Protein Microarrays Discover Angiotensinogen and PRKRIP1 as Novel Targets for Autoantibodies in Chronic Renal Disease

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Biomarkers for early detection of chronic kidney disease are needed, as millions of patients suffer from chronic diseases predisposing them to kidney failure. Protein microarrays may also hold utility in the discovery of autoantibodies in other conditions not commonly considered auto-immune diseases. We hypothesized that proteins are released as a consequence of damage at a cellular level during end-organ damage from renal injury, not otherwise recognized as self-antigens, and an adaptive humoral immune response to these proteins might be detected in the blood, as a noninvasive tracker of this injury. The resultant antibodies (Ab) detected in the blood would serve as effective biomarkers for occult renal injury, enabling earlier clinical detection of chronic kidney disease than currently possible, because of the redundancy of the serum creatinine as a biomarker for early kidney injury. To screen for novel autoantibodies in chronic kidney disease, 24 protein microarrays were used to compare serum Ab from patients with chronic kidney disease against matched controls. From a panel of 38 antigens with increased Ab binding, four were validated in 71 individuals, with (n = 50) and without (n = 21) renal insufficiency. Significant elevations in the titer of novel auto-Ab were noted against angiotensinogen and PRKRIP1 in renal insufficiency. Current validation is underway to evaluate if these auto-Ab can provide means to follow the evolution of chronic kidney disease in patients with early stages of renal insufficiency, and if these rising titers of these auto-Ab correlate with the rate of progression of chronic kidney disease. ***Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.000497, 1–8, 2011.***

Renal injury affects nearly a half-million patients in the United States and over a million people world-wide. Millions of additional patients diagnosed with chronic kidney disease (CKD)[1] are at highest risk for progression to end-stage renal disease (ESRD), and those individuals diagnosed with diabetes or hypertension are at high risk for developing this disease. The consequences of development of ESRD are severe, especially when progression occurs without early diagnosis. Early detection and initiation of potentially kidney-sparing therapies, such as angiotensin converting enzyme inhibitors and angiotensin II receptor blockers, can offer significant benefit to patients with renal injury (1, 2).

Several markers have been discovered over the past few years that predict mortality in patients with ESRD on dialysis, including increased FGF-23 (3), γ-glutamyltransferase (4), N-terminal pro-brain natriuretic peptide (5), osteoprotegerin (6), and markers of protein-energy wasting (7). Neutrophil gelatinase-associated lipocalin has been shown to be an early marker of progression from CKD to ESRD (8, 9). However, besides osteoprotegerin, few of these markers have demonstrated utility as candidates that predict worsening of kidney function toward ESRD in patients without CKD.

A chronic inflammatory state in renal injury and ESRD has been well described. C-reactive protein level, a broad marker of inflammation, is a strong predictor of mortality (10), whereas specific immune dysregulation, including dysfunction of regulatory T cells, has been shown to be present in ESRD (11). Levels of the inflammatory mediator long pentraxin 3 have been shown to be increased in the serum of patients with ESRD (12). Variants in genes coding for immune factors, including IL-10, have been associated with susceptibility to ESRD (13).

Given these studies, we hypothesized that we may be able to identify novel autoantibody biomarkers for CKD as it is likely that intracellular proteins are exposed as a consequence of damage at a cellular level from end-organ damage from chronic kidney disease. Given the increased inflammatory state, we hypothesized that some of these released proteins might not normally be exposed to B cells undergoing development and are not otherwise recognized as self-antigens. We suspected that some of these proteins could be recog-

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[1] The abbreviations used are: CKD, chronic kidney disease; ESRD, end-stage renal disease; ELISA, enzyme-linked immunoabsorbent assay; AGT, angiotensinogen; ACE, angiotensin converting enzyme.

nized as antigenic, yielding an adaptive humoral immune response that could be detected in the blood in organ injury.

To test our hypotheses and to screen for these putative auto-Ab responses, we used protein microarrays to screen sera from patients with CKD and control individuals. Protein microarrays have previously been used to identify tumor antigens (14), auto-antibody targets in rheumatologic and autoimmune disease (15), allo-antibodies in solid-organ transplantation (16), and potential markers for graft-*versus*-host disease. Rather than expecting a role for autoimmunity in renal injury, we suspected that the humoral immune response could serve as an amplifier for abnormal proteins exposed in the end-organ damage. We therefore used protein microarrays to detect the presence of auto-Ab in the sera of patients with established renal insufficiency.

In a first-stage analysis, we compared the serum antibodies from 17 patients with CKD with an estimated glomerular filtration rate $<$ 30 ml/min/1.73m^2 (17)(CKD stages 4 and 5) against seven age-matched healthy control individuals with normal renal function. We identified 38 candidate antigens showing significantly higher antibody binding levels in patients with CKD as compared with controls. We then tested four of these antibodies (anti-AGT, Anti-PRKR1P1, anti-ATG7, and anti-DUSP26; $p < 0.005$) in a second-stage, using *independent* sera obtained from 50 patients with CKD and 21 healthy age-matched controls. We found anti-angiotensinogen (anti-AGT) autoAb ($p < 0.001$) and anti-PRKRIP1 autoAb ($p < 0.013$) were significantly higher in patients with CKD, independent of etiology. Analysis of confounders for Ab signal showed no impact of gender, age, race, CKD therapy, or original diagnosis.

MATERIALS AND METHODS

Patient Characteristics and Serum Processing—Approval was obtained from the Stanford Institutional Review Board for these studies and individual informed consent for further studies was obtained from all patients and donors. A 5.0 ml volume of blood was collected from the patients into a 5-ml cryotube and let stand at room temperature for at least 30 min. The serum was separated from the clot by centrifuging at 2000 $\times$ *g* using a swinging bucket rotor and stored at −80°C until use.

Discovery Phase—Samples from 17 patients with CKD collected between August 2001 and April 2006, were included. Causes of renal insufficiency in these patients related to reflux nephropathy ($n = 4$), focal segmental glomerulosclerosis ($n = 4$) and glomerulonephritis of unknown etiology ($n = 9$). Mean age of the patients at the time of sampling was 10.6 ± 5.4 (range 1–19 years). Twenty-four percent of patients were female. Samples from seven age-matched healthy controls were included.

Protein Microarray Protocol—The ProtoArray® human protein microarray V3 (Invitrogen, PAH052401) was used for profiling serum IgG interactions with potential antigens. Each microarray contains 5056 separate proteins printed in duplicate with N-terminal glutathione S transferase (GST) epitopes expressed in Baculovirus and affinity purified under native conditions maintaining their cellular enzymatic activities/native conformations. Each protein was derived from the Ultimate ORF collection (Invitrogen, Carlsbad, CA). Microarrays were stored at −20 °C, equilibrated to 4 °C for 10 min before use, blocked for 1 hour at 4 °C with sodium phosphate blocking buffer consisting of 100 mM sodium phosphate, 200 mM NaCl, 0.08% Triton X-100, 25% glycerol, 20 mM reduced glutathione, and 1.0 mM dithiothreitol at pH 7.4. All steps were carried out at +4 °C. Microarrays were then probed for 90 min with patient serum diluted 1/500 in phosphate buffered saline-Tween (PBST) buffer consisting of 1× phosphate buffered saline (PBS), 1% bovine serum albumin and 0.1% Tween 20 at pH 7.4. Probing was followed by washing with PBST buffer five times for 10 min each and then incubated for 90 min with goat anti-human IgG (H+L) Alexa Fluor® 647 (Invitrogen, A21445). Microarrays were then washed again with PBST buffer five times for 10 min and then dipped briefly into ddH2O, prior to centrifugation for 1 min to dryness before scanning. The Axon GenePix 6.0 Scanner and Analyzer were used to acquire microarray data. Slides were scanned at 635 nm with a PMT (Photomultiplier tubes) gain of 600, a laser power of 100% and a focus point of 0 μm. The ".gal" files were obtained from a ProtoArray central portal on the Invitrogen website (www.invitrogen.com/ProtoArray) by submitting the barcode of each protein microarray.

Statistical Analysis—Given that all samples were obtained from conditions not otherwise considered as auto-immune, spot intensities on the protein microarrays were low, as expected. The average background empty spot intensity across protein microarrays ranged from 458 to 986, whereas maximum spot intensities across microarrays ranged between 1002 and 2552. The median spot-to-spot Pearson correlation across the 5056 noncontrol intensity measurements on the protein microarrays was 0.40. Spot-to-spot Pearson correlation coefficients were significantly linearly correlated with overall average intensity measurements, with a linear regression model of correlation coefficient $= -0.07 + 0.00061 * \text{average intensity}$. Spots with average intensity measurements over 700 had average spot-to-spot Pearson correlation coefficient of 0.35. Results calculated using spot-to-spot Spearman correlation coefficients were similar.

Probes were selected from the 10,112 noncontrol spots on the protein microarrays, selecting for probes (1) showing a differential increase in mean intensity from control samples to CKD patient samples of twofold or higher, (2) showing an uncorrected *t* test *p* value ≤ 0.01, (3) with a mean intensity in healthy donor samples $\leq$ the mean empty spot intensity in those samples, and (4) with a mean intensity in renal insufficiency samples $\geq$ the mean empty spot intensity in those samples. This yielded only 42 (0.4%) significant probes. Probe identifiers were matched to Entrez Gene identifiers using ALLUN (18), yielding 38 unique proteins. Four proteins were selected from these 38 based on prior biological knowledge on these proteins (see "Results") and targeted for ELISA validation in independent CKD patient and control serum.

To estimate a false discovery rate, we randomly permuted the CKD and control labels for each of the 10,112 noncontrol spots, and recalculated using the four criteria above. Across 100 shuffles, none of the 10,112 spots met the criteria, leading us to estimate the false discovery rate at or below 0.01.

As a first-pass assessment of intra-array technical variability, we separated the duplicate noncontrol spots from the 17 CKD patients and made two synthetic control groups, with each group containing 5056 spots and 17 CKD patients. Mirroring the four-step method above, we searched for probes showing a difference in mean intensity between the two groups of twofold higher or lower, an uncorrected *t* test *p* value ≤ 0.01, with a mean intensity in one group $\leq$ the mean empty spot intensity in those samples, and with a mean intensity in the other group $\geq$ the mean empty spot intensity in those samples. Only 15 proteins met these control criteria, indicating probes with significant spot-to-spot variability. None of these 15 proteins overlapped with the 38 proteins discovered above.

For each of the 10,112 noncontrol spots, we tested whether the intensities measured at that spot across the seven control patients

was normally distributed using the Shapiro-Wilk test of normality as implemented in R. A total of 9346 spots (92%) had a p value ≥ 0.05 suggesting the null hypothesis of normal distribution would not be rejected. Although the overall distribution of intensities on any one array is clearly non-normal, our data would indicate that the distribution of any one spot across a group of individuals could be viewed as normally distributed.

We found no statistically significant difference in overall max, mean, and sum of the noncontrol probes between control samples and renal insufficiency sample protein microarrays. To be parsimonious in methodology, our results were calculated without global normalization or scaling of microarrays. Even when each protein microarray was linearly scaled such that each probe measurement was divided by the sum of the overall intensity of each microarray, our results still held (though the p values were more significant).

All statistics were performed in R version 2.9.

Validation Phase—Enzyme linked immunosorbent assay (ELISA) validation for the candidate antibodies was performed on 71 independent serum samples obtained from 50 patients with CKD and 21 healthy age- and gender-matched controls with no history of CKD. The validation CKD samples were selected from a pool of hundreds of CKD patients enrolled in Lucile Packard Children's Hospital at Stanford over the past 5-year period. Our effort was to study CKD in general that included several phenotypes of CKD. Of the 50 CKD samples, 10 were from patients with aplastic, hypoplastic, or dysplastic kidneys, 10 with obstructive uropathy, 10 with focal segmental glomerulosclerosis, 8 with reflux nephropathy, 2 with idiopathic crescentic glomerulonephritis, 2 with antineutrophil cytoplasmic antibody vasculitis, and 1 each with vesicourethral reflux, type 1 membranoproliferative glomerulonephritis, systemic lupus erythematosus nephritis, C1Q nephropathy, IgA nephropathy, antineutrophil cytoplasmic antibody disease with an anti-glomerular basement membrane component, microscopic polyangiitis/pulmonary renal syndrome, and interstitial nephritis.

ELISA—An enzyme linked immunosorbent assay (ELISA) was developed to detect serum immunoglobulin binding to ATG7 (GenBank BC000091), AGT (BC011519), PRKRIP1 (BC014298), and DUSP26 (NM 024025). We followed previously published protocol (19). In brief, insect-derived purified proteins fused to glutathione S-transferase were acquired from Invitrogen (Carlsbad, CA). A titration was performed to determine the optimal amount to be coated to be coated on to the immunosorbant 96-well plate (NUNC Brand, 446612). Briefly, the 96-well microwell ELISA plates were coated with corresponding protein in 50 μl coating buffer (15 mM Na_2CO_3, 30 mM $NaHCO_3$, 0.02% NaN_3, pH 9.6) and incubated overnight at 4 °C. Standard curves were generated using anti-GST tag (mouse monoclonal IgG) (Millipore, Temecula, CA) and AP-conjugated AffiniPure Goat anti-Mouse IgG (Jackson ImmunoResearch, West Grove, PA). After washing the plates with tris buffered saline-Tween (TBST) buffer five times, the nonspecific protein binding was blocked by 100 μl, 2% dry milk in TBST buffer for 1 h at room temperature. Following the blocking step, 50 μl serum samples (40-fold diluted with 2% milk in TBST buffer) were incubated in the wells for 1 h at room temperature. The plates were washed five times with TBST buffer and incubated in 50 μl AP-conjugated AffiniPure mouse anti-human IgG (Jackson ImmunoResearch, West Grove, PA). The color was developed by using AP-pNPP Liquid Substrate System for ELISA (Sigma-Aldrich, St. Louis, MO). Absorption was measured at 405 nm with a SPECTRAMax 190 microplate reader (Molecular Devices, Sunnyvale, CA). To control for nonspecific binding, wells with no proteins coated were served as negative controls. The mean absorbance resulted by subtracting the negative control signal was used to calculate t test p values. Receiver operating characteristics (ROC) curves were generated from the ELISA data using ROCR (20).

RESULTS

To screen for putative auto-antibodies in patients with renal insufficiency, we used protein microarrays to measure serum antibodies from 17 patients with CKD and 7 control individuals. We identified 38 proteins (measured on 42 spots of the 10,112 spotted, or 0.4%) that demonstrated a significant increase in antibody binding levels as detected by secondary antibody (Table I), and chose to validate four of these antigens: angiotensinogen (AGT), protein kinase R interacting protein 1 (PRKRIP1), autophagy related 7 homolog (ATG7), and dual specificity phosphatase 26 (DUSP26).

Anti-AGT antibodies demonstrated a significant increase from a mean intensity of 354 in control samples to a mean intensity of 891 in renal insufficiency samples (3.2 times increase, t test p value = 0.005; Fig. 1*A*). As AGT is a well known drug target, and is known to be expressed in the kidney, as well as liver, lung, heart, and parts of the brain, AGT became our top candidate for validation. PRKRIP1 is also known to be well expressed in kidney and is induced by IL11 (21). IL11 can play a therapeutic role in reducing glomerular injury in mouse models of glomerulonephritis (22). Anti-PRKRIP1 antibodies showed a 2.2-fold increase, from 412 in control samples to 916 in renal insufficiency samples (t test p value = 0.0007; Fig. 1*B*).

ATG7 showed a significant 4.7 fold increase, from a mean intensity of 155 in control samples to a mean intensity of 735 in renal insufficiency samples (t test p value = 0.00008; Fig. 1*C*). ATG7 knockout mice show abnormal liver and central nervous system physiology and function (23). Though ATG7 is known to play a role in autophagy, it had not been implicated in kidney disease. Finally, levels of anti-DUSP26 increased from 278 in controls samples to 692 in renal insufficiency samples (2.5-fold increase, t test p value = 0.007; Fig. 1*D*). DUSP26 is involved in the growth of anaplastic thyroid cancer cells (24), but its potential role in kidney function or disease has not been described to date.

ELISAs were developed to detect serum immunoglobulin binding to these four candidate antigens, and were tested in independent serum obtained from 50 patients with renal insufficiency and 21 healthy control individuals. A separate published study demonstrated that there is an excellent correlation in between the fluorescent signal detected by Protoarrays and signal intensity measured using ELISA (19). We confirmed that anti-AGT autoAb were significantly higher in CKD samples (mean 0.075, standard deviation 0.028) than control samples (mean 0.021, standard deviation 0.022; 3.5 times increase in level, t test p value = 0.00143; Fig. 2*A*). Anti-AGT autoantibodies were able to distinguish CKD from control samples with an area under the receiver operating characteristic curve (area under the curve (AUC)) of 0.72 (Fig. 3).

We also found anti-PRKRIP1 autoantibodies were significantly higher in CKD samples (mean 0.17, standard deviation 0.09) than control samples (mean 0.13, standard deviation 0.05;

TABLE I

We identified 38 proteins demonstrating an increase in serum antibody binding in patients with ESRD as compared with controls (see Methods). We pursued validation for ATG7, DUSP26, PRKRIP1, and AGT; the latter two were successfully validated

Array ID	GenBank ID	T-test *p* value	Fold increase	Symbol	Name
B10R12C20	BC026229.1	0.0005	6.1	SFTPA1B	Surfactant, pulmonary-associated protein A1B
B42R03C05	BC000091.1	0.0001	4.8	ATG7	ATG7 autophagy related 7 homolog (S. cerevisiae)
B10R04C12	NM_006877.1	0.0019	4.1	GMPR	Guanosine monophosphate reductase
B22R08C20	NM_138454.1	0.0041	3.8	NXNL1	Nucleoredoxin-like 1
B29R05C10	NM_016058.1	0.0024	3.6	TPRKB	TP53RK binding protein
B37R09C15	BC007047.1	0.0000	3.5	FGL1	Fibrinogen-like 1
B33R05C18	BC024002.2	0.0011	3.5	FNDC8	Fibronectin type III domain containing 8
B38R12C16	NM_152285.1	0.0016	3.3	ARRDC1	Arrestin domain containing 1
B01R07C03	NM_005916.2	0.0076	3.2	MCM7	Minichromosome maintenance complex component 7
B14R08C04	BC058819.1	0.0066	3.2	LOC400713	Zinc finger-like
B13R04C15	BC011519.1	0.0051	3.2	AGT	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
B22R12C13	BC025682.1	0.0018	3.1	GSDML	Gasdermin-like
B11R07C19	BC028723.1	0.0015	3.1	CSNK1A1L	Casein kinase 1, alpha 1-like
B42R12C17	BC002680.1	0.0066	3.0	TRIP6	Thyroid hormone receptor interactor 6
B48R12C10	BC033731.1	0.0048	2.8	APPL2	Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 2
B39R13C06	NM_005181.2	0.0023	2.7	CA3	Carbonic anhydrase III, muscle specific
B06R05C13	BC005339.1	0.0034	2.7	TPMT	Thiopurine S-methyltransferase
B03R07C20	BC010089.1	0.0104	2.6	ASMTL	Acetylserotonin O-methyltransferase-like
B14R09C16	NM_005981.2	0.0044	2.6	TSPAN31	Tetraspanin 31
B33R13C01	NM_005258.2	0.0092	2.6	GCHFR	GTP cyclohydrolase I feedback regulator
B02R07C15	NM_006228.2	0.0010	2.5	PNOC	Prepronociceptin
B02R07C13	BC020814.1	0.0099	2.5	SUGT1L1	SGT1, suppressor of G2 allele of SKP1 like 1 (S. cerevisiae)
B28R12C05	NM_174909.1	0.0092	2.5	TMEM167	Transmembrane protein 167
B37R05C16	NM_024025.1	0.0068	2.5	DUSP26	Dual specificity phosphatase 26 (putative)
B05R09C19	BC026070.2	0.0073	2.5	TULP2	Tubby like protein 2
B24R11C01	NM_144980.1	0.0016	2.4	C6orf118	Chromosome 6 open reading frame 118
B37R09C06	NM_012321.1	0.0033	2.4	LSM4	LSM4 homolog, U6 small nuclear RNA associated (S. cerevisiae)
B03R13C14	BC070109.1	0.0036	2.4	C22orf13	Chromosome 22 open reading frame 13
B14R08C05	NM_004311.1	0.0054	2.4	ARL3	ADP-ribosylation factor-like 3
B38R07C18	BC034713.1	0.0042	2.3	DNAJC10	DnaJ (Hsp40) homolog, subfamily C, member 10
B22R08C02	BC035026.2	0.0072	2.3	FAM47B	Family with sequence similarity 47, member B
B09R09C01	NM_015955.2	0.0071	2.2	MEMO1	Mediator of cell motility 1
B43R12C15	BC014298.1	0.0007	2.2	PRKRIP1	PRKR interacting protein 1 (IL11 inducible)
B20R03C03	BC030636.1	0.0094	2.2	PREP	Prolyl endopeptidase
B37R07C16	NM_005643.2	0.0008	2.1	TAF11	TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 28kDa
B01R09C01	BC019045.1	0.0010	2.1	DOK6	Docking protein 6
B42R12C09	BC051687.1	0.0081	2.1	KLF3	Kruppel-like factor 3 (basic)
B15R05C18	NM_004869.2	0.0054	2.0	VPS4B	Vacuolar protein sorting 4 homolog B (S. cerevisiae)

1.3 times increase in level, *t* test *p* value = 0.0139; Fig. 2*B*). Anti-AGT and anti-PRKRIP1 levels were themselves strongly correlated across all the samples with chronic renal injury (Pearson correlation 0.47, *p* value 0.00056). Because of this, there was no meaningful improvement in the AUC when both anti-AGT and anti-PRKRIP1 measurements were combined using coefficients found through logistic regression (AUC 0.73).

In the 50 independent samples for validation, from patients with CKD, there was no significant difference in anti-AGT and anti-PRKRIP1 antibodies by gender (*t* test *p* values 0.91 and 0.18, respectively), race (ANOVA *p* values 0.99 and 0.27, respectively), or by age (Pearson correlation *p* values 0.18 and 0.39, respectively). There was no significant difference in anti-AGT and anti-PRKRIP1 antibodies based on etiology of ESRD, when all 15 types of renal injury were considered (ANOVA *p* values 0.93 and 0.40, respectively), or when only those 38 samples from four major etiological categories were considered (aplastic/hypoplastic/dysplastic kidneys; obstructive uropathy; focal segmental glomerulosclerosis; and reflux nephropathy; *p* values 0.57 and 0.59, respectively). There was no significant difference in anti-AGT and anti-PRKRIP1 Ab based on whether dialysis was received in the pre-transplantation period (*t* test *p* values = 0.97 and 0.71, respectively), nor whether a patient was on an anti-hypertensive therapy (*t* test *p* values 0.74 and 0.24, respectively). Only two patients were on angiotensin converting enzyme inhibitors; there was

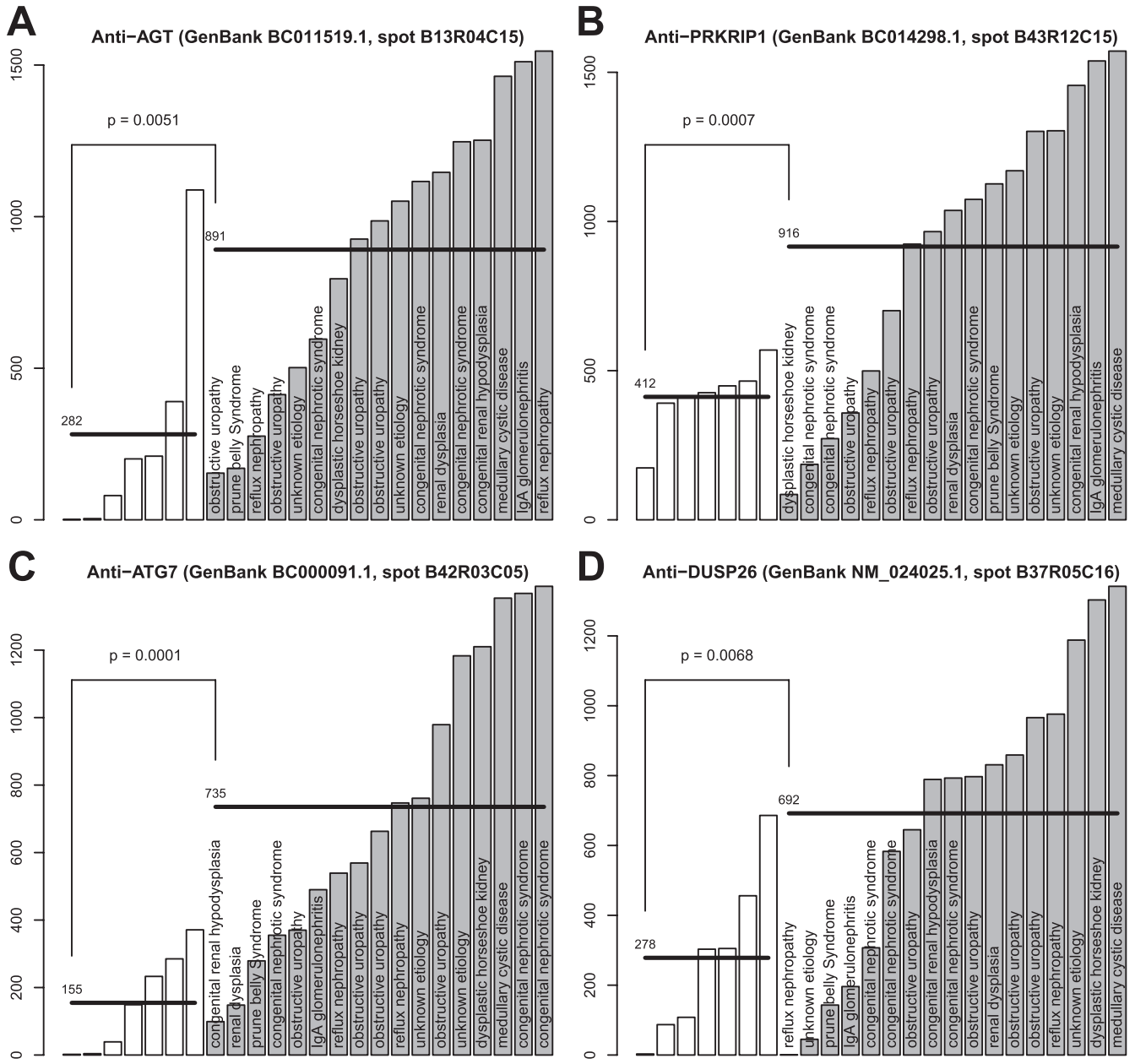

FIG. 1. **Four auto-autoantibodies were selected from 38 significant autoantibodies detected from serum samples using protein microarrays.** White bars indicate levels detected in normal healthy control samples, whereas gray bars indicate samples from patients with end-stage renal disease of various etiologies, as indicated. Mean levels for each group are shown, along with *t* test *p* values. *A,* anti-angiotensinogen (AGT). *B,* anti-protein kinase R interacting protein 1 (PRKRIP1). *C*, anti-autophagy related 7 homolog (ATG7). *D*, anti-dual specificity phosphatase 26 (DUSP26).

no significant difference in their antibody levels. Neither antibody level significantly correlated with systolic or diastolic blood pressure at the time of transplant. These data indicate that specific auto-Ab, mounted to AGT and PRKRIP1 are not likely to be biased by gender, age, race, renal insufficiency therapy, or original diagnosis, and relate instead to the common causation of kidney injury in all patients.

Our other two candidates for validation, anti-ATG7 and anti-DUSP26, did not show significant differences in levels in CKD samples compared with controls (*t* test *p* values = 0.60 and 0.075, area under the ROC curves = 0.50 and 0.63, respectively).

DISCUSSION

These results demonstrate the feasibility of using protein microarrays to discover serum auto-antibodies at increased levels in renal insufficiency from kidney injury. By comparing the serum antibody profiles of patients with CKD *versus*

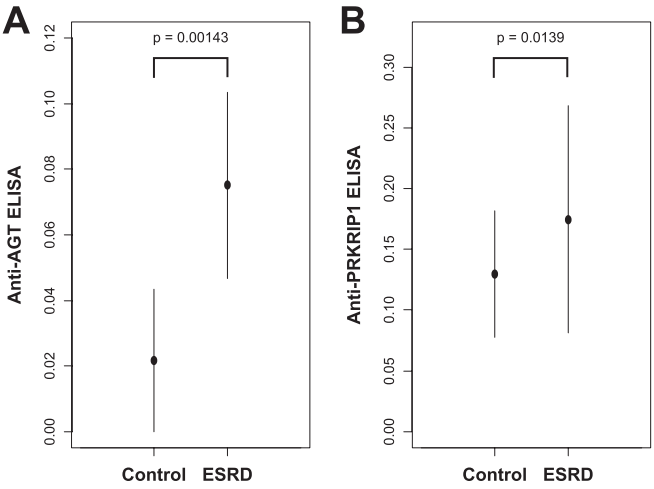

FIG. 2. **Serum ELISAs successfully validated two of four tested autoantibodies in patients with end-stage renal disease.** Mean and one standard deviation are shown. *A*, anti-angiotensinogen (AGT). *B*, anti-protein kinase R interacting protein 1 (PRKRIP1).

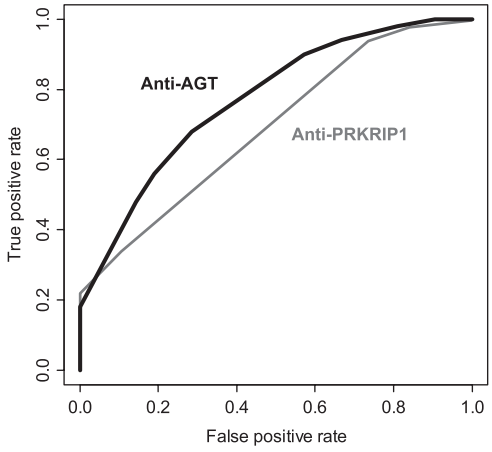

FIG. 3. **Receiver operating characteristic (ROC) curves showing the performance of anti-AGT (black) and anti-PRKRIP1 (gray) auto antibodies in distinguishing samples from patients with ESRD from control samples.** Areas under the ROC curves are 0.72 and 0.62, respectively.

healthy controls, we found and subsequently validated two auto-antibodies in CKD, a disease not normally considered as an autoimmune process: anti-AGT and anti-PRKRIP1.

Unexpectedly, we found anti-angiotensinogen antibodies significantly higher in the serum of patients with chronic renal injury. To our knowledge, neither auto-antibodies against AGT nor their association with morbidity have been previously described. Angiotensin itself is well known to play a role in kidney physiology and ESRD pathophysiology. Though most systemic angiotensinogen comes from the liver, angiotensinogen is well known to also be expressed in the kidney (25). Angiotensinogen has been shown to be necessary for normal kidney development in mice as disruption of the angiotensinogen gene leads to abnormal kidney vasculature, cortex, and tubules (26). AGT is converted to angiotensin I by renin, expressed in the kidney, and subsequently converted to angiotensin II by angiotensin converting enzyme (ACE), expressed in the lungs and renal endothelium. As angiotensin II is a potent arteriolar vasoconstrictor, patients with hypertension are commonly treated using ACE inhibitors. Several major clinical trials have shown the benefit of angiotensin II receptor blockers in preventing nephropathy because of type 2 diabetes, possibly independent of effects on blood pressure (1, 27). Angiotensin II directly stimulates the transforming growth factor β pathway in kidney (28, 29), and is known to modulate tubulointerstitial fibrosis in animal models of chronic kidney disease (30). Interestingly, circulating levels of angiotensin II have been shown to be significantly higher in hemodialysis patients with hypotension compared with those with normal blood pressure (31), whereas others could not find a difference in angiotensin II levels between control subjects and diabetics with or without nephropathy (32).

An analog to angiotensin I was successfully tested in rats nearly 10 years ago as an active immunization to prevent the increase in blood pressure from angiotensin I (33), and clinical trials on an active immunization against angiotensin I and II are underway (34–36). Anti-angiotensin II receptor auto-antibodies have been described and associated with the development of pre-eclampsia (37, 38).

Anti-PRKRIP1 is a novel auto-Ab for chronic renal injury. Although antibodies against other antigens, including strains of bacteria such as *Streptococcus pyogenes*, have been shown to be increased in ESRD (39), to our knowledge, no auto-Ab have yet been described in chronic renal injury.

There are several important limitations to consider in our study. This study is based on observational measurements made on a limited number of prevalent pretransplant patients. Measurements in additional samples may yield more positive results, especially for anti-DUSP26, which was negative in our study but demonstrated a trend toward positivity. Although we suspect the elevation of anti-angiotensinogen Ab are reflective of kidney damage, we cannot exclude that these auto-Ab are being raised because of increased amounts of serum angiotensinogen or angiotensin I from decreased functioning of ACE, nor can we ascertain the specific pathogenicity of these auto-Ab. Although we cannot ascertain where the potential antigens may be coming from, it does appear likely that both PRKRIP1 and AGT antigens are present in the organ undergoing damage. We cannot distinguish whether these auto-Ab are targeting angiotensinogen or angiotensin I, as cross-reactivity in antibodies against these two antigens has been described (33). A more detailed study is warranted to establish an antibody assay to test whether these antibodies target the full length protein or a specific fragment or epitope of the protein. In this pilot study, anti-AGT demonstrated a sensitivity of 70% at a specificity of 70%. Improvements in these assays, including further delineation of the reactive peptides, are still needed to improve the sensitivity and specificity to above 90%, where performance reaches clinical levels (40).

We also acknowledge that in this pilot study, we may not have enough samples to associate anti-AGT with differences in blood pressure or renal injury course, or to fully compensate for multiple hypothesis testing. A significant challenge with protein microarray measurements is that methodologies for correcting for multiple hypothesis testing have not yet been fully established; we feel this is less of a concern here, because of the validation phase we added to this study. We also acknowledge that the average spot-to-spot correlation of intensity measurements on the protein arrays may have been a contributing factor to our success rate in validation. As used here, auto-Ab levels measured for diagnostic purposes for non-autoimmune disease are likely to demonstrate lower intensities than auto-Ab levels traditionally measured in autoimmune diseases, so continued improvements in the reliability of measurements at these lower levels will clearly be needed.

Nonetheless, it is interesting that patients with CKD have a common auto-Ab response, regardless of the original cause of their chronic renal insufficiency. Auto-immune responses to tissue damage and disease may be more prevalent across diseases than is otherwise considered today. Future studies are still needed to ascertain whether these results are reproduced in larger patient cohorts, how early these auto-Ab are present and can be detected, whether there is a clinical distinction in patients with varying levels of these auto-Ab, and whether they play a role in therapeutic efficacy of ACE inhibitors. These two specific auto-Ab need to be tested in serially in patients with occult or early renal insufficiency to distinguish those few who progress to CKD and ESRD. Finally, as more biomarkers are being sought for other chronic conditions, the detection of auto-immune responses to changes in proteins may be easier to detect than changes in the proteins themselves.

Our results highlight that even in organ injuries from non-immune causes; there is a significant component of inflammatory and auto-immune signal that generates a repertoire of auto-immune Ab that track exquisitely with the presence or absence of underlying end-organ injury. Uncovering the repertoire of these novel auto-Ab to specific antigenic targets, can serve as detectable and effective biomarkers of specific end-organ injury. Future work will involve more extensive antibody-antigen titration for each potential biomarker reported, which is beyond the scope of the focused nature of this pilot study. Further studies are also needed to test and validate if prospective screening for these auto-Ab can provide non-invasive surrogates for detection and progression of CKD. Early detection of organ injury carries a significant social and economic impact for improved therapeutic interventions with resultant improved patient morbidity and mortality.

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