

Insulin Receptor Substrate 2 mRNA Expression in Urinary Sediment Cells as a Diagnostic Marker of Nephropathy in Diabetic Patients

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Introduction. The initiation and progression of diabetic nephropathy (DN) is complex. Quantification of mRNA expression in urinary sediment cells (USCs) has emerged as a novel strategy for studying kidney diseases. Insulin requires a family of insulin receptor substrate (IRS) proteins for its physiological effects, and many reports have highlighted the role of insulin and IRS proteins in kidney physiology and disease. This study aimed to assess *IRS2* expression in USCs of patients with diabetes mellitus, DN, and nondiabetic chronic kidney disease.

Materials and Methods. To quantify *IRS2* expression, RNA was extracted from USCs of 223 individuals comprised of diabetes mellitus, DN, and nondiabetic chronic kidney disease as well as a healthy control group. The cDNA was synthesized and comparative TaqMan real-time reverse transcript polymerase chain reaction was used in the presence of beta actin gene as a reference gene for normalization, relative to the control.

Results. Our data showed that the USCs expression of *IRS2* gene was significantly increased in the DN patients compared with other groups ($P < .001$). The *IRS2* expression was not significantly different between microalbuminuria and macroalbuminuria conditions or different stages of DN, except for the end-stage renal disease where the expression was lower.

Conclusions. In patients with DN, urinary mRNA expression of the *IRS2* gene is associated with kidney function. Our result suggests that serial measurement of urinary expression of this gene may have a value for early detection of kidney injury in diabetic patients.

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INTRODUCTION

The International Diabetes Federation Atlas for Diabetes shows that the Middle East and North Africa region of the world has the highest prevalence of diabetes mellitus (DM).¹ Despite current therapies, a variety of diabetic patients will suffer from kidney function reduction. Diabetic nephropathy (DN) is a common cause of end-stage renal disease. It is also the major cause of morbidity and premature mortality in patients suffering type

2 DM. The prevalence of DN was reported as 5% to 10% at the time of diagnosis of type 2 DM by several population-based studies.² Among Iranian patients with DM, the prevalence of DN seems to be much higher than what has been reported by other populations-based studies.³

Poor glycemic control, high blood pressure, and albuminuria are well-known risk factors for the development or the progression of DN, but these factors could not explain all of the inter-individual

variability in the rate of progression to end-stage renal disease.⁴

Nowadays, the integration of molecular approaches including transcriptome analyses has uncovered pathological mechanisms involved in the progression of DN. Identifying the molecular marker candidates may enable the characterization of patients at high risk for progression to end-stage renal disease, a molecular definition of DN and also targeted treatment to improve patient care.⁵⁻⁷

Gene expression analysis has been an important means of acquiring pathophysiologic information from renal biopsy specimens. There are practical problems that limit gene expression analysis in kidney biopsy specimens. These problems may be invasive procedure of renal biopsy, no feasibility to have serial monitoring of the same patient, nonsuitability of biopsy for all patients with kidney disease (eg, patients with bleeding tendency or solitary kidney), and possibility of distortion of the result due to sampling bias.^{8,9} It has been reported that although free RNA exists in urine, urine sediment, which contains cells shed from kidney tissue, seems to be a more suitable target for gene expression analysis.¹⁰

Recent research has highlighted the role of insulin signaling directly on kidney cells in renal damage in DN.¹¹ Insulin action requires insulin receptor tyrosine kinase phosphorylation of insulin receptor substrate (IRS) proteins to trigger key cellular effects such as glucose uptake, cell proliferation and longevity.¹² Insulin resistance is attenuation of insulin sensitivity at target tissues. The IRS proteins are cellular adaptor molecules. They mediate key metabolic actions of insulin. The IRS proteins recruit downstream effectors, when tyrosine is phosphorylated by the activated insulin receptor. These downstream effects can be categorized as phosphoinositide 3-kinase and mitogen-activated protein kinase, for elicitation of cellular responses such as glucose uptake, lipid metabolism, and cell proliferation.¹³

The IRS proteins integrate signals from the insulin and insulin-like growth factor 1 receptor with those generated by pro-inflammatory cytokines, nutrients, and neurotrophins.¹⁴ Phosphorylated IRS proteins activate multiple signaling pathways, including the PI3K and Erk cascades.^{15,16} There are three main IRS proteins in humans: IRS1, IRS2, and IRS4, of which IRS1 and IRS2 are widely

expressed in most tissues, whereas IRS4 expression is restricted to the hypothalamus-pituitary gland and thymus.¹² Although these proteins are similar in overall structure and sequence, there are distinct physiological roles for the individual IRS proteins in vivo.¹⁷ Studies have highlighted roles for IRS proteins in the kidney and suggest that insulin receptor engagement of IRS proteins is a crucial component of kidney physiology and DN.¹⁸⁻²¹

Expression of IRS2 is reported in the developing and adult kidney tubular epithelial compartments. A link between bone morphogenetic protein 7 (BMP7) and IRS2 promoter activation and signaling in kidney tubule epithelial cells was demonstrated that was considered new light on the role of IRS2 in diabetic kidney disease.¹⁷

The aim of the present study was to investigate the impact of *IRS2* gene expression in urinary sediment cells on diabetic kidney failure in a group of Iranian type 2 diabetic patients, in order to identify molecular marker candidates that may enable the characterization of patients at high risk for progression to DN. The *IRS2* gene expression was studied in urinary sediment cells (USC), which contain cells shed from kidney tissue by TaqMan real-time reverse transcription polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Study Population

This case-control study was carried out in a group of 223 Iranian individuals, including 50 with type 2 DM, 50 with DN, 50 with nondiabetic chronic kidney disease (CKD), 23 with microalbuminuria (13 diabetic and 10 nondiabetic), and 50 unaffected sex- and age-matched controls. Patients with nephropathy complications were classified into different stages of the pathology according to their glomerular filtration rate (GFR); among them 11 were in the early stages (stages 2 to 3; GFR, 30 mL/min to 89 mL/min) in each of the DN and nephropathy groups; 15 and 11 were in the late stages (stages 4 to 5, GFR, 15 mL/min to 29 mL/min) of the DN and nephropathy groups, respectively; and 24 and 28 were on hemodialysis treatment in the DN and nephropathy groups, respectively. General and lifestyle information of the patients and also family pedigrees were recorded. The protocol was approved by the Ethics Committee of the National Institute of Genetic Engineering

and Biotechnology based on Helsinki declaration. Patients and controls signed a written informed consent letter before enrolment.

Table 1 shows clinical and analytical data for the patients and control groups. The patients with type 2 DM and nephropathic patients who had an estimated GFR of 22.5 mL/min and greater received conventional treatment. Hemodialysis was done 3 times per week. Patients with bacterial infections, oncological disease, hepatitis C or B, positive human immunodeficiency virus, hepatic insufficiency, immunosuppressive therapy, and antibiotics uptake at least 1 month prior to sampling were excluded. The relevant data regarding the clinical reports from all of the participant was recorded.

Urinary Sampling

A whole-stream early-morning urine specimen was collected from each study participant and was centrifuged at 3000 g for 30 minutes at 4°C. The urinary supernatant was discarded, and the remaining cell pellet was resuspended in 1.5 mL diethyl pyrocarbonate-treated phosphate-buffered saline and was then centrifuged at 13 000 g for 5 minutes at 4°C.

Microalbuminuria and macroalbuminuria were defined as abnormal increase in albumin excretion rate within the specific range of 30 mg to 299 mg and 300 mg and greater of albumin per of gram

of creatinine, respectively. The type 2 diabetic patients were classified as 3 groups according to the albuminuria condition: macroalbuminuria (DN), microalbuminuria, and normoalbuminuria.

RNA Extraction and Complementary DNA Synthesis

Total RNA was purified from cell pellet of the urine with the RNX Plus Kit (CinnaGen, Iran), which is a guanidine/phenol solution for total RNA isolation from homogenized sample. Through the action of guanidine salt in RNA isolation procedure, simultaneously DNA and protein were precipitated in phenol phase. Aqueous phase contains all types of high-quality genomic RNA. Two microgram of total RNA was digested by 2 µg of DNase 1 (Fermentas USA) to remove genomic DNA contamination, and then 1 µg of RNA was used for complementary DNA synthesis, with Precision qScript™ Reverse Transcription Kit (Primerdesign, UK). All the steps were done following the manufacturer's instructions. The RNA concentration and purity were confirmed using the relative absorbance ratio at 260/280 on a nanodrop 2000 (Thermo, Wilmington, USA).

Standard Curve Construction

Amplification efficiency for each primer pairs and relative probes was determined by the amplification

Table 1. Clinical Data of Patients With Diabetic Nephropathy, Type 2 Diabetes Mellitus, and Nondiabetic Nephropathy, Compared With a Healthy Control Group*

Characteristic	Patients			Control Group	P
	Diabetic Nephropathy	Diabetes Mellitus	Nondiabetic Nephropathy		
Number of participants	50	50	50	50	...
Age, y	60.10 ± 11.23	58.2 ± 9.8	58.4 ± 10.9	57.11 ± 1.97	...
Sex					
Male	32	22	35	25	
Female	18	28	15	25	< .05
Body mass index, kg/m ²	25.9 ± 3.4	28 ± 0.4	25.95 ± 0.37	25.59 ± 0.96	< .05
Glomerular filtration rate categories, mL/min					
< 15	24	0	28	0	
15 to 29	15	0	11	0	
30 to 89	11	0	11	0	
> 89	0	50	0	50	< .001
Glomerular filtration rate, mL/min	...	106.0 ± 20.2	...	114.1 ± 28.2	...
Fasting plasma glucose, mg/dL	240.0 ± 40.8	230.0 ± 32.5	90.0 ± 9.3	89.0 ± 9.8	< .001
Glycated hemoglobin, g/dL	7.5 ± 1.8	9.2 ± 1.8	5.0 ± 0.7	5.0 ± 0.5	< .001
Serum creatinine, mg/dL	8.3 ± 3.3	0.8 ± 0.2	9.1 ± 3.7	0.8 ± 0.2	< .001
Duration range of diabetes	1 mo to 20 y	10 to 20 y

*Values are mean ± standard deviation or frequency.

of a linear standard curve (from 0.24 ng to 1000 ng) of total complementary DNA assessed by ultraviolet spectrophotometer. Standard curves showed good linearity and amplification efficiency (100%) for each primer set of experimental *IRS2* gene and reference (*beta actin*) gene.

Real-time Reverse Transcript Polymerase Chain Reaction Analysis

All PCRs were performed using an ABI 7500/7500 fast real-time system (USA). For each sample, 500 ng/ μ L of total complementary DNA was used. Complementary DNA was mixed with 0.4 μ M of each forward and reverse primers and 0.25 μ M of each TaqMan probe (Table 2) with 10 μ L of TaqMan master mix (ABI, USA) to a final reaction volume of 20 μ L. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 minutes and 45 cycles at 95°C for 10 seconds and 60°C for 30 seconds and 72°C for 20 seconds. The experiments were performed with duplicates for each data point. Each sample was previously run with *beta actin* primers without reverse transcription to detect genomic DNA contamination; moreover, negative test controls were assayed in each reaction and for each primer set to detect DNA contamination of reagents. Using the $2^{-\Delta\Delta CT}$ method,²² the data were presented as the fold change in gene expression normalized to an endogenous reference gene (*beta actin*) and relative to the controls. Two-fold and more RNA expression considered as upregulation, between 0.5- and 2-fold as normal and 0.5-fold and less as downregulation.

Statistical Analyses

Statistical computations were performed using the SPSS software (Statistical Package for the Social

Table 2. Sequences of Designed Oligonucleotides Used as Real-time Polymerase Chain Reaction Primers and Probes*

Gene Name	Primer Sequence (5'-3')
IRS2	F: GGCCACCATCGTGAAAGAGT
	R: GCTGAAACAGTGCTGAGCGTC
	FAM ATCTGTCTGGCTTTATCACCAGGATGTCACA TAMRA
Beta actin	R:GCATTTGCGGTGGACGAT
	F:CAGCAGATGTGGATCAGCAAG
	FAM AGGAGTATGACGAGTCCGGCCCC TAMRA

*Primers and probe were designed using Primer express software version 3.0 and ordered to synthesis to Bioneer Company (South Korea).

Sciences, version 16.0, SPSS Inc, Chicago, IL, USA). The comparison of the data between patient and control groups was carried out using the analysis of variance test. The Student *t* test was performed for comparisons between the two groups. For all analyses, differences were accepted as statistical significant at a *P* less than .05. Numerical data are presented as mean \pm standard deviation.

RESULTS

Demographic Characteristics of Study Population

Table 1 summarizes the demographic, clinical, and biochemical data for the different groups of patients and controls. Except in the nephropathy groups with more male participants (*P* < .05), no other significant differences were observed in terms of age and sex among other patients and controls. The mean age of the patients (DN, type 2 DM, and nephropathy groups) were 60.10 ± 11.23 years in the DN group, 58.2 ± 9.8 years in the type 2 DM group, and 58.4 ± 10.9 in the nephropathy group, compared to 57.11 ± 9.97 years in the controls.

Body mass index was significantly higher in the diabetic group compared to the other study groups (*P* < .05). The differences in the routine biochemical parameters were as expected: creatinine and urea levels were higher in the nephropathy complication groups both with and without DM when compared to the other groups (*P* < .001). Glycated hemoglobin and fasting blood glucose levels were higher in both of the diabetic and DN compared to the nephropathy and control groups (*P* < .001). The duration of DM was 10 to 20 years in the type 2 DM group.

Urinary Sediment Cell *IRS2* mRNA Expression

As shown in Figure 1, the *IRS2* mRNA expression was dramatically higher in USCs of the DN patients compared with the diabetic patients and nondiabetic CKD and control groups (*P* < .001). The average expression of the *IRS2* in USCs of the DN patients was 8.5 ± 2.6 -fold higher than that in the control groups.

Urinary Sediment Cell *IRS2* mRNA Expression and Nephropathy

The type 2 diabetic patients consisted of 50 with macroalbuminuria (DN), 13 with microalbuminuria, and 50 with normoalbuminuria. The mean

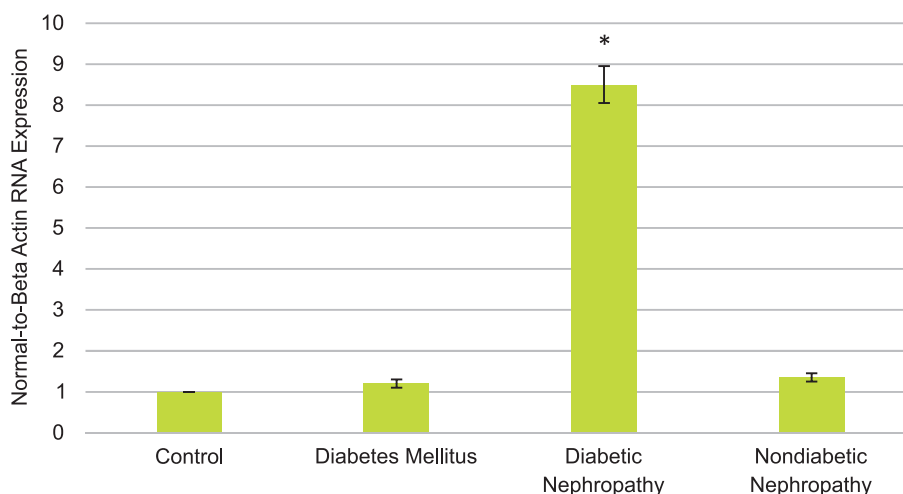


Figure 1. Real-time reverse transcript polymerase chain reaction analysis of urinary sediment cells *IRS2* mRNA expression in type 2 diabetes mellitus, diabetic nephropathy, and nondiabetic chronic kidney disease, compared with the controls. Results are expressed as fold number increase versus control assumed as $1 \pm$ standard deviation. The *IRS2* values were previously normalized to *beta actin* RNA values.

* $P < .001$

albumin-creatinine ratio as 620.44 ± 237.72 mg/g, 82.24 ± 50.76 mg/g, and 4.20 ± 2.17 mg/g in the macroalbuminuria, microalbuminuria, and normoalbuminuria among the diabetic patients, respectively. Similarly the nondiabetic CKD patients were categorized into 50 macroalbuminuria and 10 microalbuminuria patients with a mean albumin-creatinine ratio of 572.76 ± 203.3 mg/g and 76.99 ± 39.16 mg/g, respectively. The urinary albumin-creatinine ratio was 1.3 ± 0.3 mg/g in the control group. As shown in Figure 2 (top), the *IRS2* mRNA expression in nephropathy patients with hyperglycemia was significantly higher than their counterparts without hyperglycemia ($P < .001$). There were no significant differences in *IRS2* RNA expression in USCs between the patients with microalbuminuria and macroalbuminuria.

Figure 2 (bottom) shows patients with nephropathy and DN by GFR. The *IRS2* mRNA expression in USCs of the patients with DN in all stage groups were significantly higher than that in the patients with nephropathy with no sign of hyperglycemia and in the control group. There were no significant differences between stages 2 to 3 and stages 4 to 5 in USCs' *IRS2* mRNA expression; however, the end-stage group's USCs *IRS2* expression was lower ($P < .001$).

DISCUSSION

Our data indicated that *IRS2* expression was

significantly higher in USCs of the patients with DN compared with the patients with type 2 DM and nondiabetic CKD and the healthy controls. The USC's *IRS2* expression was at the normal range in those with type 2 DM and nondiabetic CKD. These data suggest that upregulation of *IRS2* expression detectable in USCs is a feature of diabetic kidney disease, and hyperglycemia may be the primary driver of increased *IRS2* levels.

Our data was in line with a study done by Hookman and colleagues¹⁷; they reported in a cohort of patients with DN and a range of CKD severity that *IRS2* mRNA levels were elevated approximately 9-fold in kidney tissue samples. They stated that *IRS2* was expressed in the kidney epithelium and might play a role in the downstream protective events triggered by BMP7 in the kidney.¹⁷ Our data showed that the expression of *IRS2* mRNA in USCs was about 8.5-fold higher in the patients with DN than the controls.

Two main IRS proteins widely expressed in humans are the *IRS1* and *IRS2*. Their central role is in the insulin signaling pathway. In a study reported by Lavin and coworkers, male mice lacking *IRS1* or *IRS2* represented elevated blood glucose or type 2 DM, respectively, but for reasons yet to be identified, female *IRS2* (-/-) mice did not develop type 2 DM.²¹ Our data in the present study showed that *IRS2* mRNA expression detectable in USCs was somehow at the normal range in

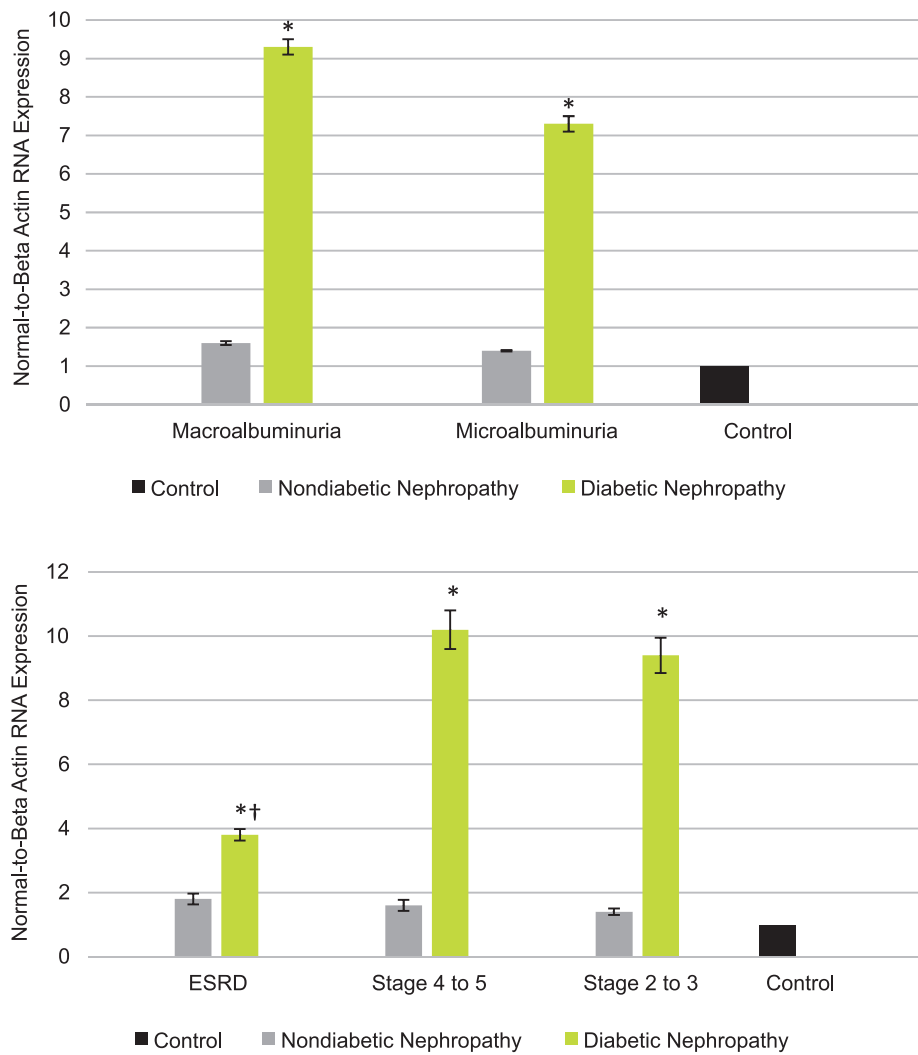


Figure 2. Comparison of urinary sediment cells *IRS2* mRNA expression in different stages of nephropathy among patients with and without hyperglycemia.
 * $P < .001$ compared with nephropathy and control groups
 † $P < .001$ compared with stage 2 to 3 and stage 4 to 5

type 2 diabetic patients, both male and female, compared with other study groups. It may be due to preservation of *IRS2* insulin signaling in renal tubules, while insulin signaling via *IRS1* is inhibited. Insulin signaling via *IRS2* continues to stimulate sodium reabsorption in the proximal tubule and causes sodium retention, edema, and hypertension. The *IRS1* signaling deficiency in the proximal tubule may impair *IRS1*-mediated inhibition of gluconeogenesis, which could induce hyperglycemia by preserving glucose production. In the glomerulus, the impairment of *IRS1* signaling deteriorates the structure and function of podocyte and endothelial cells, possibly causing DN.¹³

Our data showed that high expression of *IRS2*

mRNA detectable in USC was observed only in patients with DN. It was previously stated that *IRS2* expression in the kidney epithelium might play a role in the downstream protective events triggered by BMP7 in the kidney.¹⁷ An epistatic signaling pathway involving BMP7 and *IRS2* may exist in the developing kidney. The BMP7-mediated upregulation of *IRS2* may involve promoter upstream of the *IRS2* transcription start site. A series of conserved Smad4 transcription factor modules in the *IRS2* proximal promoter was identified by bioinformatics analysis in human, mouse, and rat. It was suggested that canonical pSmad1/5/8-to-Smad4 signaling may be involved in BMP7-mediated regulation of *IRS2* transcription.

The IRS2 is phosphorylated on tyrosine residues in response to insulin and insulin-like growth factor-1 as well as other growth factors.¹⁷ Upregulation of IRS2 levels in the diabetic kidney may be in part a protective mechanism to enhance insulin-mediated cell survival, or to limit the tubular epithelial cell function damages induced by glucose. As shown in Figure 2 (bottom), the expression of *IRS2* in end-stage renal disease, when the kidney function is highly damaged, was significantly decreased compared with other nephropathy stages. This may be due to tubular epithelial cells failure to limit the glucose-induced damages.

As DN is a progressive disease from inflammation to fibrosis, the main obstacle in the management of DN patients is the absence of early clinical signs before the kidney enters an irreversible dysfunction stage. The most common biomarkers for CKD are serum creatinine, urea nitrogen, and proteinuria. However, those biomarkers are influenced by many factors such as patient's age, diet, and infection conditions, especially those that could not accurately reflect the severity of renal fibrosis in early stages.²³ In the past few years, with the development of reliable RNA extraction techniques from urinary sediment and real-time quantitative RT-PCR, measurement of mRNA expression in urinary sediment has become an emerging tool for the study of kidney diseases.

Expression of *IRS2*, as our data showed, was upregulated in DN patients regardless of albumin excretion. This expression was not dependent on stages of the disease. In other words, it can be determined even at early stages of the disease. This upregulation may be considered as a candidate of nephropathy detection in diabetic patients.

Further experiments are required to fully elucidate the role of *IRS2* in the pathogenesis of diabetic kidney disease. As the regulation of *IRS2* was altered in almost all the stages of DN, our data suggest that *IRS2* may serve as a novel biomarker for DN. Future studies involving larger numbers of patients will confirm this hypothesis.

CONCLUSIONS

We can conclude that specific upregulation of *IRS2* in the kidney tubules of DN patients, detectable in USCs even at early stages of the disease, indicates a novel role for *IRS2* as a marker or mediator of human DN progression. Furthermore, this

phenomenon may propose *IRS2* RNA expression in USCs as a potential noninvasive biomarker for detection of nephropathy in diabetic patients.

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CONFLICT OF INTEREST

None declared.

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