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Identification, Confirmation, and Replication of Novel Urinary MicroRNA Biomarkers in Lupus Nephritis and Diabetic Nephropathy

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Abstract

BACKGROUND—The prevalence of chronic kidney disease (CKD) is increasing, leading to significant morbidity and mortality. Kidney biopsy remains the gold standard for diagnosing the underlying etiology of CKD, but the procedure carries complication risks. The aim of this study was to identify novel noninvasive biomarkers correlating with kidney function and histopathology in biopsyproven CKD patients.

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METHODS—We profiled 2402 urinary microRNAs (miRNAs) to identify and confirm differentially expressed miRNAs associated with kidney function and histopathology in patients with diabetic nephropathy (n = 58) or lupus nephritis (n = 89), important etiologies of CKD, compared with healthy controls (n = 93 and 119, respectively). Top performing miRNAs were then measured in 2 independent multi-institutional cohorts of patients with diabetes mellitus with (n = 74) or without nephropathy (n = 71) and systemic lupus erythematosus with (n = 86) or without (n = 37) nephritis.

RESULTS—In patients with diabetic nephropathy, miR-2861, miR-1915-3p, and miR-4532 were down-regulated (>10-fold, $P < 0.0001$) and were associated with estimated glomerular filtration rate ($P < 0.01$) and interstitial fibrosis/tubular atrophy ($P < 0.05$). The *c*-statistics for miR-2861, miR-1915-3p, and miR-4532 were 0.91, 0.86, and 0.85, respectively. In lupus nephritis patients, miR-3201 and miR-1273e were down-regulated (>3-fold, $P < 0.0001$) and associated with endocapillary glomerular inflammation ($P < 0.01$), with *c*-statistics of 0.97 and 0.91, respectively.

CONCLUSIONS—We have identified novel miRNAs that correlate with histopathological lesions and functional markers of kidney damage to facilitate sensitive, specific, and noninvasive detection of diabetic nephropathy and lupus nephritis.

Chronic kidney disease (CKD)[9] affects >10% of the adult US population and frequently leads to cardiovascular disease (1), progressive kidney dysfunction (2), and mortality (3, 4). Diabetic nephropathy (DN) is the leading cause of end-stage renal disease worldwide (3), and lupus nephritis (LN) carries a 10-year mortality up to 50% in patients who cannot achieve remission (5). Current clinical biomarkers of renal function and injury, such as estimated glomerular filtration rate (eGFR), proteinuria, and urinary sediment analysis, help to anticipate various etiologies of underlying CKD but may not pinpoint a specific diagnosis or degree of activity (6, 7). Kidney biopsy provides valuable information regarding specific diagnosis, prognosis, and treatment decisions for patients with various causes of CKD (8). Histopathological information dictates treatment decisions in LN and also provides important prognostic information for both LN and DN (9–11). However, kidney biopsies are an invasive procedure with nonnegligible complications (major complications: 2.4%) (12). Noninvasive markers of kidney disease etiology would advance clinical medicine by obviating the need for an invasive procedure, improve diagnostic accuracy in patients who do not undergo biopsy, and potentially allow for surrogate endpoint-based therapy.

Extracellular microRNAs (miRNAs) are present in body fluids and are known to influence gene expression and regulation (13, 14). Abundant expression, lower complexity, tissue specificity, stability, and evolutionary conservation are some of the qualities that make extracellular miRNAs attractive as noninvasive biomarkers to reflect pathophysiological conditions and disease states (15). Circulating and secretory miRNAs demonstrate promise as biomarkers not only in kidney disease (16–18) but also in cancer subtypes (19), liver disease (20), multiple sclerosis (21), and cardiovascular disease (22).

[9]Nonstandard abbreviations: CKD, chronic kidney disease; miRNA, microRNA; DN, diabetic nephropathy; LN, lupus nephritis; eGFR, estimated glomerular filtration rate; HE, healthy (study group); UW, University of Washington; OSU, Ohio State University; BKBx, Boston Kidney Biopsy cohort; DM, diabetes without kidney disease (study group); SLE, systemic lupus erythematosus; FDR, false discovery rate; SCr, serum creatinine; AUC, area under the curve; OR, odds ratio; IFTA, interstitial fibrosis and tubular atrophy; Ct, threshold cycle.

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The objective of this study was to identify noninvasive miRNA biomarkers reflective of kidney pathology to help elucidate diagnosis, prognosis, and disease monitoring in patient care as well as in clinical trial designs. Although DN and LN are distinct entities, we leveraged the spectrum of histopathology in patients with biopsy-proven DN and LN, in addition to their renal function parameters, to identify differentially expressed urinary miRNAs compared with healthy controls (HE), and to validate the top performing candidates in 2 independent multi-institutional cohorts.

Methods

STUDY POPULATION

All participants were patients or healthy volunteers recruited at Brigham &Women's Hospital (discovery and confirmation cohort), University of Washington (UW), or The Ohio State University (OSU) (replication cohorts) (Table 1).

Urine samples in the discovery and confirmation cohort were from patients with biopsy-proven DN and LN obtained from the Boston Kidney Biopsy cohort (BKBx) on the day of kidney biopsy. BKBx is a prospective observational study of individuals undergoing native kidney biopsy at 3 tertiary care hospitals in Boston, MA, that began in September 2006. Urine samples were selected from enrolled adults (age > 18 years) with a primary diagnosis and clinicopathologic phenotype representative of DN and active LN. Urine samples from Boston healthy volunteers were obtained from the PhenoGenetics Project, a study of the impact of genetic variation in healthy individuals. Deidentified human kidney paraffin tissue samples from patients with varying degrees of kidney fibrosis were obtained from the Department of Pathology at Brigham&Women's Hospital and Beth Israel Deaconess Medical Center.

In the DN replication cohort (UW), midstream, clean-catch, spot urine samples were collected from participants with type 1 and 2 diabetes. DN was defined as either a urine albumin to creatinine ratio ≥300 mg/g or an eGFR <60 mL/min/1.73 m^2 and urine albumin to creatinine ratio ≥30 mg/g. Diabetes without kidney disease (DM) was defined as eGFR ≥90 mL/min/1.73 m^2 and urine albumin to creatinine ratio <300 mg/g after ≥30 years of type 1 diabetes or ≥15 years of type 2 diabetes. Healthy patients' samples were obtained from healthy volunteers near UW. In the LN replication cohort (OSU), spot urine samples were collected from patients with LN immediately before kidney biopsy between August 2005 and March 2016. Only patients with LN flare (Class II–V) were included in this study. Urine samples from systemic lupus erythematosus patients without nephritis (SLE) were collected during the Ohio SLE Study. The Ohio SLE Study was a prospective, observational cohort of clinically and phenotypically well-characterized, multiracial SLE patients in which urine was systematically collected from and stored for all patients. Healthy patient samples were obtained from healthy volunteers near OSU.

The Institutional Review Boards approved the protocols for recruitment and sample collection, which was performed with written consent of the participants at each respective site.

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Further information regarding sample processing is located in the Materials and Methods section of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue9.

EVALUATION OF HISTOPATHOLOGY

Each patient enrolled in the BKBx cohort was adjudicated for a primary clinicopathologic diagnosis and histopathologic lesions in a meeting between 2 renal pathologists along with 2 nephrologists. During the biopsy meeting, each biopsy is reviewed on light microscopy and scored in consensus-based semiquantitative measures of 13 different categories of histopathological features of inflammation, fibrosis, vascular, and tubular injury (see Fig. 1 in the online Data Supplement).

STUDY DESIGN

We performed a sequential approach with the following 3 main phases: global profiling, confirmation, and replication (Fig. 1). In the global profiling, 2402 different miRNAs (based on the latest miRbase version 21) were screened in the following 4 pooled samples: HE, LN, DN, and renovascular kidney disease (HTN) (Fig. 1A). After primary confirmation in the same pooled samples, precandidates specific of each etiology were confirmed in an expanded set of patients with biopsy-proven DN (n = 58) and LN (n = 89) in the BKBx cohort (Fig. 1B). Top performing candidates were then measured at UW (DN, n = 74) and OSU (LN, n = 86), and final candidates were selected (Fig. 1C). We evaluated the renal tissue expression by in situ hybridization of selected final candidates using kidney biopsy samples from HE (n = 5). The details of miRNA profiling, final selection approach, urine miRNA expression analysis, and miRNA in situ hydridization are provided in the Material and Methods section of the online Data Supplement.

STATISTICS

For the normalization of miRNA urine levels, Ct (threshold cycle) values were normalized using the geometric mean of technical controls (miRNA reverse transcription control and positive PCR control). To adjust for urine dilution, relative levels of miRNA expression (2^{-dCt}) were divided by urinary creatinine. For pairwise comparisons analysis, *P* values were corrected for false discovery rate (FDR) using the Benjamini–Hochberg adjusted Student *t*-test (5% FDR). ANOVA was used to determine differences between groups in the replication phase, followed by Tukey post hoc analysis for multiple comparisons. Spearman rank correlation analysis was applied to evaluate the relationship between urine miRNAs and renal function biomarkers [proteinuria, serum creatinine (SCr), and eGFR]. Associations between urine miRNAs and histopathological scores were investigated using the Wilcoxon rank-sum test for 2 group comparisons and the Kruskal–Wallis test (>2 groups), followed by the Dunn test for multiple comparisons. To evaluate the classification power of the final candidates, logistic regression and area under the curve (AUC) values from ROC curves were calculated in the BKBx, OSU, and UW cohorts. We calculated odd ratios (OR) with 95% CI and AUC-ROC values. We considered a *P* value ≤0.05 to be significant. Statistical analysis was performed using STATA software version 13.1 (StataCorp LP) and SAS software version 9.4 (SAS Institute).

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Results

DISCOVERY AND CONFIRMATION OF URINARY MiRNA BIOMARKERS USING A BIOPSY-PROVEN CKD COHORT

First, we conducted global urinary miRnome profiling measuring 2402 miRNAs in pooled urine samples (n = 10/group) from the BKBx cohort that included patients with DN, LN, renovascular kidney disease, and HE (Table 1, Fig. 1A). The 4 groups were compared on the basis of the fold change of expressed miRNAs (with qPCR Ct <28). The comparative analysis led to the identification of 365 miRNAs that were measured individually in the same previously pooled patient samples (n = 40) (see Fig. 2 in the online Data Supplement). Further, 26 precandidates were selected that displayed differential expression (significant fold change >1.5) when comparing arbitrary units (2^{-dCt}/urinary creatinine) between DN/renovascular kidney disease and HE. Then, 18 precandidate miRNAs were selected using the same differential expression criteria between LN and HE (see Fig. 3 in the online Data Supplement). The DN and renovascular kidney disease groups were combined because of their similar histopathological profiles in terms of tubulointerstitial and vascular disease. A detailed description of the discovery and evaluation phases is provided in the Material and Methods section of the online Data Supplement.

Next, these miRNA precandidates were measured in a larger group of patients from the BKBx cohort (Table 1). Best performing candidates were selected based on (*a*) significant differences (FDR < 0.05) compared with the HE group and (*b*) significant correlation with any of the renal function biomarkers, such as proteinuria, SCr, and eGFR, or significant association with any of the prespecified histopathological parameters (Fig. 1B). In DN patients, 15 miRNAs were differentially present in urine compared with in HE controls (Fig. 2A); 11 urine miRNAs correlated with at least 1 of the renal function markers (Fig. 2B), and 9 were associated with histopathological lesions (Fig. 2C). In total, 16 of 18 urine miRNAs were significantly different in LN patients compared with HE controls (Fig. 3A). Seven miRNAs correlated with at least 1 of the renal function markers (Fig. 3B), and 12 were associated with histopathological lesions (Fig. 3C).

miRNAs that passed the selection criteria were ranked by fold change, and the first 12 were selected for each CKD group (DN and LN) to continue to the replication phase (for fold changes and ranking details, see Table 1 in the online Data Supplement).

REPRODUCIBILITY AND ROBUSTNESS OF TOP MiRNA CANDIDATES USING INDEPENDENT REPLICATION CKD COHORTS

Differentially expressed candidate urine miRNAs were tested by measuring miRNAs in 2 independent cohorts of patients with type 1 or type 2 diabetes mellitus with or without nephropathy and in patients with SLE with and without nephritis (Table 1, Fig. 1C). In the UW cohort, urine miR-1915-3p, miR-2861, and miR-4532 levels were similar in the control groups (HE and DM) but were significantly lower in DN patients (P < 0.0001 for all; Fig. 4A). Urine levels of miR-4536-3p and miR-6747-3p were higher in DN patients than in HE (P < 0.0001) and DM (P = 0.001) controls, but miR-6747-3p did not have any significant difference between DN and DM patients (Fig. 4A). In the OSU cohort, urine miR-1273e,

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miR-204-5p, and miR-3201 levels were similar in the control groups (HE and SLE) but significantly different in LN patients (miR-1273e, miR-3201: $P < 0.0001$ and miR-204-5p: $P = 0.0001$; Fig. 4B). Urine miR-30c-5p levels were significantly higher in LN patients compared with HE (P = 0.001) and SLE (P = 0.01) controls but were also significantly higher in SLE compared with HE controls (P = 0.04) (Fig. 4B). For fold change details in both cohorts, see Table 2 in the online Data Supplement.

We evaluated the association of the top performing miRNAs with the dichotomous outcomes of DN vs control groups (HE and DM separately) and LN vs control groups (HE and SLE separately) (Fig. 5). When comparing the outcome of DN vs HE, miR-2861 (OR, 0.25; 95% CI, 0.16–0.39), miR-1915-3p (OR, 0.37; 95% CI, 0.26–0.51), and miR-4532 (OR, 0.40; 95% CI, 0.29–0.54) were closely associated with HE, whereas miR-6747-3p (OR, 2.70; 95% CI, 1.85–3.92) and miR-4536-3p (OR, 3.03; 95% CI, 1.95– 4.72) were more closely related with DN (Fig. 5A). The associations were in the same direction when comparing DN vs DM, but the ORs were weaker and *c*-statistics lower for miR-6747-3p and miR-4536-3p in their relation to DN (Fig. 5B). The *c*-statistics remained strong when comparing DN vs HE and DN vs DM for miR-2861 (0.91 and 0.91), miR-1915-3p (0.87 and 0.86), and miR-4532 (0.83 and 0.85). Comparing the outcome of LN vs HE revealed that miR-3201 (OR, 0.10; 95% CI, 0.04–0.27) and miR-1273e (OR, 0.09; 95% CI, 0.09–0.25) were strongly associated with HE, but miR-204-5p (OR, 4.69; 95% CI, 2.48–8.87) and miR-30c-5p (OR, 2.99; 95% CI, 1.72–5.18) were strongly associated with LN (Fig. 5C). Although the associations were in the same direction when comparing LN vs SLE, miR-204-5p and miR-30c-5p had weaker ORs and lower *c*-statistics in their relation to LN (Fig. 5D). The *c*-statistics remained strong when comparing LN vs HE and LN vs SLE for miR-3201 (0.97 and 0.96) and miR-1273e (0.91 and 0.86). To view the association between individual candidate miRNAs for the diagnosis of DN or LN in all cohorts, see Figs. 4 and 5, respectively, in the online Data Supplement.

ASSOCIATION OF THE TOP MiRNA CANDIDATES WITH KIDNEY FUNCTION MARKERS AND HISTOPATHOLOGY

In DN patients, miR-2861, miR-1915-3p, and miR-4532 were associated with SCr and eGFR in the BKBx and UW cohorts (Figs. 2B and 4C). In addition, miR-2861, miR-1915-3p, and miR-4532 were all associated with interstitial fibrosis and tubular atrophy (IFTA) and tubulointerstitial inflammation in the fibrotic regions on the histopathological assessment in the BKBx cohort (Fig. 2C). Patients with >50% IFTA grades had decreased urinary levels of miR-2861, miR-1915-3p, and miR-4532 compared with patients with mild (25%) and moderate (<50%) IFTA (see Fig. 6 in the online Data Supplement). miR-1915-3p had lower urinary levels for each increasing grade of IFTA (see Fig. 6 in the online Data Supplement). These miRNAs showed mild correlation with proteinuria but this was not significant in the BKBx cohort. However, they had a significantly negative correlation with proteinuria in the UW cohort (Fig. 4C). Neither miR-6747-3p nor miR-4536-3p was associated with renal function parameters in the BKBx or UW cohorts (Figs. 2B and 4C). Both were significantly associated with the presence of tubulointerstitial inflammation in the preserved regions on the histopathological assessment in the BKBx cohort (Fig. 2C). For

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histopathological characteristics of the BKBx cohort, see Fig. 1 in the online Data Supplement.

In LN patients, neither miR-3201 nor miR-1273e was associated with renal function parameters in the BKBx or OSU cohorts (Figs. 3B and 4D). However, in the BKBx cohort, both were strongly associated with the presence of endocapillary glomerular inflammation (P = 0.008 and $P = 0.01$, respectively) (Fig. 3C). Patients with moderate-to-severe endocapillary glomerular inflammation had decreased urinary levels of miR-3201 and miR-1273e compared with patients with none or mild grades of this pathological feature (see Fig. 7 in the online Data Supplement). In addition, lower levels of miR-3201 and miR-1273e were associated with proliferative LN when compared with nonproliferative LN ($P = 0.02$ and $P = 0.03$, respectively) (see Fig. 8 in the online Data Supplement). In the BKBx cohort, miR-204-5p was positively associated with eGFR ($r_S = 0.22$, $P = 0.05$), and this relationship was stronger in the OSU cohort ($r_S = 0.37$, $P = 0.0005$) (Figs. 3B and 4D). Notably, higher scores of arterial (P = 0.05) and arteriolar sclerosis (P = 0.04) were associated with lower levels of miR-204-5p in the BKBx cohort (see Fig. 3C here and Fig. 7 in the online Data Supplement). miR-30c-5p moderately correlated with proteinuria ($r_S = 0.2$, $P = 0.05$) in the BKBx cohort; however, this relationship was stronger and significant in the OSU cohort ($r_S = 0.31$, $P = 0.005$) (Fig. 4D). Additionally, miR-30c-5p had an association with the presence of cellular crescents (P = 0.05), acute tubular injury (P = 0.03), arterial sclerosis (P = 0.02), and arteriolar sclerosis (P = 0.05) (Fig. 3C).

RENAL EXPRESSION OF TOP MiRNA CANDIDATES IN HEALTHY KIDNEY TISSUE

We investigated the expression of the top miRNA candidates using in situ hybridization in healthy kidney tissue specimens (see Materials and Methods and Fig. 9A in the online Data Supplement). Renal expression of miR-2861, miR-1915-3p, and miR-4532 was detected in the nucleus and cytoplasm of the tubular and glomerular epithelial cells (see Fig. 9, B, C, and D, respectively, in the online Data Supplement). miR-1273e expression had a strong nuclear staining in all renal cell types (see Fig. 9E in the online Data Supplement). miR-204-5p expression was detected in the nucleus of tubular epithelial cells and was more prominent in distal tubules (see Fig. 9F in the online Data Supplement). miR-3201 was not detected by in situ hybridization in the kidneys.

Discussion

We present the largest human study to date to identify miRNA biomarkers of DN and LN derived from associations with both histopathological lesions on kidney biopsy and functional markers of kidney damage. Five miRNAs (miR-2861, miR-1915-3p, miR-4532, miR-4536, and miR-6747) in DN and 4 miRNAs (miR-3201, miR-1273e, miR-204-5p, and miR-30c-5p) in LN replicated in independent multi-institutional cohorts when compared with healthy and disease controls. Three DN miRNAs (miR-2861, miR-1915-3p, and miR-4532) and 2 LN miRNAs (miR-3201, miR-1273e) showed strong *c*-statistics in their ability to discriminate between patients with renal disease vs either healthy or disease controls, and were associated with the most characteristic histopathological lesions. In addition, we confirmed the expression of 4 of 5 of these top miRNA candidates in healthy

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kidney tissue. These results identify new urine miRNAs as sensitive and specific noninvasive biomarkers carrying a histopathologic signature of DN or LN and may obviate the need for invasive tests.

The Kidney Disease Outcome Quality Initiative suggests eGFR and proteinuria as indicators of kidney function and glomerular injury, respectively (23). Although baseline eGFR and proteinuria are predictors of renal function decline, they lack the ability to discriminate between disease states or identify histopathological lesions (24–26). Urinary miRNAs offer a unique opportunity as noninvasive biomarkers given their proximity to injury (tissue specificity), ease of collection, stability at room temperature, and preservation during multiple freeze–thaw cycles (27, 28). miRNA dysregulation may lead to CKD via disruption of podocyte homeostasis, extracellular matrix, and cellular adhesion proteins, as well as transforming growth factor-β-mediated fibrosis (29). Argyropoulos et al. showed 27 urinary miRNA profiles among varying stages of DN in patients with type 1 diabetes (30). Delić et al. identified 16 miRNAs differentially present in urinary exosomes when comparing patients with type 2 diabetes with nephropathy vs healthy controls (31). Less is known about the role of urine miRNAs in glomerular diseases, but miR-146a and miR-155 were up-regulated in the urine and renal tissue of patients with biopsy-proven IgA nephropathy compared with healthy controls (32). Few studies examined urine miRNAs in those with LN, but miR-29c may be an early marker of renal fibrosis in LN patients (33).

In this study, miR-2861, miR-1915-3p, miR-4532, miR-4536-3p, and miR-6747-3p may help identify patients with DN. miR-4536-3p and miR-6747-3p were not associated with renal function parameters or typical histopathological findings on renal biopsy. Although miR-4536-3p and miR-6747-3p were more statistically suggestive of DN compared with HE, they performed much more weakly when compared with patients with DM. MiR-2861, miR-1915-3p, and miR-4532 were the most down-regulated miRNAs in the urine of DN patients and positively correlated with eGFR. Moreover, the decreased expression of these miRNAs in the urine of DN patients was associated with increased levels of IFTA and tubulointerstitial inflammation. The association between miR-2861, miR-1915-3p, and miR-4532 followed a near dose–response relationship with IFTA highlighted by the lower urinary levels per increasing grade of IFTA, and may suggest a role for fibrosis formation in DN. Previous studies reported the up-regulation of miR-2861 and miR-1915-3p in patients with type 2 diabetes with microalbuminuria (31) and down-regulation of miR-1915-3p in focal segmental glomerulosclerosis patients (34). However, these miRNAs were assessed in urinary exosomes; very few patients were enrolled in these studies ($n < 20$), and none of them had biopsy-proven DN. Although miR-30e-5p did not replicate in the UW cohort, a strong and significant correlation between this miRNA and the levels of proteinuria in DN patients was constant in both the BKBx and UW cohorts.

MiR-3201, miR-1273e, miR-204-5p, and miR-30c-5p have not been previously reported in patients with biopsy-proven LN. miR-204-5p may have a role in chronic injury given its histologic relationship to vascular disease and correlation with SCr and eGFR. The lack of statistical correlation with IFTA may be because of the relatively low levels of fibrosis in this cohort of LN patients. However, miR-204-5p's inability to have discriminatory power between LN and SLE may also support its role as a marker of chronic injury as opposed to

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LN activity. miR-1273e and miR-3201 were associated with proliferative LN, which may be driven by the association with endocapillary glomerular inflammation in the BKBx cohort, but not associated with renal function parameters in the BKBx or OSU cohorts. The association with glomerular pathology but lack of an association with renal function parameters may suggest a regulatory role for these miRNAs in glomerular inflammation, warranting further study. miR-30c-5p was associated with proteinuria and cellular crescents in LN. The members of the miR-30 family are important regulators of podocyte homeostasis and pathogenesis abundantly expressed in the kidney where their deregulation is associated with podocyte injury as described in patients with primary focal segmental glomerulosclerosis (35, 36). Members of this family have a similar sequence, but their genes are localized on different chromosomes, making a different gene-expression pattern possible, depending on different stimuli (37). Our findings suggest miR-30c-5p and miR-30e-5p are involved in the pathophysiological mechanism of proteinuria in CKD.

Our study has some limitations to consider. First, we cannot verify that all healthy samples were from patients free of any disease or that DM/SLE patients did not have early changes suggestive of DN/LN. However, all centers performed an exhaustive attempt to collect samples from healthy patients and used current clinical guidelines to choose disease controls (DM/SLE). Second, although the study relied on kidney biopsy findings, the clinical indication for biopsy likely introduces some confounding by indication since we are unable to identify some earlier histopathological features of DN, which does not allow for distinguishing between the glomerular and vascular phenotypes of DN. Third, the cross-sectional design is unable to address causality or prediction of subsequent outcomes. Fourth, our scope did not cover the confirmation of the function and the targets of the top miRNA candidates, and further information will help to elucidate their role in the pathophysiology of CKD. Last, although this represents the largest study to date with the use of validation cohorts, these markers need to be further investigated in larger cohorts of DN and LN patients, particularly to evaluate the prognostic potentials of these candidate miRNAs.

In summary, we performed global urinary miRNA profiling and identified unique, sensitive and specific, noninvasive biomarkers for detection of DN and LN in patients. The miRNA biomarkers were confirmed and replicated in multi-institutional cohorts and were associated with conventional markers of kidney dysfunction and biopsy-adjudicated pathology. Deeper understanding of these noninvasive miRNAs may improve our capacity for disease monitoring and patient stratification, or possibly lead to their use as therapeutic targets in clinical trials for DN and LN patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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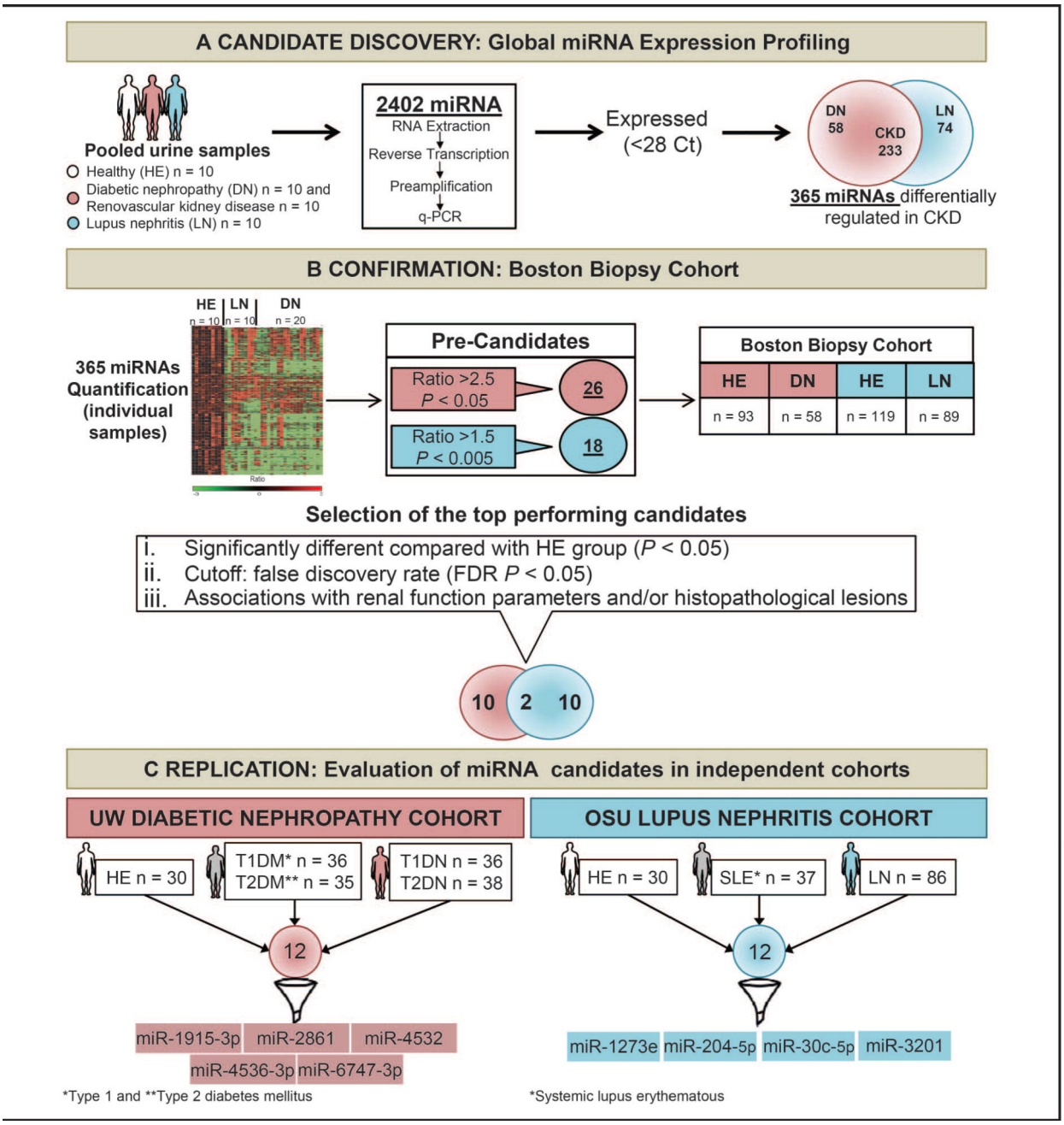

Fig. 1. Schematic overview of the strategy for discovery of urinary miRNAs as biomarkers for CKD

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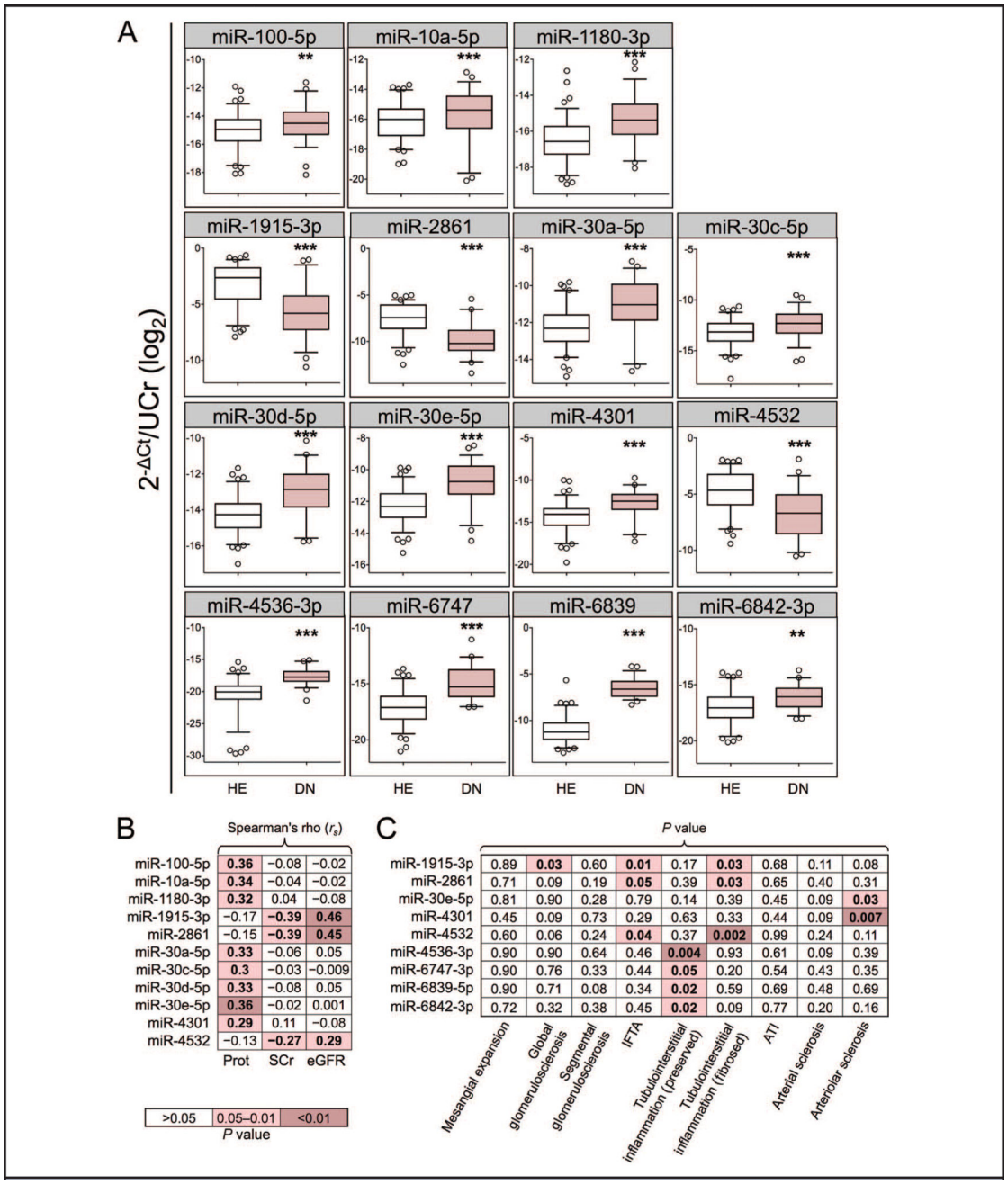

Fig. 2. Selection of the best performing urinary miRNAs for DN in the BKBx cohort

miRNAs differentially present in DN (n = 99) compared with healthy controls (HE = 119) (*t*-test, FDR $P < 0.05$) (A). Box plot represents the median ±25th and 75th percentiles, and whiskers are determined by the 5th and 95th percentiles (**$P < 0.01$; ***$P < 0.001$). Spearman rank correlation analysis between miRNAs and renal function parameters: proteinuria (Prot), SCr and eGFR, as well as Spearman correlation coefficients (r_S) are presented in the boxes (B). Associations between miRNAs and renal histopathological parameters; *P* values are presented in the boxes (Wilcoxon rank-sum test for 2 independent observations, and Kruskal–Wallis followed by Dunn test for multiple comparisons) (C).

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Note: for panels B and C, only miRNAs that had significant correlation or association are shown.

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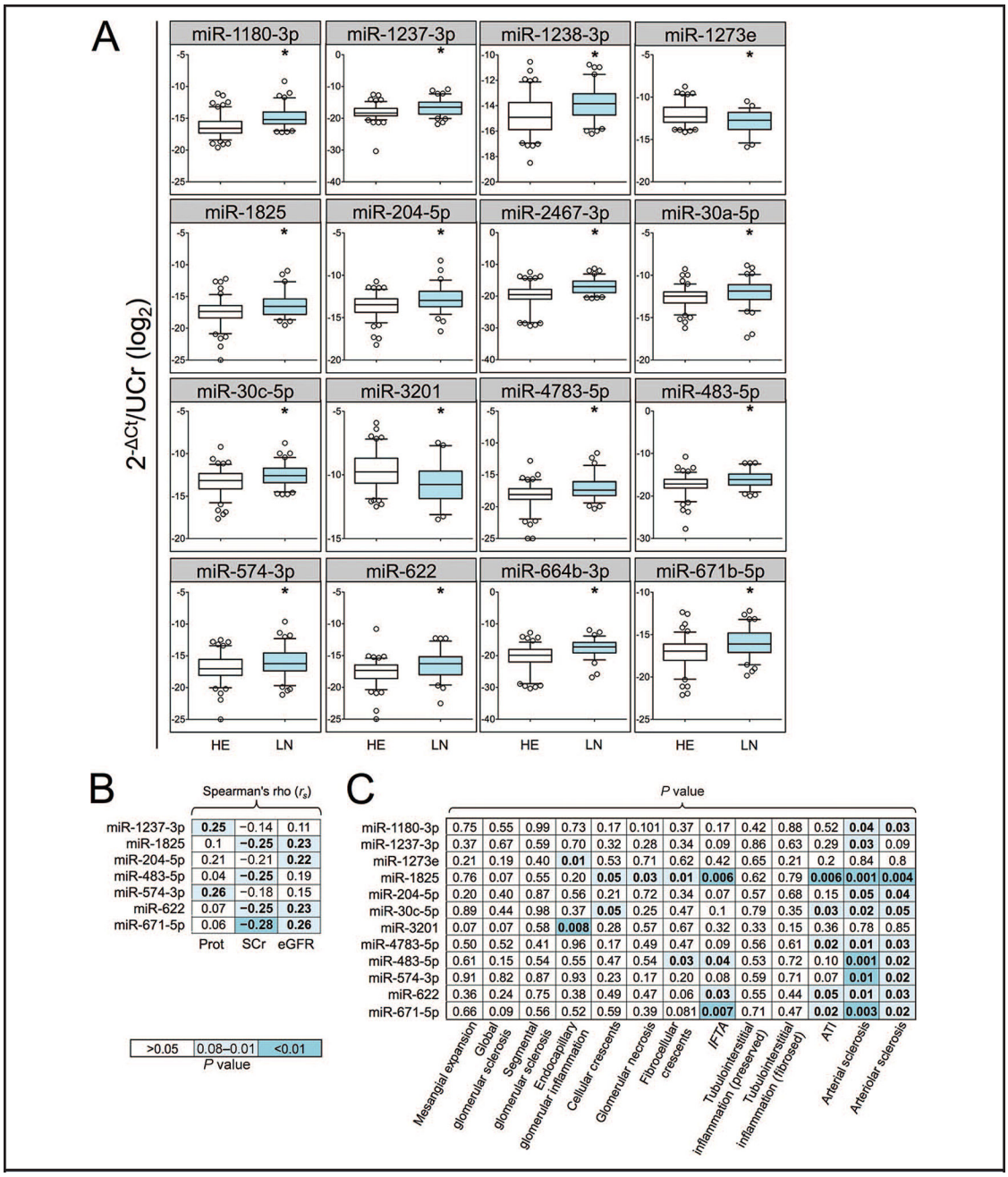

Fig. 3. Selection of the best performing urinary miRNAs for LN in the BKBx cohort

miRNAs differentially present in LN (n = 99) compared with healthy controls (HE = 119) (*t*-test, FDR $P < 0.05$) (A). Box plot represents the median ±25th and 75th percentiles, and whiskers are determined by the 5th and 95th percentiles (*$P < 0.05$; **$P < 0.01$). Spearman rank correlation analysis between miRNAs and renal function parameters: proteinuria (Prot), SCr and eGFR, as well as Spearman correlation coefficients (r_s) are presented in the boxes (B). Associations between miRNAs and renal histopathological parameters; *P* values are presented in the boxes (Wilcoxon rank-sum test for 2 independent observations, and

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Kruskal–Wallis followed by Dunn test for multiple comparisons) (C). *Note*: for panels B and C, only miRNAs that had significant correlation or association are shown.

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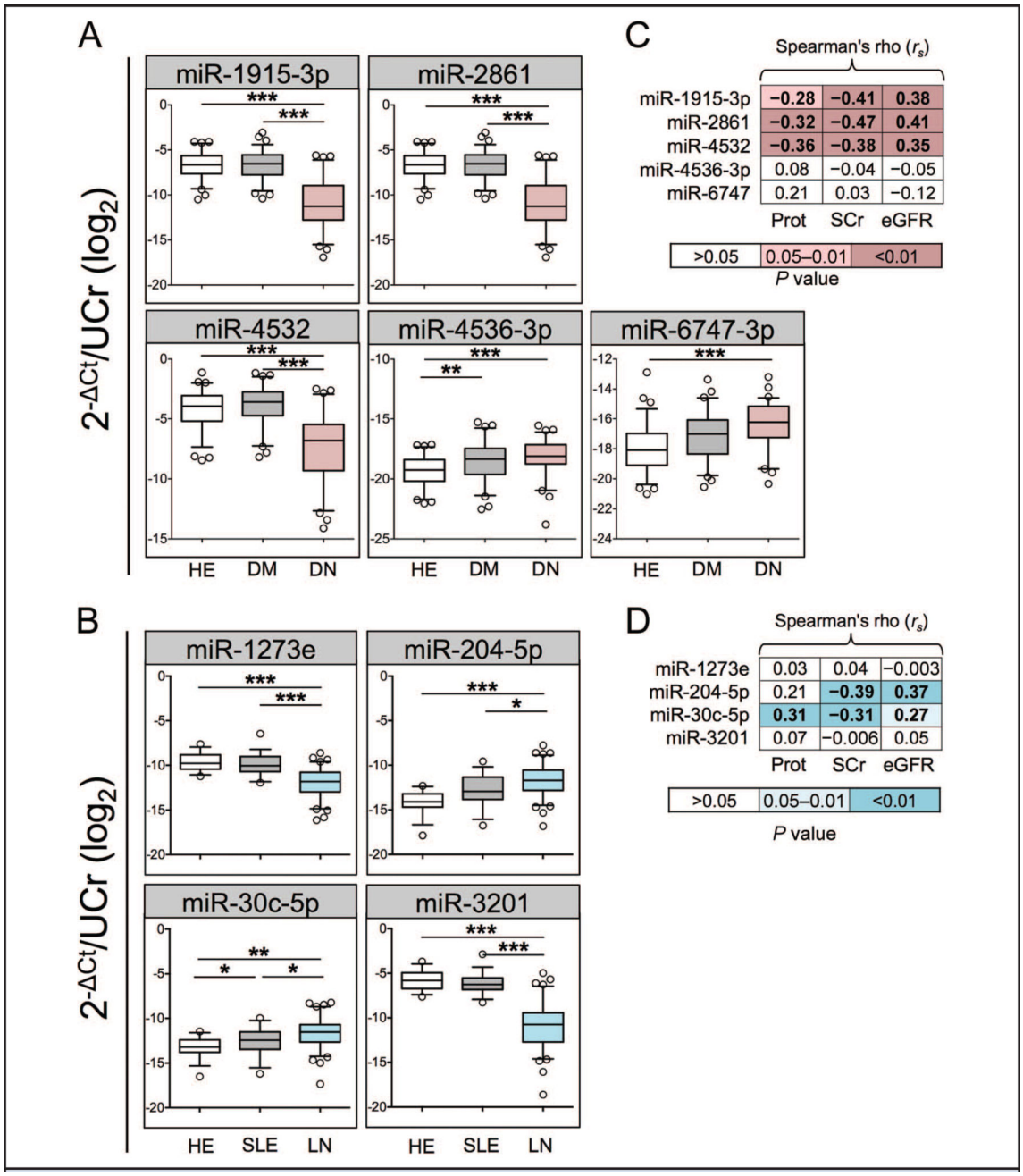

Fig. 4. Biomarker replication in the UW DN and OSU LN cohorts

miRNAs differentially present in DN (n = 74) compared with the control groups: healthy (HE = 30) and diabetes mellitus (DM = 71) (A). miRNAs differentially present in LN (n = 86) compared with the control groups: healthy (HE = 86) and systemic lupus erythematosus (SLE = 37) (B). Box plot represents the median±25th and 75th percentiles, and whiskers are determined by the 5th and 95th percentiles (*P< 0.05, **P < 0.01, ***P < 0.001; ANOVA, Tukey multiple comparison). Spearman rank correlation analysis between miRNAs and renal function parameters in the DN and LN groups, respectively. Spearman correlation coefficients (r_S) are presented in the boxes (C and D).

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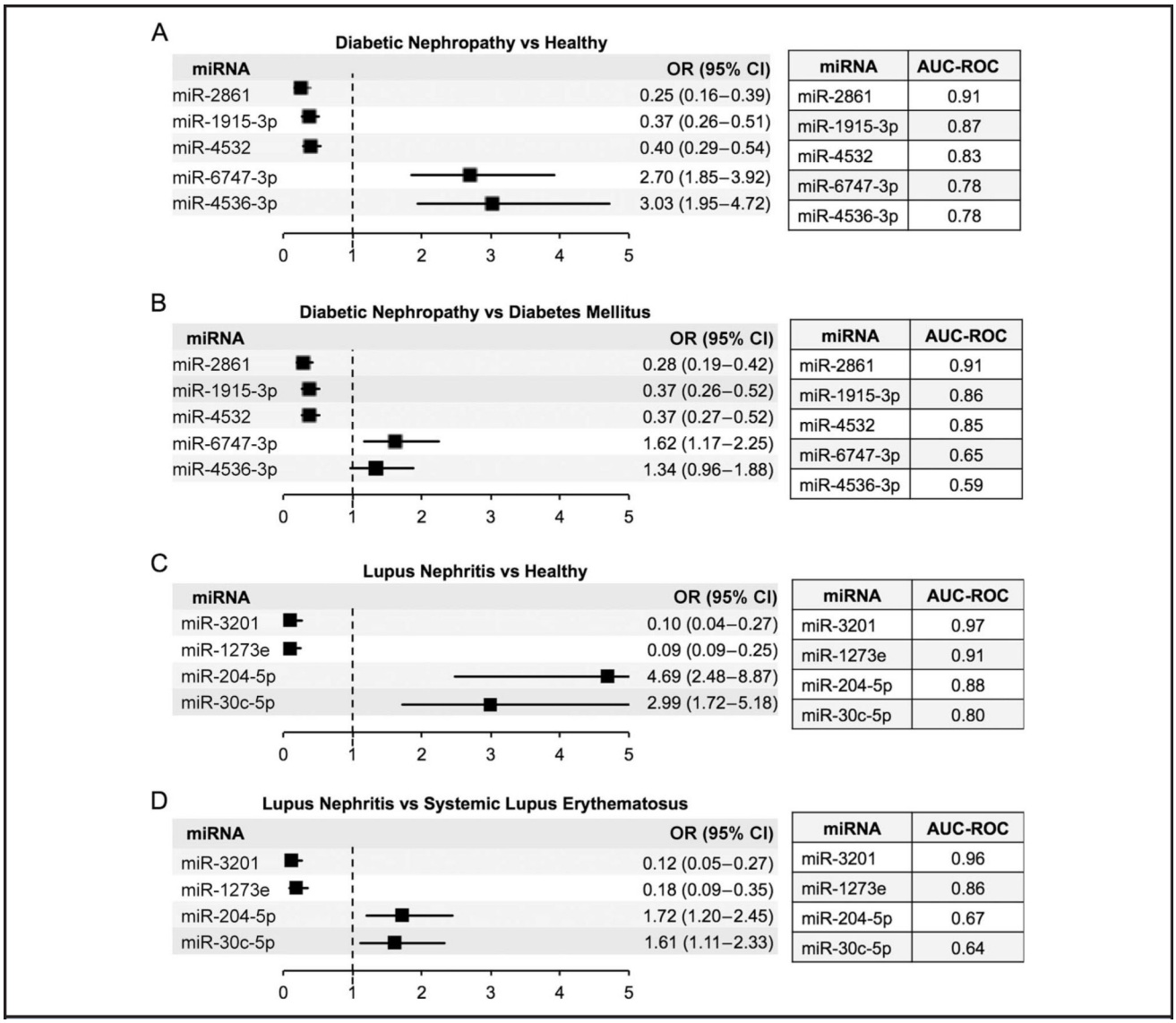

Fig. 5. Association of individual miRNAs with DN and LN in the replication cohorts

Forest plot of individual miRNAs and AUC-ROC of replicating miRNAs associated with outcome of DN vs HE in the UW cohort with unadjusted ORs and 95% CIs (A). Forest plot of individual miRNAs and AUC-ROC of replicating miRNAs associated with outcome of DN vs DM in the UW cohort with unadjusted ORs and 95% CIs. miR-6747-3p and miR-4536-3p replicated more weakly in this cohort compared withDNvs HE in the UW cohort (B). Forest plot of individual miRNAs and AUC-ROC of replicating miRNAs associated with outcome of LN vs HE in the OSU cohort with unadjusted ORs and 95% CIs (C). Forest plot of individual miRNAs and AUC-ROC of replicating miRNAs associated with outcome of LN vs SLE in the OSU cohort with unadjusted ORs and 95% CIs (D). miR-204-5p and miR-30c-5p replicated more weakly in this cohort compared with LN vs HE in the OSU cohort.

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Table 1

Demographic and clinical characteristics of CKD patients in the discovery, confirmation, and replication cohorts.[a]

CKD	n	Age (years)	Sex (%F[b])	Race (%W)	Proteinuria (g/g Cr)	eGFR (mL/min/1.73 m^2)	SCr (mg/dL)
Discovery cohort							
HE	10	34.5 ± 15.3	50.0	50.0	NA	NA	NA
DN	10	60.4 ± 13.5	50.0	40.0	5.5 (0.8–11.5)	34.5 (29.0–65.0)	2.6 ± 1.7
HTN[b]	10	58.5 ± 14.2	40.0	80.0	0.6 (0.2–3.1)	32.0 (29.0–49.0)	2.3 ± 1.6
LN	10	38.4 ± 9.6	100.0	30.0	3.0 (0.8–3.3)	97.5 (77.0–123.0)	0.7 ± 0.2
Confirmation stage: Boston Kidney Biopsy cohort (BKBx)							
HE	119	36.6 ± 13.5	47.5	57.5	NA	NA	NA
DN	58	59.4 ± 14.0	48.3	48.3	4.7 (2.0–7.2)	32.0 (22.3–45.0)	2.4 ± 1.4
LN	89	38.8 ± 13.0	85.4	32.6	2.1 (1.2–3.3)	90.9 (57.4–116.6)	1.2 ± 1.0
Replication stage: UW diabetic nephropathy cohort							
HE	30	54.4 ± 13.6	66.2	83.1	0.009 (0.003–0.008)	NA	NA
DN	74	54.7 ± 13.0	33.8	70.3	0.42 (0.12–1.29)	35.5 (21.0–51.0)	2.5 ± 2.3
DM	71	54.5 ± 12.5	43.7	76.1	0.006 (0.004–0.01)	93.4 (79.0–106.0)	0.8 ± 0.2
Replication stage: OSU lupus nephritis cohort							
HE	30	43.4 ± 13.1	63.3	33.3	NA	NA	NA
LN	86	34.3 ± 10.2	80.2	46.5	2.0 (1.2–3.2)	79.1 (44.0–96.3)	1.3 ± 0.9
SLE	37	41.7 ± 13.7	40.5	62.9	NA	98.0 (86.0–109.0)	1.0 ± 0.8

[a]Age and SCr are presented as mean ± SD. Proteinuria and eGFR are presented as median (IQR range).

[b]F, female; W, white; HTN, renovascular kidney disease.