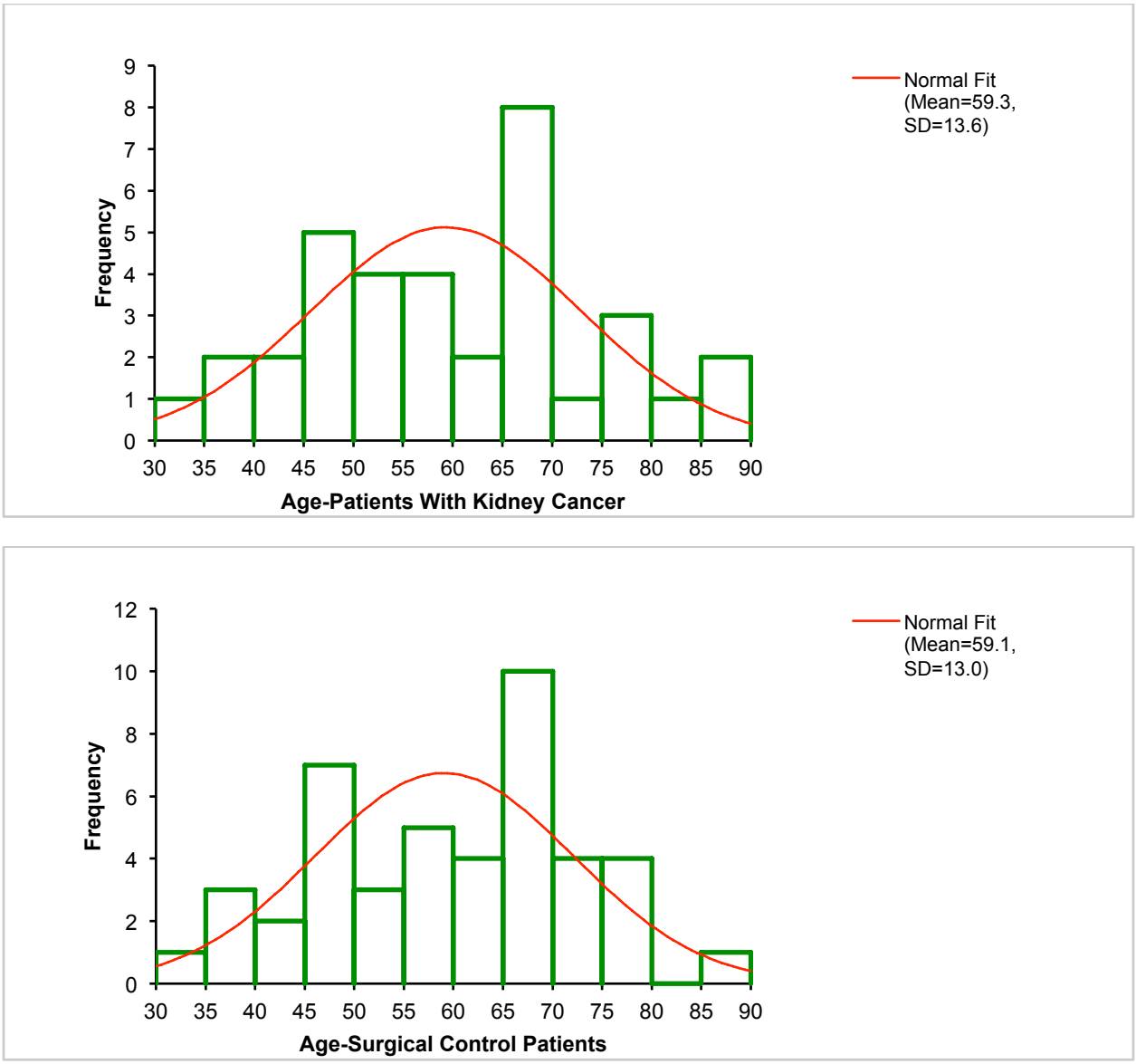

Figure 1. Histogram of age in the cohort of 35 patients with clear cell and papillary subtypes of kidney cancer (upper) and the 44 patient surgical control cohort (lower).

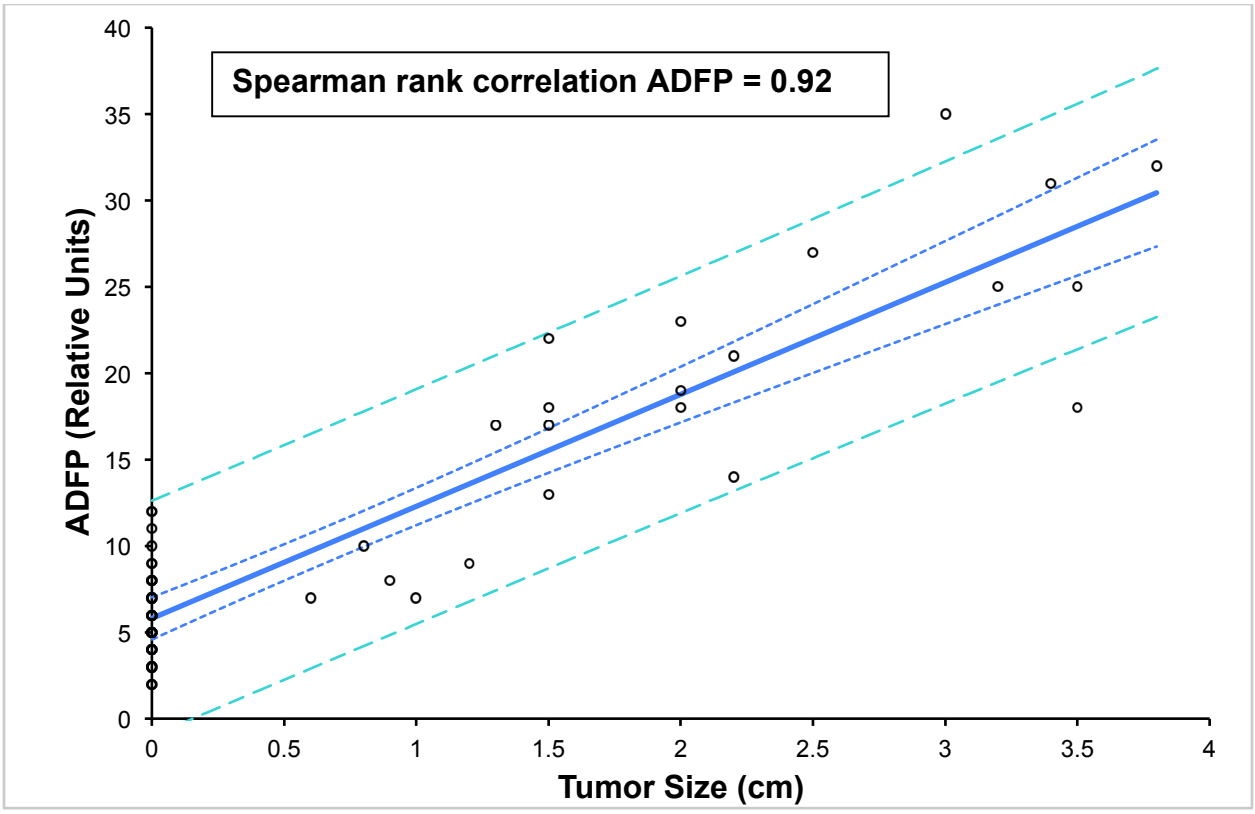

Figure 2. Regression analysis of urinary ADFP levels in patients with stage T1a clear cell and papillary kidney cancer and surgical control patients (zero tumor size).

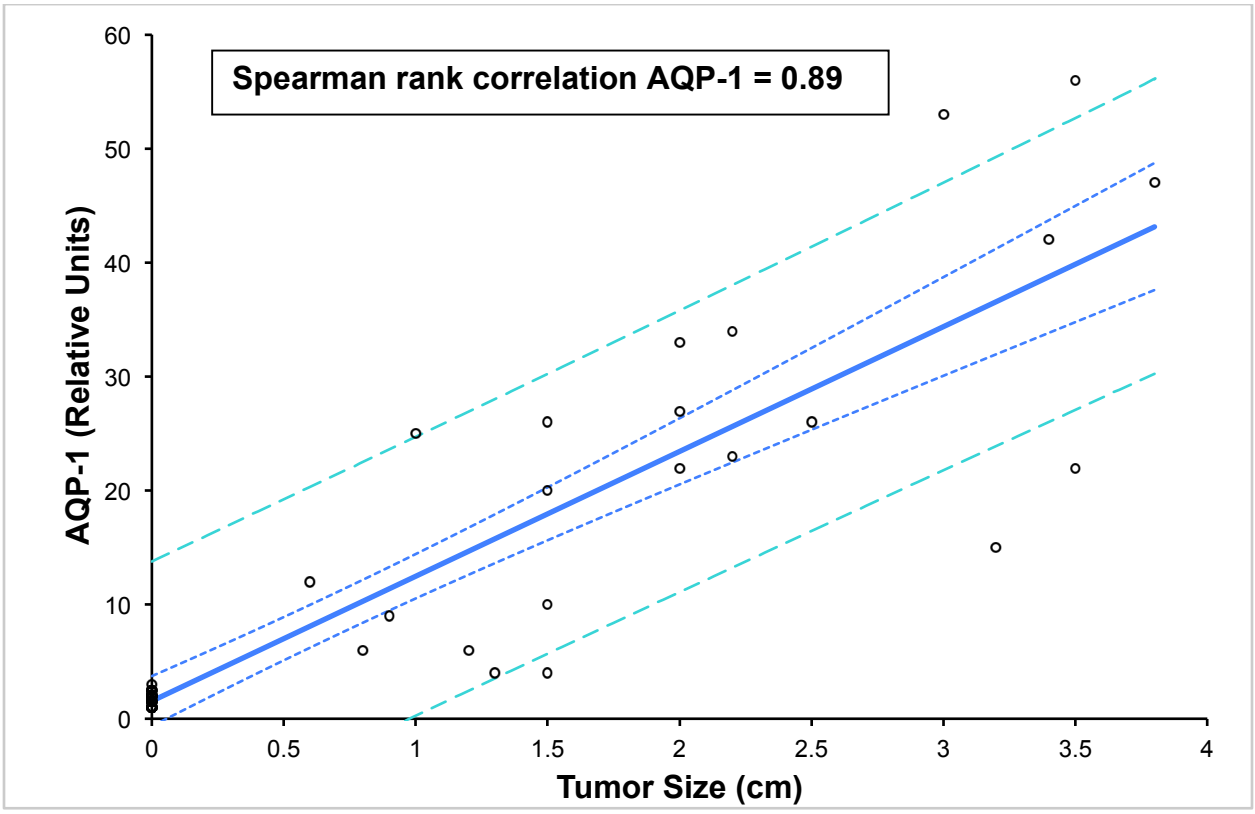

Figure 3. Regression analysis of urinary AQP-1 levels in patients with stage T1a clear cell and papillary kidney cancer and surgical control patients (zero tumor size).

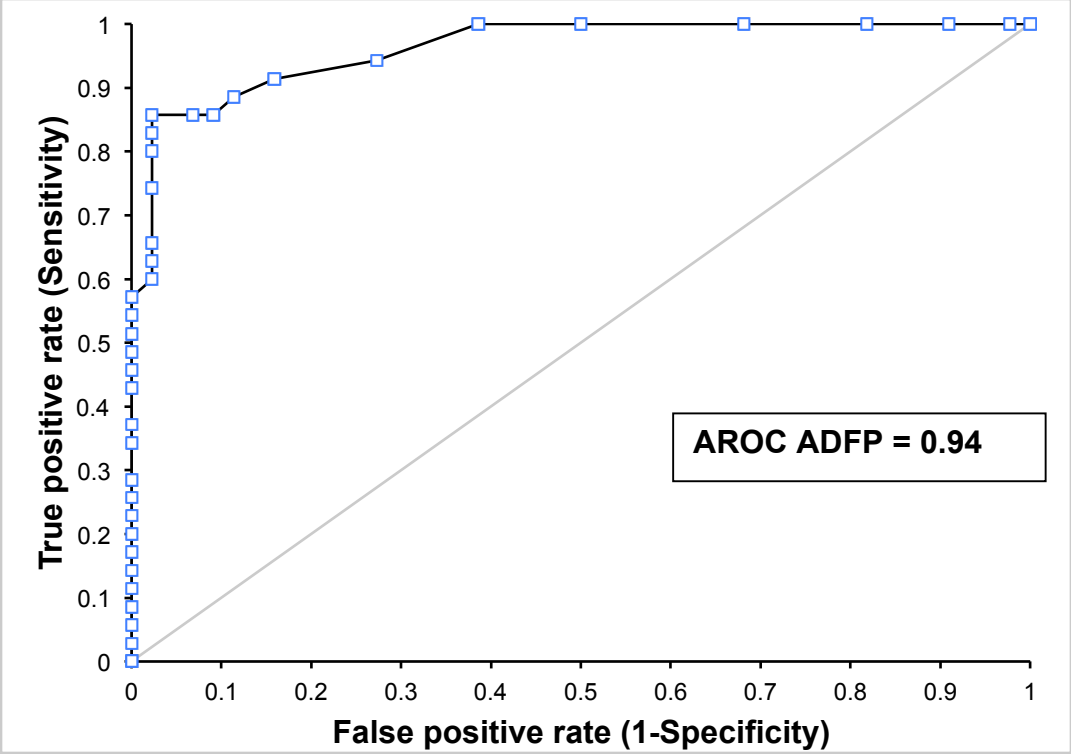

Figure 4. Receiver operating characteristic curve analysis of urinary ADFP levels in patients with stage T1a clear cell and papillary kidney cancer and surgical control patients.

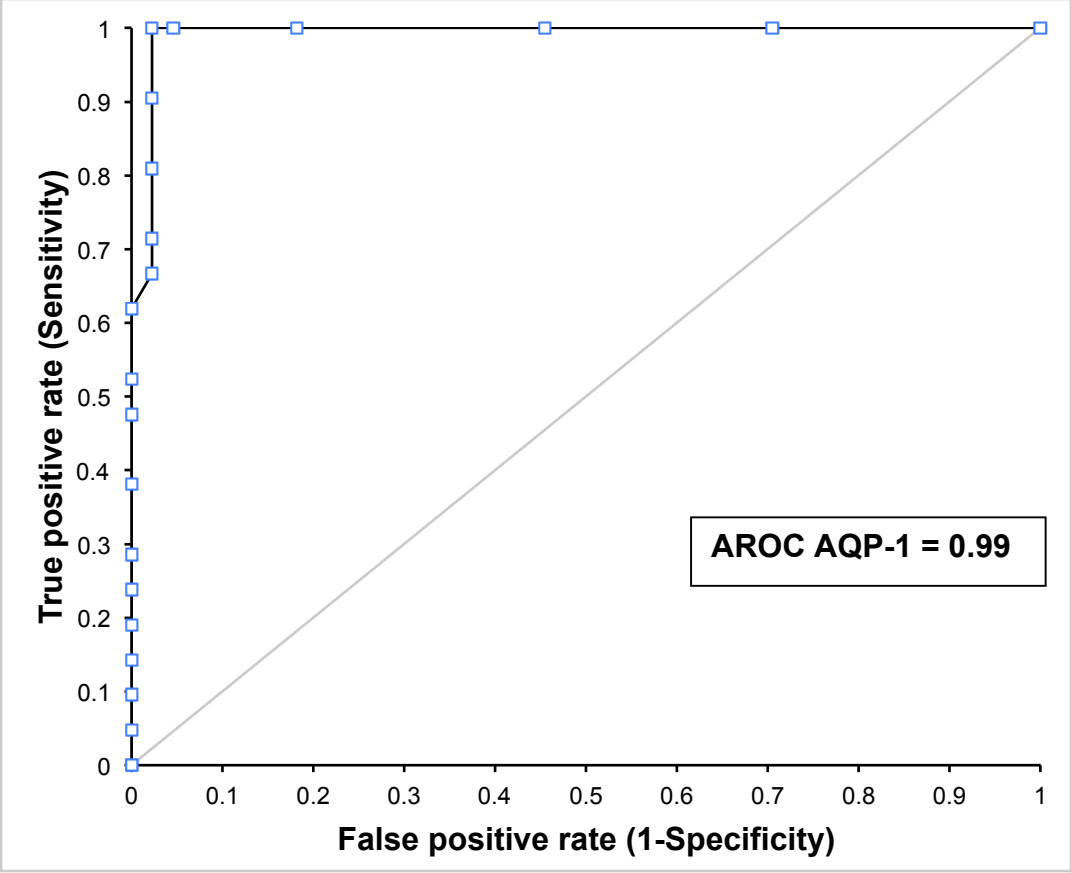

Figure 5. Receiver operating characteristic curve analysis of urinary AQP-1 levels in patients with stage T1a clear cell and papillary kidney cancer and surgical control patients.