CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Urinary Exosomes and Exosomal *CCL2* mRNA as Biomarkers of Active Histologic Injury in IgA Nephropathy

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IgA nephropathy (IgAN) features variable renal pathology and a heterogeneous clinical course. Our aim was to search noninvasive biomarkers from urinary exosomes for IgAN patients; membrane nephropathy and minimal change disease were included as other glomerulopathy controls. Transmission electron microscopy and nanoparticle tracking analysis confirmed the size and morphology characteristic of urinary exosomes. Exosome markers (Alix and CD63) as well as renal cell markers [aquaporin 2 (AQP2) and nephrin] were detected, which indicate the renal origin of urinary exosomes. Exosome excretion was increased markedly in IgAN patients compared with controls and correlated with levels of proteinuria and tubular injury. More important, urinary exosome excretion correlated with greater histologic activity (mesangial hypercellularity, crescents, and endocapillary hypercellularity). Profiling of the inflammation-related mRNA revealed that exosomal chemokine (C-C motif) ligand 2 (*CCL2*) was up-regulated in IgAN patients. In a validation study, *CCL2* was exclusively highly expressed in IgAN patients compared with healthy controls as well as minimal change disease and membrane nephropathy patients. Also, a correlation between exosomal *CCL2* and estimated glomerular filtration rate levels was found in IgAN. Exosomal *CCL2* was correlated with tubulointerstitial inflammation and C3 deposition. High *CCL2* levels at the time of renal biopsy were associated with subsequent deterioration in renal function. Thus, urinary exosomes and exosomal *CCL2* mRNA are promising biomarkers reflecting active renal histologic injury and renal function deterioration in IgAN. (*Am J Pathol* 2018, 188: 2542–2552; <https://doi.org/10.1016/j.ajpath.2018.07.017>)

IgA nephropathy (IgAN) is one of the most common primary renal diseases worldwide and is an important cause of end-stage renal disease.¹ However, patients with IgAN show variable clinical presentations, which range from asymptomatic microscopic hematuria to rapidly progressive glomerulonephritis or nephrotic syndrome.^{2,3} Besides, although IgAN runs a relatively benign course in most patients, up to 40% of patients progress to end-stage renal disease over the course of 30 to 40 years. Thus, biomarkers for predicting outcomes and identifying patients at higher risk of progression are essential for better management of IgAN.

Currently, renal biopsy and pathologic assessment remain the standard diagnostic approach in IgAN.^{1,4} Biopsy

specimens meeting IgAN diagnostic criteria have a range of pathologic changes that are reflected in the variable clinical course of IgAN.³ Given the invasive nature and nontrivial postoperative complications of biopsy, novel noninvasive biomarkers associated with kidney histologic damage are urgently needed for clinical practice.

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Exosomes are nano-sized extracellular vesicles of endosomal origin, secreted into the extracellular space through fusion of multivesicular bodies with the plasma membrane.^{5,6} Urine has been a promising source for identifying novel biomarkers for kidney disease.⁷ Since the first description of exosomes in the urine in 2004,⁸ urinary exosomes have become an attractive target for biomarker discovery in kidney disease. Urinary exosomes may be produced by epithelial cells facing the urinary space, including podocytes and renal tubular epithelial cells.^{8,9} Thus, the urinary exosome could represent a promising liquid biopsy that reflects the pattern and/or severity of renal histologic injury. The discovery of exosomes as natural carriers of RNA (miRNA and mRNA) information from parent cells has raised great interest in exosomal RNAs as potential biomarkers for renal disease. *CD2AP* mRNA and miR-29c in urinary exosomes may be used as biomarkers that reflect chronic kidney disease progression and the severity of fibrosis.^{10,11} Other laboratories have shown that exosomal miRNA may also be a potential biomarker of chronic kidney disease, diabetic kidney disease, and lupus nephritis.^{9,12–14} However, the role of urinary exosomal RNAs in the monitoring of IgAN has not been tested.

Tubular and interstitial inflammation plays a crucial role not only in the onset but also in the progression of IgAN.¹⁵ Numerous lines of evidence have demonstrated that an immunohistochemical evaluation of tubulointerstitial inflammation is a powerful tool in determining the prognosis in IgAN.^{16–18} Inflammatory responses are involved in the pathogenesis of IgAN at distinct phases and in characteristic lesions, possibly via the recruitment and activation of distinct types of leukocyte populations.¹⁹ Interestingly, the exosomal chemokine (C-C motif) ligand 2 (*CCL2*) plays an important role in the development of tubulointerstitial inflammation.²⁰ In this study, we profiled the expression of a panel of cytokines and chemokines as well as their receptors in urinary exosomes to evaluate their potential role as noninvasive biomarkers of IgAN. Patients with IgAN showed augmented urinary excretion of exosomes with exaggerated expression of *CCL2* mRNA compared with other molecules. Further study showed that *CCL2* mRNA levels correlated with more severe histologic activity and tubular injury. More important, the exosomal *CCL2* mRNA may represent a promising biomarker for deterioration of renal function and active tubulointerstitial injury in IgAN.

Materials and Methods

Patients and Normal Controls

For detection of exosomal inflammation-related mRNA expression, in the preliminary screening test, a group of six IgAN patients and six healthy controls were enrolled. In the validation study, 55 patients with IgAN and 24 normal

Table 1 Human Subject Characteristics of Healthy Controls and IgAN Patients

Characteristic	Healthy controls (n = 24)	IgAN patients (n = 55)
Male/female ratio	11:13	31:24
Age, years	38.2 ± 13.8	38.6 ± 13.3
BUN, mmol/L	4.15 (3.53–4.96)	5.70 (4.40–7.96)***
SCr, μmol/L	78 (68.25–89.5)	97 (73.5–127.5)*
UA, μmol/L	307 (274–341)	349 (276–418)
eGFR, mL/minute per 1.73 m ²	86.2 (78.7–97.3)	73.8 (46.7–91.9)*
Proteinuria, g/24 hours	<0.3	2.45 (1.32–5.45)*

eGFR has been calculated using the modification of diet in renal disease creatinine formula (mL/minute per 1.73 m²). Data are expressed as means ± SD or median (interquartile range).

*P < 0.05, ***P < 0.001 (*U*-test).

BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; IgAN, IgA nephropathy; SCr, serum creatinine; UA, uric acid.

individuals were included. In addition to healthy controls, patients with membranous nephropathy (MN; *n* = 16) or minimal change disease (MCD; *n* = 9) were recruited as other glomerulopathy controls. All cases of IgAN were proved by biopsy in the Kidney Institute of the Zhongda Hospital, Southeast University (Nanjing, China). The inclusion criteria included the following: patients with biopsy-proved IgAN and age ≥18 years. The exclusion criteria were as follows: patients with malignancy or severe liver injury; patients who were pregnant or in the lactation period; patients who had undergone renal replacement therapy, such as kidney transplantation or dialysis; and patients with acute or chronic infection. The glomerular filtration rate was estimated by the modification of diet in renal disease equation. Twenty-four normal individuals were included without proteinuria, hypertension, diabetes, impaired renal function, or chronic inflammatory diseases. The baseline clinical characteristics, including blood urea nitrogen, serum creatinine, uric acid, proteinuria, and urinary neutrophil gelatinase-associated lipocalin, were recorded. All of the studies were approved by the Ethics Committee of Zhongda Hospital, Southeast University.

Urine and blood samples were collected from 36 IgAN patients after renal biopsy, with a median of 18.5 months of follow-up.

Urine Sample Preparation and Exosome Isolation

The first morning urine was collected. Samples were centrifuged for 20 minutes at 2000 × *g* and 13,500 × *g* at 4°C (model 5804R; Eppendorff, Hamburg, Germany) to remove cellular debris and eliminate large microvesicles, respectively. Supernatants were then ultracentrifuged for 2 hours at 200,000 × *g* at 4°C (Beckman Instruments, Fullerton, CA), and urinary exosomes were resuspended in 100

μL phosphate-buffered saline. The pellet was frozen at -80°C for later analysis.

Exosome Characterization and mRNA Measurement

The exosome pellets were diluted five times with phosphate-buffered saline and then applied to 200-mesh nickel grids. Samples were stained with 2% phosphotungstic acid for 5 minutes and air dried. Exosomes were detected using a transmission electron microscope (HT 7700; Hitachi High-Tech, Tokyo, Japan) at 80 kV. ZetaView Nanoparticle Tracking analysis (Particle Metrix, Meerbusch, Germany) was performed, as reported before.²⁰ The protein from exosomes was extracted by RIPA Lysis and Extraction Buffer (Thermo, Wilmington, DE) with protease inhibitor and phosphatase inhibitor. Urine exosome was quantified using the EXOCET assay (System Biosciences, Mountain View, CA), which measured the activity of exosome acetylcholinesterase.²¹ Exosome quantification was also normalized to urine creatinine to control for variation in urine flow rate.

Exosome markers (Alix and CD63) were detected by Western blot analysis. Antibodies, including mouse anti-Alix (1:500; Santa Cruz Biotechnology, Dallas, TX), mouse anti-CD63 (1:1000; Abcam, Cambridge, MA), goat anti-nephrin (1:400; Santa Cruz Biotechnology), and mouse anti-AQP2 (1:500; Santa Cruz Biotechnology), were used.

Total RNA was extracted from urinary exosomes with the miRNeasy micro kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Kusatsu, Japan) was used for reverse transcription, according to the manufacturer's protocol. Urinary exosomal mRNA levels were quantified by real-time RT-PCR on the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA).

Evaluation of Renal Histopathological Lesions

Two experienced pathologists (P.-S.C. and H.-F.N.) who were masked to the patients' information performed the assessment of the histopathological damage using the

Figure 1 Urinary exosome excretion is increased in IgA nephropathy (IgAN) patients. **A:** Representative electron micrograph of exosomes in urine from IgAN patients. Boxed area is shown at higher magnification below. **B:** Size distribution of urinary exosomes analyzed by nanoparticle tracking analysis. The mean diameter of exosomes was 96.3 ± 41.6 nm. **C:** Expression of exosomal markers (Alix and CD63) and renal cell markers (AQP2 and nephrin) was assessed by Western blot analysis in protein extracted from isolated urinary exosomes of healthy controls and IgAN patients. Note a significant increase in urinary exosome excretion normalized to urine creatinine (UCr) in IgAN patients. **D:** Urinary exosomal protein was quantified by EXOCET assay, which measured the activity of exosome acetylcholinesterase, and results were normalized to UCr in 15 healthy controls (HCs) and 29 biopsy-proven IgAN patients. Urinary exosome protein production was significantly increased in IgAN patients. **E:** The amount of exosomes was markedly increased in the cohort of severe proteinuria (>3.5 g/24 hours) compared with patients with nonsevere proteinuria (≤ 3.5 g/24 hours). *** $P < 0.001$, **** $P < 0.0001$. Scale bars: 200 nm (A, top panel); 100 nm (A, bottom panel).

Figure 2 Urinary exosome excretion is correlated with levels of proteinuria and tubular injury in IgA nephropathy (IgAN) patients. **A:** Normalized concentration of urinary exosome amount in IgAN patients positively correlates with levels of proteinuria at the time of renal biopsy. **B:** No significant correlation is found between urinary exosome excretion and estimated glomerular filtration rate (eGFR) in IgAN patients. **C:** Plots of correlation between neutrophil gelatinase-associated lipocalin (NGAL) and proteinuria in IgAN patients. **D:** Normalized urinary exosome quantification correlates closely with levels of NGAL in IgAN patients. **Lines** show the linear correlation for each of the two variables. UCr, urine creatinine.

updated Oxford MEST-C scoring system.²² The mesangial score was recorded as the number of cells per mesangial area (M1 or M0). The endocapillary hypercellularity was scored as presence (E1) or absence (E0). The segmental sclerosis score was measured as absent (S0) or present (S1). Interstitial fibrosis or tubular atrophy was classified according to the area with tubulointerstitial damage: <25% (T0), 25% to 50% (T1), or >50% (T2).²² Tubulointerstitial inflammation was semiquantitatively graded into normal (no significant infiltrations), mild (up to three local infiltrations

involving up to 25% of the interstitium), and marked (any local infiltrations involving >25% of interstitium or diffuse infiltration).^{16,18} C3 deposition was observed and scored as follows: –, +, ++, and +++. Crescent lesions were scored as C0 (no crescents), C1 (crescents in <25% of glomeruli), and C2 (crescents in ≥25% of glomeruli).

Infiltration of macrophages within the kidney was demonstrated by immunoperoxidase staining of CD68. The primary monoclonal antibodies used were anti-CD68 antibodies (1:100; ab-201340; Abcam).

Figure 3 Correlation between urinary exosome excretion and kidney histologic injury. **A:** Patients with endocapillary hypercellularity (E = 1) show increased urinary exosome production compared with those without endocapillary hypercellularity (E = 0). **B:** No differences in normalized urinary exosome are found between IgA nephropathy patients with (S = 1) or without (S = 0) segmental glomerulosclerosis. **C:** No difference is found in patients with different tubular atrophy and interstitial fibrosis severity [area with tubulointerstitial damage: <25% (T = 0), 25% to 50% (T = 1), or >50% (T = 2)]. **D:** Patients with crescents (C1/C2) show increased exosome excretion compared with patients without crescents (C0). * $P < 0.05$, *** $P < 0.001$ (t -test). UCr, urine creatinine.

Table 2 Human Subject Characteristics of Healthy Controls and IgAN Patients for Screening of Inflammation-Related Genes in Urinary Exosomes

Characteristic	Healthy controls	IgAN patients
Male:female, <i>n</i>	3:3	3:3
Age, years	27 (26.7–30.5)	36.5 (22.3–42.5)
BUN, mmol/L	3.5 (3.4–3.8)	5.15 (3.37–9.13)
SCr, μmol/L	64.5 (59.0–75.7)	98.5 (79.0–128.7)*
UA, μmol/L	255 (209–287)	314 (266–374)
eGFR, mL/minute per 1.73 m ²	99.1 (83.7–114.2)	74.3 (50.1–91.7)*
Proteinuria, g/24 hours	<0.3	1.41 (0.78–7.57)*

Values are expressed as median (interquartile range).
*P < 0.05 (*U*-test).
BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; IgAN, IgA nephropathy; SCr, serum creatinine; UA, uric acid.

Statistical Analysis

Statistical analysis was conducted by SPSS version 18.0 (SPSS Inc., Chicago, IL) and GraphPad Prism 5 (GraphPad Software, San Diego, CA). All of the data were shown as means ± SD or median (interquartile range), whether the data were normally or abnormally distributed. The relative amount of mRNA to glyceraldehyde-3-phosphate dehydrogenase was described by the equation ($2^{-\Delta Ct}$), where ΔCt refers to Ct-mRNA minus Ct–glyceraldehyde-3-phosphate dehydrogenase. In the screening analysis, the value of $2^{-\Delta Ct}$ was log 2 transformed and analyzed by the heat map package in R version 3.2.5. *t*-Test, *U*-test for non-parametric

data, or one-way analysis of variance was used to compare the data between different groups. Spearman’s correlation was performed to analyze the correlation between two groups of data.

Results

Urinary Exosome Excretion Is Increased in IgAN Patients

Exosomes were purified from the urine of 55 IgAN patients and 24 healthy controls matched by age and sex. The basic clinical characteristics of these patients are listed in Table 1. IgAN patients showed significantly higher levels of blood urea nitrogen, serum creatinine, and proteinuria, with reduced values of estimated glomerular filtration rate (eGFR), compared with healthy controls (*P* < 0.05).

Urinary exosomes were characterized by using transmission electron microscopy, ZetaView nanoparticle tracking analysis, and Western blot analysis (using Alix and CD63 as exosome markers). Transmission electron microscopy showed homogeneous size of vesicles, and the diameter was 96.3 ± 41.6 nm, as measured by nanoparticle tracking analysis (Figure 1, A and B). Exosome markers Alix and CD63 were readily detected by Western blot analysis. More important, tubular epithelial marker AQP2 and podocyte marker nephrin were detected in urinary exosomes, which confirmed the renal origin of the purified exosomes. Interestingly, urinary exosome excretion was significantly enhanced in IgAN patients compared with

Table 3 Primers for Quantitative RT-PCR

Primer	Forward	Reverse
Homo CCL-2	5'-CCTTCATTCCTCCCAAGGGCTC-3'	5'-GGTTTGCTTGCTCCAGGTGGT-3'
Homo CCL-3	5'-TCTTCCTAACCAAGCGAAGC-3'	5'-TTGGCAACAACAGTCCATA-3'
Homo CCL-5	5'-CGCTGTCATCCTCATTGCTA-3'	5'-CCAGACTTGCTGTCCCTCTC-3'
Homo CCL-21	5'-AGAGAGACCGAGGAGGGAGA-3'	5'-TTTGGGTGGGAAGACAGAAC-3'
Homo CCL-22	5'-CTGTTCCCATCAGCGATTCC-3'	5'-GACCCAGAAGTGGGATGTGT-3'
Homo CXCL-1	5'-ACTCAAGAATGGGCGGAAAG-3'	5'-TCCTAAGCGATGCTCAAACA-3'
Homo CXCL-2	5'-CTCAAGAATGGGCAGAAAGC-3'	5'-AAACACATTAGGCGCAATCC-3'
Homo CXCL-8	5'-TGCAGCTCTGTGTGAAGGTG-3'	5'-ACTTCTCCACAACCCCTCTGC-3'
Homo CXCL-10	5'-AAGGATGGACCACACAGAGG-3'	5'-AGCAGGGTCAGAACATCCAC-3'
Homo CCR2	5'-CGGTGCTCCCTGTCTATAAAT-3'	5'-CCCAAAGACCCACTCATTTG-3'
Homo CCR5	5'-AACCAGGCGAGAGACTTGTG-3'	5'-CAATGCCGTCAAGGTTCTTC-3'
Homo CXCR-2	5'-TCAGTGTGGACCGTTACCTG-3'	5'-CACGATGAAGCCAAAGGACT-3'
Homo CXCR-4	5'-TCATCAAGCAAGGGTGTGAG-3'	5'-GAGAGGATCTTGAGGCTGGA-3'
Homo IL-1β	5'-GCATCCAGCTACGAATCTCC-3'	5'-TCGTTATCCCATGTGTCGAA-3'
Homo IL-6	5'-CCTTCCAAAGATGGCTGAAA-3'	5'-AGCTCTGGCTTGTTCTCTCAC-3'
Homo IL-10	5'-CCACGCTTCTTAGCTGTGA-3'	5'-CTCCGAGACACTGGAAGGTG-3'
Homo IL-18	5'-CAGACCTTCCAGATCGCTTC-3'	5'-ATAGAGGCCGATTTCTCTGG-3'
Homo IL-22	5'-AGGCTCAGCAACAGGCTAAG-3'	5'-TTCAGCTTGTCTCTGGTCAA-3'
Homo IFN-γ	5'-AAGTAGGCAGCCAACCTAAGC-3'	5'-AAGCACCAGGCATGAAATCT-3'
Homo TNF-α	5'-CCCAGGGACCTCTCTCTAATC-3'	5'-TGAGGTACAGGCCCTCTGAT-3'
Homo GAPDH	5'-CTCTGCTCCTCCTGTTCGAC-3'	5'-GCGCCCAATACGACCAATC-3'

CCL, chemokine (C-C motif) ligand; CXCR, C-X-C chemokine receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Homo, homo sapiens; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α.

Figure 4 Expression profile of inflammatory response-related genes in exosomes from IgA nephropathy (IgAN) patients and healthy controls (HCs). Twenty cytokines and chemokines as well as receptors were screened in IgAN patients and matched controls. The expression patterns are presented as cluster heat maps established by the heat map package in R version 3.3.5. $n = 6$ IgAN patients and HCs. CCL, chemokine (C-C motif) ligand; CXCR, C-X-C chemokine receptor; IFN- α , interferon- α ; TNF- α , tumor necrosis factor- α .

healthy controls, as evidenced by Western blot analysis of exosome markers and renal cell markers (Figure 1C). Besides, exosome quantification was performed in 29 IgAN patients and 15 healthy controls via EXOCET assay. The result confirmed remarkable increased exosome production in IgAN patients compared with controls (Figure 1D). The mass of exosomes was significantly higher in the urine from patients with severe proteinuria (>3.5 g/24 hours) compared with those without severe proteinuria (<3.5 g/24 hours) in IgAN (Figure 1E).

Urinary Exosome Excretion Is Correlated with Levels of Proteinuria and Tubular Injury in IgAN Patients

Further analysis showed that the amount of exosomes correlated closely with levels of proteinuria ($r = 0.935$, $P < 0.05$) (Figure 2A). However, no correlation was found between urinary exosome excretion and eGFR in IgAN patients (Figure 2B). Because tubular epithelial cells are directly exposed to filtered protein in conditions of proteinuria and are the major cellular targets of

proteinuria toxicity,³ the relationship between urinary exosome excretion and tubular injury was next examined. Urinary protein levels of neutrophil gelatinase-associated lipocalin, a marker of tubular damage, were significantly correlated with proteinuria, as expected ($r = 0.678$, $P < 0.0001$) (Figure 2C); and a strong positive association was found between urinary exosome excretion and neutrophil gelatinase-associated lipocalin in IgAN patients ($r = 0.594$, $P < 0.05$) (Figure 2D). These data suggest that urinary exosome production may attribute to proteinuria and the consequent tubular injury accruing from protein-overload toxicity.

Correlation between Urinary Exosome Production and Kidney Histologic Injury in IgAN

To further understand the relevance of exosomes to kidney histologic injury in IgAN, renal biopsy specimens were scored according to the Oxford Classification of IgA nephropathy 2016.²² Representative kidney histologic injury,

Figure 5 Exosomal chemokine (C-C motif) ligand 2 (CCL2) mRNA is up-regulated in IgA nephropathy (IgAN) patients and correlates with estimated glomerular filtration rate (eGFR). **A:** The relative expression of CCL2 mRNA in urinary exosomes of IgAN patients is significantly higher than in healthy controls (Ctrls) as well as minimal change disease (MCD) and membranous nephropathy (MN) patients. **B** and **C:** Lines show the linear correlation between CCL2 mRNA in urinary exosomes and eGFR in all IgAN patients and in patients with eGFR <60 mL/minute per 1.73 m^2 . $n = 55$ IgAN patients (**A**); $n = 24$ healthy controls (**A**); $n = 9$ MCD patients (**A**); $n = 16$ MN patients (**A**). *** $P < 0.001$ versus healthy controls. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 6 Exosomal chemokine (C-C motif) ligand 2 (*CCL2*) mRNA correlates with tubulointerstitial inflammation and fibrosis in the kidney. **A** and **B**: No differences in urinary exosomal *CCL2* mRNA are found between IgA nephropathy (IgAN) patients with (E = 1 or S = 1) or without (E = 0 or S = 0) endocapillary hypercellularity (**A**) or segmental glomerulosclerosis (**B**). **C**: Patients with >50% area of tubular atrophy and interstitial fibrosis (T = 2) show the highest expression of exosomal *CCL2* mRNA (T = 0 indicates <25%; and T = 1, 25% to 50%). **D**: No significant difference is found between patients with (C = 1/2) or without (C = 0) crescents (cellular, fibrocellular, and fibrosis). **E**: The levels of *CCL2* mRNA in urinary exosomes of patients with normal (0%), mild (<25%), or marked (>25%) interstitial inflammation. Patients with marked interstitial inflammation show higher levels of urinary exosomal *CCL2* mRNA. **F**: Urinary exosomal *CCL2* mRNA expression of IgAN patients with different degrees of C3 deposition in mesangial areas. The cohort with +++ C3 complement deposition shows the highest expression of urinary exosomal *CCL2* mRNA among all IgAN patients. *n* = 12 patients with normal interstitial inflammation (**E**); *n* = 25 patients with mild interstitial inflammation (**E**); *n* = 8 patients with marked interstitial inflammation (**E**). *P < 0.05, **P < 0.01. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

assessed by periodic acid-Schiff and Masson staining, is shown in [Supplemental Figure S1](#). Among the enrolled patients, all presented with M1 lesions (>50% of glomeruli hypercellular) and more urinary exosome excretion was observed compared with controls ([Figure 1D](#)); thus, no further subgroup was analyzed. When the cohort was stratified on the basis of endocapillary score, patients with endocapillary hypercellularity (E1) presented with increased urinary exosome production compared with patients without endocapillary hypercellularity (E0) (*P* < 0.001) ([Figure 3A](#)). However, no difference was found in patients with different degrees of segmental glomerulosclerosis and tubular atrophy/interstitial fibrosis ([Figure 3, B and C](#)). Patients with crescents (C1/C2) showed increased exosome excretion compared with those without crescents (C0) ([Figure 3D](#)). Thus, exosome release associates with more severe and active histologic activity (mesangial hypercellularity, endocapillary hypercellularity, and crescent).

Exosomal Expression of Inflammation-Related Genes in IgAN Patients

The expression profiles of inflammation-related genes were first analyzed in urinary exosomes from a screening set of IgAN patients and controls by real-time PCR (*n* = 6 for

each group). The clinical characteristics of patients and controls are listed in [Table 2](#). Twenty key inflammatory cytokines and chemokines as well as their receptors were included in the analysis ([Table 3](#)). Among them, *IL6* was not detectable in all samples, whereas most other genes showed low expression. By contrast, *CCL2* was robustly up-regulated in all IgAN samples, with a 10-fold induction compared with controls ([Figure 4](#)).

Validation of Exosomal *CCL2* mRNA in IgAN Patients

To validate findings from the screening set, expression of *CCL2* mRNA was further analyzed in exosome samples from 55 IgAN patients and 24 healthy controls. To determine the specificity of exosomal *CCL2* in IgAN patients, 16 MN and 9 MCD patients were also included as other glomerulopathy controls in this part. The basic clinical characteristics are shown in [Supplemental Table S1](#). The groups of MCD and MN patients did not differ in age, sex, renal function, and levels of proteinuria compared with IgAN patients, although they exhibited significant proteinuria compared with healthy controls. Results confirmed that exosomal *CCL2* is extremely highly overexpressed in IgAN patients compared with normal controls, as well as in MCD and MN patients ([Figure 5A](#)). Besides, a notable inverse correlation between exosomal *CCL2* mRNA and eGFR was

observed, especially in patients with eGFR <60 mL/minute per 1.73 m² ($r = -0.624$, $P < 0.05$) (Figure 5, B and C).

Exosomal *CCL2* mRNA Correlates with Tubulointerstitial Inflammation

To further explore the correlation between exosomal *CCL2* mRNA with renal pathology, the levels of exosomal *CCL2* mRNA were investigated in groups of patients stratified by MEST-C score. Levels of *CCL2* mRNA expression did not vary between patients with and without endocapillary hypercellularity (Figure 6A) or segmental glomerulosclerosis (Figure 6B). However, *CCL2* showed higher levels of expression in patients with more severe tubular atrophy and interstitial fibrosis ($P < 0.01$ versus T0, $P < 0.01$ versus T1) (Figure 6C). No significant differences in *CCL2* were found between patients with or without crescents (Figure 6D).

Tubulointerstitial inflammation was scored and stratified into three groups: normal, mild, and marked.^{16,18} *CCL2* mRNA levels were progressively higher with increasing severity of tubulointerstitial inflammation ($P < 0.01$ versus normal, $P < 0.01$ versus mild) (Figure 6E). *CCL2* mRNA levels also increased with increasing glomerular C3 deposition (Figure 6F) ($P < 0.05$ versus C3-, $P < 0.01$ versus C3+, and $P < 0.05$ versus C3++).

Thus, exosomal *CCL2* mRNA is closely correlated with tubulointerstitial inflammation.

Urinary Exosomes and Exosomal *CCL2* mRNA as Biomarkers of IgAN

To assess the diagnostic accuracy of exosomes and exosomal mRNA in urine, receiver operating characteristic curves were generated. Both urinary exosome quantification and *CCL2* mRNA expression could efficiently discriminate IgAN from healthy controls, with areas under the receiver operating characteristic curve of 0.925 (95% CI, 0.847–1) and 0.81 (95% CI, 0.676–0.945), respectively. However, compared with urine exosome, exosomal *CCL2* mRNA showed superiority in discriminating between IgAN patients with marked versus non-to-mild interstitial inflammation. The area under the receiver operating characteristic curve was 0.828 (95% CI, 0.642–1.0) for exosomal *CCL2* and 0.691 (95% CI, 0.478–0.905) for exosomal protein (Figure 7).

Urinary Exosome and Exosomal *CCL2* mRNA as Predictors of IgAN Progression

In total, 36 patients were monitored longitudinally, with an average follow-up period of 18.5 months after renal biopsy. The clinical characteristics of the patients at baseline and follow-up are shown in Table 4. Levels of proteinuria were significantly reduced during follow-up. Urinary exosomes from 14 patients among the follow-up group were analyzed.

Urinary exosome excretion decreased with the reduction in proteinuria (Figure 8, A and B). At the end of the follow-up, renal function, as measured by eGFR, declined in 25 patients (69.4%). The remaining patients maintained stable renal function. Baseline urinary exosomal *CCL2* expression was higher in patients whose IgAN progressed compared with the stable group, suggesting that *CCL2* may be a reliable biomarker for predicting IgAN progression (Figure 8C).

Discussion

The clinical presentations of patients with IgAN are highly variable, ranging from asymptomatic microscopic hematuria to rapidly progressive glomerulonephritis. Noninvasive

Figure 7 Urinary exosomes and exosomal chemokine (C-C motif) ligand 2 (*CCL2*) mRNA as biomarkers of IgA nephropathy (IgAN). **A:** Discrimination of IgAN from controls. Receiver operating characteristic (ROC) curves were generated using urinary exosome quantification and *CCL2* mRNA. The areas under the ROC curve (AUCs) were 0.925 (95% CI, 0.847–1) and 0.81 (95% CI, 0.676–0.945) for exosome number and *CCL2*, respectively. **B:** Discrimination of interstitial inflammation. ROC curves of urinary exosome quantification and *CCL2* mRNA were generated for distinguishing the severity of interstitial inflammation among IgAN patients. The AUCs were 0.828 (95% CI, 0.642–1.0) and 0.691 (95% CI, 0.478–0.905) for exosomal *CCL2* and exosome quantification, respectively. $n = 29$ IgAN patients using urinary exosome quantification (**A**); $n = 15$ healthy controls using urinary exosome quantification (**A**); $n = 55$ IgAN patients using *CCL2* mRNA (**A**); $n = 24$ healthy controls using *CCL2* mRNA (**A**).

Table 4 Human Subject Characteristics of IgAN Patients with Follow-Up

Characteristic	Baseline	Follow-up
Male:female, <i>n</i>	18:18	18:18
Age, years	40.1 ± 13.5	40.1 ± 13.5
BUN, mmol/L	5.6 (4.3–7.0)	6.2 (4.6–9.4)
SCr, μmol/L	91.5 (75.5–136.2)	104 (82–198)
UA, μmol/L	341 (271–433)	363 (242–590)
eGFR	78.5 (42.3–93.5)	70.5 (31.4–87.3)
Proteinuria, g/24 hours	2.67 (1.32–6.45)	0.69 (0.25–1.26)*
Follow-up period, months	18.5 (10–33)	

Data are expressed as means ± SD or median (interquartile range).

*P < 0.001 (*U*-test).

BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; IgAN, IgA nephropathy; SCr, serum creatinine; UA, uric acid.

biomarkers reflecting histologic changes are, therefore, crucial for predicting disease progression and determining therapeutic strategy. Although persistent proteinuria can reflect active capillary and podocyte lesions, proteinuria by itself cannot discriminate between active inflammation and chronic damage.^{18,23} In this study, we explored the role of urinary exosome protein and exosomal inflammatory response–related mRNA in the diagnosis of IgAN and their correlation with renal histologic damage.

First, it was found that urinary exosome excretion was elevated in IgAN patients compared with healthy controls. Moreover, exosome excretion was strongly correlated with levels of proteinuria and the severity of tubular injury. In the follow-up study, a decline in exosome excretion paralleled the attenuation of proteinuria. Regarding specific renal pathologies, exosome excretion was increased in patients with endocapillary hypercellularity and higher severity of crescents. Thus, it is possible that in proteinuric conditions, higher levels of proteinuria with tubular injury and active proliferative lesions (higher mesangial score, crescent, and

endocapillary hypercellularity) contribute to exosome release. The increased exosome production might be originating from the proliferative cells. Then, the release of exosomes seems to decrease as injury progresses toward end stage with renal fibrosis. Thus, urinary exosome may be a sensitive fluid-biopsy indicator of disease activity in IgAN patients.

Previously, increased exosome release has been reported in hypoxia, acidic pH, heat shock, and oxidative stress conditions.^{24,25} Megalin/cubulin-mediated albumin reabsorption and subsequent lysosome rupture are observed in tubular epithelial cells exposed to albumin.²⁶ Tubular epithelial cells under bovine serum albumin exposure show increased exosome release, consistent with our findings in clinical samples.²⁰ During exosome biogenesis, multivesicular bodies fuse with the plasma membrane and exosome is released. Alternatively, multivesicular bodies can fuse with lysosomes for degradation.²⁷ In conditions of proteinuria, it was suspected that proteinuria may increase exosome release because of the dysfunction of lysosomes in tubular epithelial cells. However, the mechanisms by which exosome excretion is enhanced in conditions of proteinuria require further investigation. As an alternative, it is possible that the proliferation of renal epithelial cells in patients with greater histologic activity may contribute to exaggerated exosome formation and release.

Second, among 20 selected inflammatory response–related genes, mRNA for *CCL2* was markedly induced in urinary exosomes from IgAN patients compared with controls. More important, levels of exosomal *CCL2* mRNA correlated with the severity of tubular atrophy and interstitial fibrosis, and interstitial inflammation, especially macrophage infiltration documented by renal biopsy. A significant inverse correlation between *CCL2* and eGFR was found in IgAN patients. Because tubulointerstitial damage is a key histopathological risk factor for deterioration in renal function, urinary exosomal *CCL2* may be a useful predictive biomarker for progression of IgAN. Accordingly, in our

Figure 8 Urinary exosomal protein and chemokine (C-C motif) ligand 2 (*CCL2*) mRNA during follow-up of IgA nephropathy patients. Urine samples were collected from patients at the time of renal biopsy (baseline) and on follow-up at an average of 18.5 months. **A:** Proteinuria significantly decreases after treatment compared with baseline. **B:** Urinary exosomes were obtained from a subset of patients during the follow-up period. In these patients, urinary exosomal excretion reduces compared with baseline. **C:** Baseline urinary exosomal *CCL2* expression is higher in progressive patients [estimated glomerular filtration rate (eGFR) decline] compared with the stable group (no decline of eGFR). *n* = 36 (**A**); *n* = 14 (**B**); *n* = 25 eGFR decline group (**C**); *n* = 11 no decline of eGFR group (**C**). *P < 0.05, **P < 0.01 (*t*-test or *U*-test for non-parametric data). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UCr, urine creatinine.

follow-up cohort, higher baseline exosomal *CCL2* levels associated with progressive renal function deterioration. Thus, exosomal *CCL2* correlates specifically with renal interstitial injury and may have value as a predictor of disease progression in IgAN.

Monocyte chemotactic peptide-1 (*CCL2*) is a well-studied chemokine that promotes macrophage infiltration and the development of renal disease. Eardley et al²⁸ found that the recruitment of macrophages by *CCL2* links albuminuria to the development of renal injury in the setting of IgAN. Accordingly, urinary *CCL2* has been shown to be a prognostic marker of end-stage renal disease for patients with IgAN.²⁹ Urinary *CCL2* from primary patients with glomerulonephritis has correlated with tubular atrophy and interstitial fibrosis in these diseases.³⁰ In this study, we determined that urinary exosomal *CCL2* mRNA is a novel biomarker of tubulointerstitial injury in IgAN. More important, it might be a specific marker to discriminate IgAN from MCD and MN, although validation studies are needed in a large cohort of patients. However, the underlying mechanism through which exosomal *CCL2* participates in disease pathogenesis will require further investigation. Nevertheless, it was found that exosomal *CCL2* mRNA correlated with levels of C3 deposition, and complement activation may orchestrate chronic inflammation in IgAN. Immune complex deposition triggers complement activation, driving mesangial cells to proliferate and produce cytokines and chemokines. Thus, C3 deposition might underpin the initial inflammatory response, including the generation of exosomal *CCL2* that can further amplify kidney inflammation.

Conclusion

Herein, we have demonstrated that patients with IgAN have augmented urinary excretion of exosomes corresponding to higher renal pathology severity and proteinuria. *CCL2* mRNA pacing in urinary exosomes correlates closely with tubulointerstitial injury and progressive deterioration in renal function. Thus, urinary exosomes and *CCL2* mRNA may serve as novel and promising biomarkers to predict IgAN progression.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.07.017>.

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