Hyperinsulinemia contributes to impaired-glucose-tolerance-induced renal injury *via* mir-7977/SIRT3 signaling

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Abstract

Background: Increasing evidence indicates that impaired glucose tolerance (IGT) is independently associated with chronic kidney disease, but the characteristics and underlying mechanisms remain largely unknown.

Methods: Here, the cross-sectional study was performed to study the characteristics of IGT-induced renal injury (IGT-RI). Furthermore, urine microRNA profile was evaluated and microRNAs involved in tubular injury were determined by *in-vitro* experiments.

Results: It was found that 12.1% of IGT patients had microalbuminuria, which we termed "IGT-RI." Overall, 100% of patients with IGT-RI exhibited reabsorption dysfunction and 58.3% had structural damage in the renal tubules. Two-hour postprandial insulin, retinol-binding protein, and N-acetyl-β-glucosaminidase were significantly associated with microalbuminuria and they were independent risk factors for IGT-RI. The expression of mir-7977 was altered in IGT-RI patients and may be involved in cellular response to oxidative stress. In proximal tubule epithelial cells *in vitro*, a high level of insulin increased the expression of mir-7977 and decreased that of sirtuin 3 (SIRT3), leading to oxidative stress. Overexpression of mir-7977 further decreased SIRT3 expression, whereas inhibition of mir-7977 had the opposite effect. Furthermore, mir-7977 can bind to the 3′-untranslated region of *SIRT3* mRNA and inhibit its expression. Moreover, inhibition of SIRT3 reduced the expression of cubilin and the endocytosis of albumin.

Conclusions: In conclusion, IGT-RI mainly manifests as tubular injury, especially reabsorption dysfunction. Compensatory hyperinsulinemia may be involved. A high level of insulin can activate mir-7977/SIRT3 signaling, resulting in tubular injury by inducing oxidative stress as well as reabsorption dysfunction by inhibiting the expression of cubilin, ultimately contributing to IGT-RI.

Keywords: hyperinsulinemia, impaired glucose tolerance, microRNA, renal injury

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Introduction

With a rapid increase in the prevalence of diabetes mellitus, diabetic kidney disease (DKD) has become the leading cause of end-stage renal disease (ESRD).[1] Therefore, the early prevention and treatment of DKD is of great importance. Microalbuminuria (MAU) is the main clinical criterion for early detection of DKD, but even after active treatment, a large proportion of patients will gradually develop into macroalbuminuria and even ESRD.[2] We also found that a series of pathophysiological changes including renal hypertrophy, abnormal blood pressure and renal tubular dysfunction have occurred long

before MAU in patients with type 2 diabetes mellitus (T2DM).[3] Thus it is essential to find effective biomarkers for the early prediction and diagnosis of the initiation of DKD.

T2DM is a gradual development process from normal glucose tolerance, impaired glucose tolerance (IGT) to hyperglycemia. Patients with prediabetes might have obvious pathophysiological abnormalities, such as insulin resistance and hyperinsulinemia. Although the compensatory elevated insulin is helpful to control blood glucose, it may be harmful to the heart, kidneys, and other tissues. Increasing evidence indicates that IGT is independently associated with DKD.[4,5] IGT might have harmful effects on the kidneys[6] and can increase the risk of developing chronic kidney disease.[7] In addition, it has been found that MAU occurs in 13.1% of IGT patients.[8] The Australian Diabetes, Obesity, and Lifestyle Study (AusDiab) reported a MAU prevalence of 11.1% in IGT patients.[9] Otsuka Long Evans Tokushima Fatty (OLETF) rats are an ideal model for studying type 2 DKD. Using electron microscopy, we have demonstrated that the main presentation of renal injury (RI) in the IGT stage in OLETF rats is tubular injury, manifested as follows: the array of tubular epithelial cells is irregular, brush-border tubular epithelial cells are shed off, and inflammatory cells infiltrate the tubulointerstitium.[10] However, further research is needed to explore the exact pathogenesis and specific biomarkers for early diagnosis of IGT-induced RI (IGT-RI).

MicroRNAs are endogenously produced short noncoding RNAs, 21–25 nucleotides in length.[11] By binding to the 3′-untranslated region of target mRNAs, microRNAs can induce mRNA degradation or, more frequently, repress protein translation.[12] Multiple miRNAs participate in the pathogenesis of DKD and can be used as potential biomarkers for early diagnosis of DKD, with significantly higher sensitivity and specificity than 24-h urinary microalbumin (UMA), estimated glomerular filtration rate (eGFR), and other indicators.[13] However, whether microRNAs can be used for effective diagnosis of IGT-RI and how microRNAs participate in this disorder are largely unknown.

Studies have indicated that sirtuin 3 (SIRT3) is closely related to insulin resistance and hyperinsulinemia.[14,15] Moreover, SIRT3 has a protective effect in DKD,[16] and it protects renal tubules from oxidative stress and high glucose-induced RI.[17] However, it is unknown whether SIRT3 involved in IGT-RI.

Therefore, in this research, first, we studied the characteristics and risk factors of renal injury in IGT patients; then, we setup the profile of urine microRNAs and explored the hyperinsulinemia associated mechanism underlying IGT-RI.

Materials and methods

Study population

This was a cross-sectional study. In total, 284 subjects who underwent annual physical examination in Tianjin Haibin People's Hospital (Tianjin, China) between March 2016 and August 2016 were included in our study, including 146 healthy subjects, 99 patients with IGT, 20 patients with T2DM and 19 patients with DKD. The exclusion criteria were: patients with a history of kidney disease, such as chronic glomerulonephritis, IgA nephropathy, lupus nephritis, polycystic kidney disease, hypertensive nephropathy, or gout-associated nephropathy; patients with cancer, severe cardiovascular disease, liver and kidney dysfunction, mental disorder, autoimmune disease, and acute diabetic complications, such as ketoacidosis, abnormal glucose tolerance caused by other endocrine system disease or exogenous hormones, urinary infection and hematuria, poorly controlled hypertension, fever, or extreme physical activity; pregnant, lactating, and menstruating women; patients aged >70 or <30 years old; and patients with type 1 diabetes mellitus. None of the IGT patients treated with medications. IGT and T2DM were defined according to American Diabetes Association's guidelines.[18] DKD was defined as eGFR $\geqslant 90\,ml/min/1.73\,m^2$ and $30\,mg/24\,h \leqslant UMA < 300\,mg/24\,h$.[19] IGT-RI was defined as eGFR $\geqslant 90\,ml/min/1.73\,m^2$ and $30\,mg/24\,h \leqslant UMA < 300\,mg/24\,h$ in IGT patients.

Data collection

Blood samples were drawn from all patients after a 12-h overnight fast. Then, 24-h urine samples and first-morning midstream urine samples were collected. Biochemical indicators were detected using an automatic biochemical analyzer. The CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) formula[20] was used to calculate the eGFR, 24-h UMA was measured by immunoturbidimetry, retinol-binding protein (RBP)

was measured by immunity transmission turbidity, N-acetyl-β-glucosaminidase (NAG) was measured by an MNP-G1CNAC substrate method, β2-microglobulin (β2-MG) was measured by latex immunoturbidimetry, and α-galactosidase (GAL) was measured by a CNP-GAL method. The reference ranges determined by the manufacturers of the kits for RBP (Beijia, Shanghai, China), NAG (Gcell, Beijing, China), GAL (Huili, Jilin, China), and β2-MG (Gcell) are 0–0.7 mg/l, 0.3–12 U/l, 0–15 U/l, and 0–0.3 mg/l, respectively. Abnormal elevation of β2-MG or RBP was regarded as reabsorption dysfunction and abnormal elevation of NAG or GAL was regarded as structural damage in renal tubular epithelial cells. All specimens were tested in the Department of Clinical Laboratory at Tianjin Medical University Chu Hsien-I Memorial Hospital. Informed consent was obtained from all participants. Ethical approval was given by the medical ethics committee of Tianjin Medical University Chu Hsien-I Memorial Hospital with the following reference number: DXBYYhMEC2016-6.

Microarray analysis and qPCR verification

Urine microRNAs were extracted using an miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) and detected by Agilent miRNA microarray analysis conducted by Shanghai Biotechnology Corporation (Tianjin, Shanghai). We analyzed two samples per patient group, and each sample was a mixture of samples from five patients. The mean values of each group were compared and a fold change of $\geqslant 2$ or $\leqslant 0.5$ was considered differential expression. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses were used to study the potential signaling pathways of differentially expressed microRNAs. For each patient group, 20 differentially expressed microRNAs were verified by polymerase chain reaction (PCR) using a Taqman MicroRNA Reverse Transcription Kit, Taqman Universal PCR Master Mix, and a probe (all from ABI, Carlsbad, CA, USA). The primers used in this study were synthetized by GenScript Corp (Sangon, Shanghai, China), and their sequences are listed in Supplementary Table 1. MiRNA levels were normalized to those of cel-miR-39 in urine and U6 in cells, and mRNA expression was normalized to that of GAPDH. The $2^{-\Delta\Delta Ct}$ method was used for quantification.

Cell culture

HK-2 human renal tubular epithelial cells (American Type Culture Collection, Rockville, MD) were cultured in DMEM/F12 (Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C in 5% CO_2. Cells were stimulated with insulin (Novo Nordisk, Copenhagen, Denmark) at 0 ng/ml (used as a blank control), 5 ng/ml (physiological concentration), or 50 ng/ml (high concentration) for 24 h.[21] Cells were treated with mimics control, miR-7977 mimics, inhibitor control and miR-7977 inhibitor (GenePharma, Shanghai, China), or transfected with siRNA control and sirtuin 3 (SIRT3) siRNA (RiboBio, Guangzhou, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Luciferase reporter assay

A luciferase reporter assay was conducted using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). HEK-293T cells (1.5×10^4/well) were seeded into 96-well plates (BIOFIL, Guangzhou, China). After 12 h, the cells were transiently cotransfected with pRL-TK plasmid (Promega) containing a Renilla luciferase gene for internal normalization and various constructs containing pMIR-SIRT3 (Promega) and pMIR-SIRT3-mut (Vazyme Biotech Co., Ltd., Nanjing, China). Cells were lysed and assayed for luciferase activity using a Dual-Luciferase Reporter Assay System (Promega) 36 h after transfection. One hundred microliters of protein extracts were analyzed in a luminometer.

Western blotting

Total proteins from HK-2 cells were isolated, separated by polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene fluoride membrane (Millipore, Boston, MA). The membrane was incubated overnight with primary antibodies against sirtuin 3 (SIRT3; diluted to 1:1000, Cell Signaling Technology), super oxide dismutase (SOD2), acetyl k68-SOD2 (Ac-SOD2), and cubilin (all diluted to 1:1000, Abcam). Primary antibody diluent was purchased from Solarbio (Beijing, China). Following incubation with secondary anti-mouse/rabbit antibody (Sungene Biotech, Tianjin, China), protein bands were visualized using electrochemiluminescence (ECL) blotting detection reagent (Advansta, Menlo Park,

CA, USA). Immunocomplexes were quantified *via* densitometry using Image J software.

Immunofluorescence

HK-2 cells were washed twice with PBS for 5 min each. The cells were fixed with 4% paraformaldehyde for 15 min, blocked with goat serum for 1 h, and incubated overnight with primary antibodies against SIRT3 and cubilin (Biorbyt, Cambridge, UK). Then, the slides were incubated with goat anti-rabbit IgG/FITC (1:100; Proteintech, China). Nuclei were stained with DAPI (Solarbio). Fluorescence was captured using a Leica DMI4000 B automated inverted microscope equipped with a Leica DFC300 FX camera (Leica, Wetzlar, Germany) and evaluated using Image-Pro Plus 6.0 analysis software (Media Cybernetics, Rockville, MD, USA).

Detection of reactive oxygen species

Reactive oxygen species (ROS) were detected according to the instructions using a H2DCFDA ROS probe (KeyGen Biotech, Jiangsu, China). The fluorescence intensity of each picture was determined in three arbitrarily selected regions using Image-Pro Plus 6.0 analysis software. The ROS data were normalized by cell number. Each green dot in the picture represents a cell.

Endocytosis assay of HK-2 cells

HK-2 cells were seeded into 24-well plates (BIOFIL, Guangzhou, China) and treated with tetramethylrhodamine isothiocyanate bovine-lyophilized powder albumin (TRITC-albumin; Sigma-Aldrich) at 500 μg/ml. The plate was incubated in the dark for 24 h. Albumin uptake by the cells was observed under a laser confocal microscope and evaluated using Image-Pro Plus software (Version X; Media Cybernetics, Silver Springs, MD, USA). Albumin endocytosis was normalized to the total cell number.

Statistical analyses

SPSS19.0 (SPSS, Chicago, IL, USA) statistical software was used for statistical analysis. Quantitative data with a normal distribution are expressed as the mean ± standard deviation (SD). Quantitative data with a non-normal distribution are expressed as the median (first quartile, third quartile). One-factor analysis of variance was used to analyze differences between groups for data with a normal distribution, whereas non-parametric tests were used to analyze data with non-normal distribution. Spearman's correlation was used to evaluate correlations among indicators. Multivariate logistic regression was employed to predict independent risk factors. Power Analysis and Sample Size software (PASS 15, Kaysville, Utah, USA) was used to estimate the power of sample size in the study. A value of $p < 0.05$ was considered statistically significant.

Results

General characteristics of IGT-RI patients

In total, 12.1% (12/99) of IGT patients had microalbuminuria, which we termed "IGT-RI". General characteristics of the five study groups, including 146 NC subjects, 87 patients with IGT, 12 patients with IGT-RI, 20 patients with T2DM and 19 patients with DKD are shown in Table 1. No significant differences in age, gender, body mass index (BMI), waist-to-hip ratio, blood pressure and eGFR were observed in the IGT-RI group compared with the IGT group. The levels of fasting plasma glucose and insulin were not significantly different between the IGT and IGT-RI groups. In addition, in IGT-RI group, 2-h postprandial glucose did not differ from that in the IGT group, whereas 2-h postprandial insulin was nearly 47% higher than that in the IGT group ($p < 0.05$). Serum lipids, uric acid, and creatinine were not significantly different between the IGT and IGT-RI groups. In the T2DM group, triglyceride levels were higher than those in the NC, IGT, and IGT-RI groups ($p < 0.05$). In the DKD group, systolic blood pressure, diastolic blood pressure, serum uric acid, and triglyceride were higher than those in the NC group ($p < 0.05$).

The prevalence of tubular injury is increased in IGT-RI patients

UMA, NAG, RBP, GAL, and β2-MG increased gradually in the five study groups. In the IGT-RI group, UMA, NAG, RBP, and GAL levels were higher than those in the NC and IGT groups ($P < 0.05$) (Table 2). Among the 12 IGT-RI patients, NAG was abnormally elevated in six patients and GAL was elevated in one patient, indicating that 58.3% (7/12) of the patients had structural damage. RBP was elevated in 100% of

Table 1. General characteristics in IGT-RI patients.

	NC $n=146$	IGT $n=87$	IGT-RI $n=12$	T2DM $n=20$	DKD $n=19$	*p* value
Age (year)	46.23 ± 7.74	47.33 ± 7.27	45.94 ± 7.43	47.70 ± 8.9	52.11 ± 10.78	0.217
Male/female (case)	66/80	28/59	5/7	8/12	10/9	0.103
Height (cm)	166.31 ± 7.85	164.77 ± 7.01	158.58 ± 35.55	164.65 ± 9.14	168.22 ± 6.74	0.076
Weight (kg)	69.71 ± 15.56	69.34 ± 14.57	69.73 ± 14.72	71.55 ± 17.04	73.33 ± 7.31	0.920
BMI (kg/cm^2)	25.06 ± 4.26	25.40 ± 4.20	25.83 ± 3.49	26.18 ± 4.67	25.96 ± 2.77	0.796
Waist/hip	0.86 ± 0.09	0.86 ± 0.05	0.88 ± 0.06	0.90 ± 0.05	0.91 ± 0.04	0.22
SBP (mmHg)	127.50 ± 15.73	126.99 ± 22.45	129.00 ± 14.28	141.80 ± 19.03	145.64 ± 24.34*#	0.010
DBP (mmHg)	82.16 ± 11.46	84.09 ± 14.74	87.08 ± 11.09	90.60 ± 7.89	93.4 ± 10.91*△	0.028
FPG (mmol/l)	5.34 ± 0.49	5.54 ± 0.52	5.59 ± 0.55	6.66 ± 1.06*#△	11.83 ± 3.80*#△$	0.000
FINS (U/l)	9.38 ± 5.83	10.40 ± 7.14	10.38 ± 5.07	15.49 ± 10.58*#△	10.46 ± 7.00$	0.007
2hPG (mmol/l)	6.13 ± 0.79	8.52 ± 0.99*	8.52 ± 0.82*	12.72 ± 1.23*#△	12.53 ± 1.30*#△	0.000
2hPINS (U/l)	46.81 ± 23.37	66.82 ± 14.57	97.97 ± 22.84*#	86.60 ± 37.58*#	42.05 ± 16.09△$	0.000
ALT (U/l)	20.87 ± 11.08	23.46 ± 12.62	23.17 ± 11.57	23.91 ± 14.58	18.41 ± 8.97	0.670
AST (U/l)	18.85 ± 6.04	19.52 ± 6.36	18.39 ± 5.04	21.27 ± 10.27	17.00 ± 6.90	0.491
BUN (mmol/l)	58.77 ± 14.27	56.87 ± 18.64	52.35 ± 11.32	59.49 ± 16.29	61.23 ± 13.45	0.893
SUA (umol/l)	296.83 ± 90.48	308.90 ± 90.91	306.32 ± 112.15	349.66 ± 104.94	365.44 ± 86.76*	0.048
SCr (umol/l)	58.77 ± 14.27	55.58 ± 14.57	54.48 ± 11.63	59.49 ± 16.29	61.23 ± 13.45	0.403
eGFR (ml/min/1.73m^{-2})	125.08 ± 30.24	134.12 ± 36.35	136.54 ± 37.18*	124.96 ± 37.13	119.12 ± 34.32	0.049
TG (mmol/l)	1.49 ± 1.01	1.64 ± 0.90	1.82 ± 0.91	2.10 ± 1.20*#△	2.50 ± 1.08*#△	0.02
TC (mmol/l)	4.16 ± 0.95	4.83 ± 0.92	4.71 ± 1.02	4.95 ± 0.85	5.14 ± 1.29	0.591
LDL-C (mmol/l)	2.72 ± 0.73	2.92 ± 0.73	2.72 ± 0.72	2.86 ± 0.67	3.08 ± 1.12	0.811
HDL-C (mmol/l)	1.21 ± 0.34	1.19 ± 0.27	1.08 ± 0.22	1.16 ± 0.23	1.08 ± 1.38	0.506

Data are expressed as mean ± SD. 2hPINS, 2 h postprandial insulin; 2hPG, 2 h postprandial glucose; ALT, alanine aminotransferase; AST, aspartate transaminase; BMI, body mass index; BUN, blood urea nitrogen; DBP, diastolic blood pressure; DKD, diabetic kidney disease; eGFR, estimate glomerular filtration rate; FINS, fasting insulin; FPG, fasting plasma glucose; HDL-C, high density lipoprotein cholesterol; IGT, impaired glucose tolerance; IGT-RI, IGT-renal injury; LDL-C, low density lipoprotein cholesterol; NC, normal control; SBP, systolic blood pressure; SCr, serum creatinine; SUA, serum uric acid; T2DM, type 2 diabetes mellitus; TC, total cholesterol; TG, triglyceride.
*: compared with NC, $p<0.05$; #: compared with IGT, $p<0.05$; △: compared with IGT-RI, $p<0.05$; $: compared with T2DM, $p<0.05$. The 2hPG and 2hPINS were detected after a 75-g oral glucose tolerance test.

IGT-RI patients, indicating that reabsorption dysfunction existed in all patients (Figure 1a), but β2-MG was normal in all patients. Among the other 87 IGT patients, NAG was abnormally elevated in 20 patients and GAL was elevated in two patients, indicating that 25.3% (22/87) of these

Table 2. Tubular injury in IGT-RI patients.

	NC	IGT	IGT-RI	T2DM	DKD	*p* value
UMA	5.90 (2.90, 11.15)	14.30 (7.10, 19.95) *	49.5 (33.7, 64.60)*#	19.30 (1.70, 16.60)*	89.40 (71.23, 193.48)*#△$	0.000
NAG	5.70 (3.20, 8.30)	7.50 (4.25, 11.50)*	21.80 (9.52, 26.80)*#	10.10 (8.35, 16.30)*	24.009 (14.27, 30.85)*#△$	0.000
RBP	0.30 (0.14, 0.46)	0.78 (0.35, 1.01)*	1.47 (1.30, 1.89)*#	0.94 (0.76, 1.13) *	1.80 (0.38,8.45)*#$	0.000
GAL	2.40 (1.20, 4.50)	2.30 (1.20, 4.65)	3.75 (2.40, 9.62)*#	3.00 (1.90, 4.40) *	5.50 (3.65, 9.10)*#△$	0.000
β2-MG	0.06 (0.03, 0.10)	0.03 (0.00, 0.08)	0.05 (0.03, 0.09)	0.04 (0.01, 0.08)	0.30 (0.10, 2.98)*#△$	0.001

Data are expressed as median (first quartile, third quartile).
*: compared with NC, $p < 0.05$; #: compared with IGT, $p < 0.05$; △: compared with IGT-RI, $p < 0.05$; $: compared with T2DM, $p < 0.05$.
β2-MG, β2-microglobulin; GAL, α–galactosidase; NAG, N-acetyl-β-glucosaminidase; RBP, retinol binding protein; UMA, urine microalbumin; NC, normal control; IGT, impaired glucose tolerance; IGT-RI, IGT-renal injury; T2DM, type 2 diabetes mellitus; DKD, diabetic kidney disease.

patients had structural damage. RBP was elevated in 47 patients, and β2-MG was elevated in one patient who had elevated RBP, indicating that 54.0% (47/87) of these patients had reabsorption dysfunction. Seventeen of these patients (19.5%) had both structural damage and reabsorption dysfunction (Figure 1b).

Two-hour postprandial insulin and tubular injury correlate with UMA and contribute to IGT-RI

In the NC, IGT, IGT-RI groups, UMA was significantly correlated with RBP ($r = 0.720$, $p < 0.001$) and NAG ($r = 0.619$, $p < 0.001$), but not with GAL ($r = 0.030$, $p = 0.651$) and β2-MG ($r = 0.064$, $p = 0.341$; Figure 1c–f). Two-hour postprandial insulin was significantly correlated with UMA ($r = 0.609$, $p < 0.001$; Figure 1g). In addition, 2-h postprandial insulin was significantly correlated with RBP ($r = 0.498$, $p < 0.001$) and NAG ($r = 0.340$, $p < 0.001$), but not with GAL ($r = 0.035$, $p = 0.699$) and β2-MG ($r = 0.034$, $p = 0.613$; Figure 1h–k). The independent risk factors of IGT-RI were 2-h postprandial insulin (odds ratio [OR] 1.035, 95% confidence interval [CI]: 1.007–1.064, $p = 0.014$), RBP (OR 11.682, 95% CI: 1.637–83.350, $p = 0.014$), NAG (OR 1.195, 95% CI: 1.030–1.386, $p = 0.019$; Table 3).

Urine microRNA profiling of IGT-RI patients

Microarray analysis (GEO accession number: GSE121221) revealed that four miRNAs, including mir-7977, mir-3162-3p, mir-7975, and mir-5100, were significantly upregulated, and three miRNAs, including mir-193b-5p, mir-1275, and mir-211-3p, were significantly downregulated in the IGT-RI group compared to the NC group (Figure 2a, b). Using KEGG pathway enrichment analysis, we concluded that the differentially expressed microRNAs are involved in the regulation of insulin signaling (Figure 2c). In addition, these microRNAs may regulate the response to oxidative stress according to GO enrichment analysis (Supplementary Table 2). As mir-7977 was the most abundant and showed the strongest increase in expression in IGT-RI based on the microarray data, we verified this microRNA by TaqMan quantitative polymerase chain reaction (qPCR) in 20 patients per group. Mir-7977 expression findings were consistent with the microRNA profiling data (Figure 2d).

At a high level, insulin activates mir-7977/SIRT3/SOD2/ROS in HK-2 cells

Based on a TargetScan database search, we discovered that SIRT3 may be a target gene of mir-7977. SIRT3 reduces ROS and thus, oxidative stress, by directly deacetylating SOD2. Therefore, we assumed that a high level of insulin may induce renal tubular injury through mir-7977/SIRT3/SOD2/ROS signaling. To test this hypothesis, we treated HK-2 cells with 0 ng/ml, 5 ng/ml (physiological level), or 50 ng/ml (high level) insulin. Mir-7977 expression was significantly elevated by approximately 2.5 times in the high-level group compared with the other two groups ($p < 0.05$) (Figure 3a). In addition, SIRT3 expression was significantly reduced in the high-level group compared with the other groups as indicated by qPCR, immunofluorescence staining, and

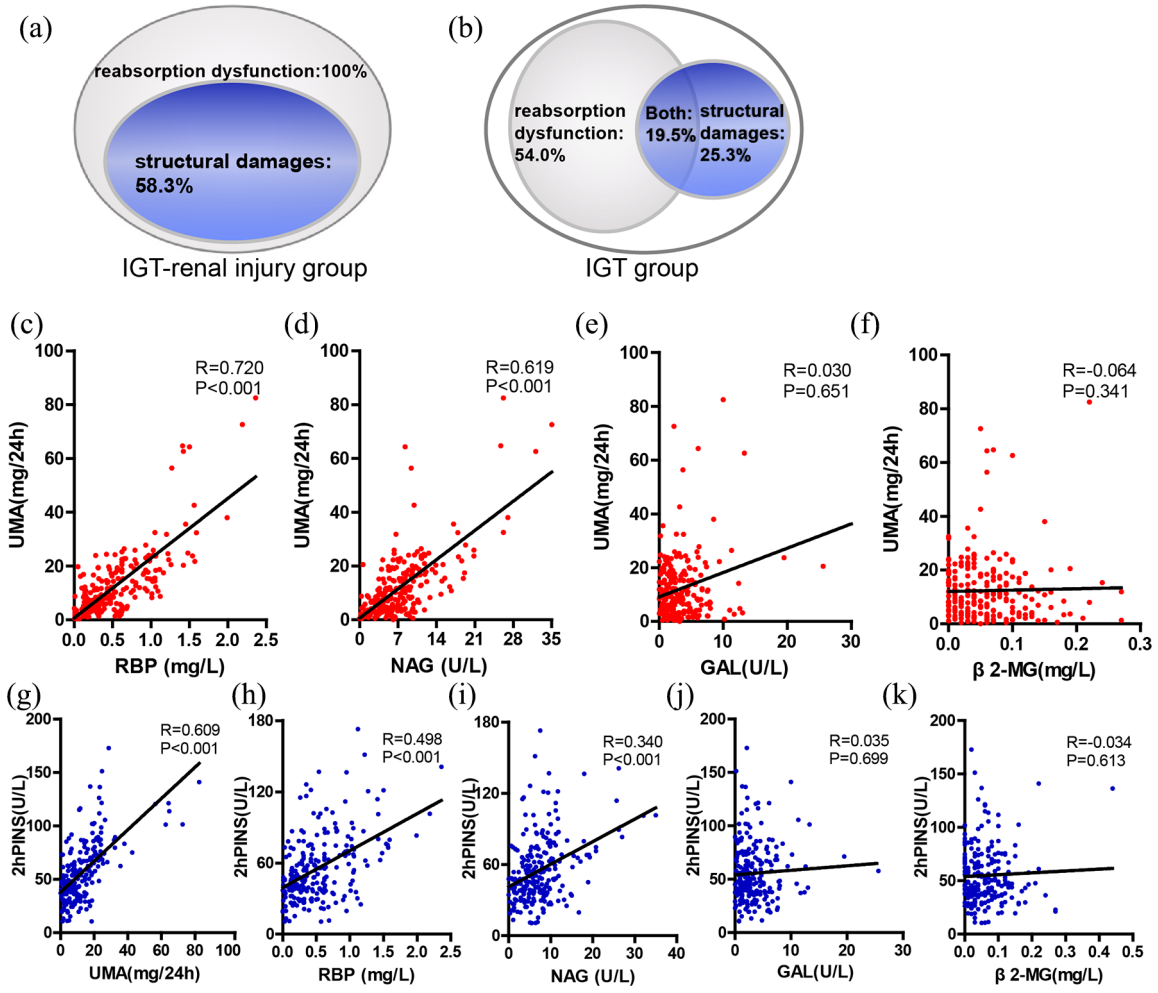

Figure 1. Correlation between renal injury and 2-h postprandial insulin. (a) Rate of RI in the IGT-RI group. (b) Rate of RI in the IGT group. (c) Correlation between UMA and RBP. (d) Correlation between UMA and NAG. (e) Correlation between UMA and GAL. (f) Correlation between UMA and β2-MG. (g) Correlation between 2hPINS and UMA. (h) Correlation between 2hPINS and RBP. (i) Correlation between 2hPINS and NAG. (j) Correlation between 2hPINS and GAL. (k) Correlation between 2hPINS and β2-MG.
2hPINS, 2-h postprandial insulin; β2-MG, β2-microglobulin; GAL, α-galactosidase; NAG, N-acetyl-β-glucosaminidase; RBP, retinol-binding protein; UMA, urine microalbumin.

Table 3. Risk factors of IGT-RI.

	B	Wald	*p* value	OR	95%CI
2hPINS(U/l)	0.034	6.042	0.014	1.035	1.007–1.064
RBP (mg/l)	2.458	6.011	0.014	11.682	1.637–83.350
NAG (U/l)	0.178	5.500	0.019	1.195	1.030–1.386

B, regression coefficient; Wald, chi-square value; OR, odds ratio; 95%CI, 95% confidence interval; 2hPINS, 2 h postprandial insulin; NAG, N-acetyl-β-glucosaminidase; RBP, retinol binding protein.

western blotting (Figure 3b–e). The ratio of Ac-SOD2 to SOD2 was significantly elevated after treatment with 50 ng/ml insulin (Figure 3d, f). ROS were significantly increased after treatment with 50 ng/ml insulin as indicated by an H2DCFDA assay (Figure 3g, h).

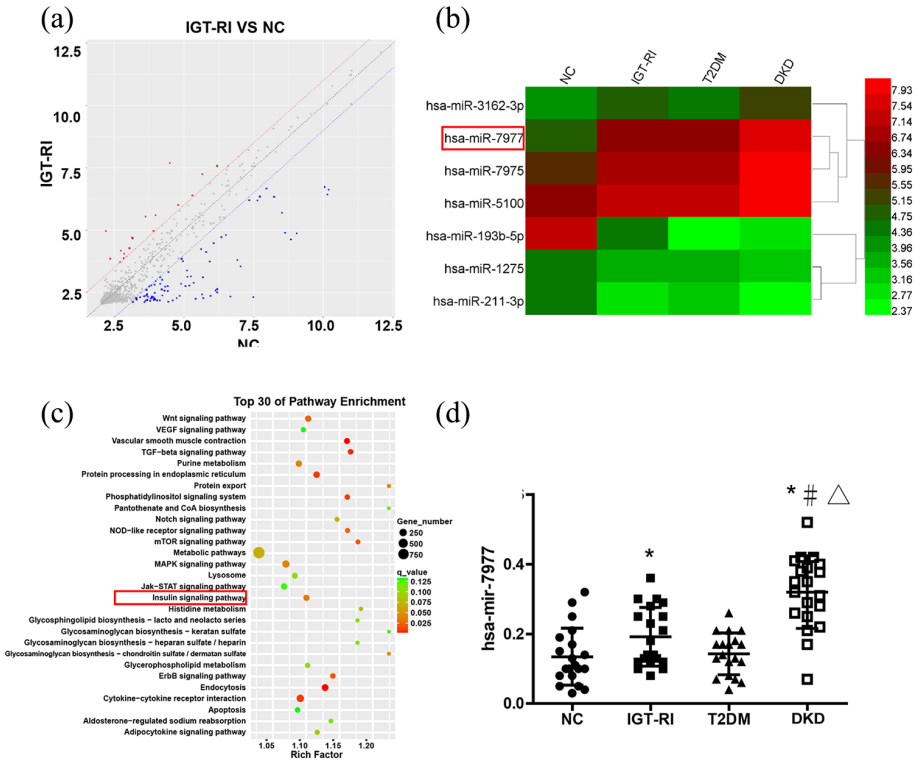

Figure 2. Urine microRNA profiles of the different study groups. (a) Scatter plot of urine microRNA array data. (b) Heatmap of urine microRNA array data. (c) KEGG pathway enrichment analysis of microRNA array data. (d) quantitative polymerase chain reaction results of miR-7977 expression in urine. Data are expressed as mean ± SD. *$p < 0.05$ compared with NC, #$p < 0.05$ compared with IGT-RI, △$p < 0.05$ compared with T2DM.
NC, normal control; IGT-RI, IGT-renal injury; T2DM, type 2 diabetes mellitus; DKD, diabetic kidney disease.

MiR-7977 is involved in SIRT3/SOD2/ROS signaling in HK-2 cells

To study the potential role of miR-7977 in SIRT3/SOD2/ROS signaling, we first overexpressed miR-7977 in HK-2 cells, using miR-7977 mimics. Overexpression of miR-7977 suppressed the expression of SIRT3 ($p < 0.05$), as indicated by immunofluorescence staining and western blotting (Figure 4a–d). In addition, overexpression of miR-7977 increased the ratio of Ac-SOD2 to SOD2 and the ROS level (Figure 4c–g; $p < 0.05$). Next, HK-2 cells were treated with miR-7977 inhibitor to suppress its expression. Compared with the inhibitor control, miR-7977 inhibitor significantly upregulated the expression of SIRT3 and reduced the ratio of Ac-SOD2 to SOD2 and ROS ($p < 0.05$; Figure 5a–g).

Mir-7977 directly targets SIRT3

We conducted a luciferase reporter assay to evaluate whether SIRT3 is a direct target of miR-7977. We constructed reporters carrying either human SIRT3 with the wild-type 3′-UTR or a mutant with four nucleotides swapped in the region corresponding to the miR-7977 seed. When cotransfected with synthetic miR-7977 mimics into HEK-293T cells, the wild-type reporter exhibited reduced luciferase activity, whereas the mutant reporter did not (Figure 5h, i). These results demonstrated that SIRT3 is a direct target of miR-7977.

At a high level, insulin reduces the expression of cubilin and endocytosis of albumin in HK-2 cells

Given that IGT patients mainly exhibited reabsorption dysfunction in tubules, we assumed that MAU may be a consequence of tubular reabsorption dysfunction in these patients. Cubilin is a key transporter protein for reabsorption of albumin by renal tubular epithelial cells. Cubilin mRNA and protein levels were lower in HK-2 cells treated with 50 ng/ml insulin than in cells treated with 5 ng/ml insulin ($p < 0.05$;

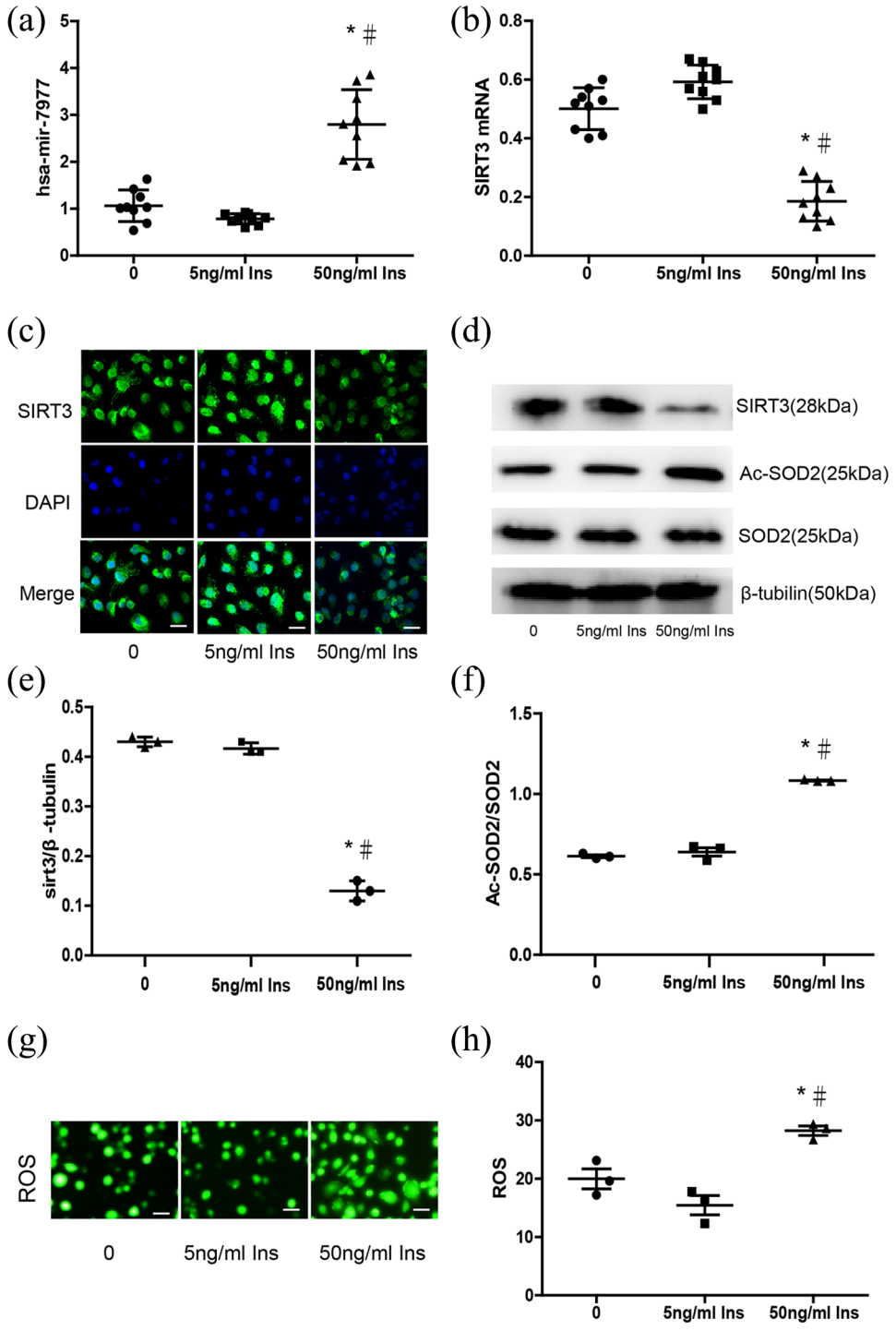

Figure 3. Effects of insulin on the mir-7977/SIRT3/SOD2/ROS signal pathway. (a) qPCR results of miR-7977 in HK-2 cells after treatment with the indicated concentrations of insulin for 24 h. (b) qPCR results of SIRT3 mRNA in HK-2 cells after treatment with the indicated concentrations of insulin for 24 h. (c) Immunofluorescence staining of SIRT3 in HK-2 cells. (d) Western blot analysis of SIRT3, Ac-SOD2, and SOD2 in HK-2 cells. (e) Quantification of SIRT3 protein expression in d. (f) Quantification of Ac-SOD2/SOD2 protein expression in HK-2 cells. (g) Level of ROS in HK-2 cells after treatment with the indicated concentrations of insulin for 24 h. (h) Quantification of results in g. The scale bar represents 20 μm. Data are expressed as mean ±SD.
*$p < 0.05$ compared with blank control group, #$p < 0.05$ compared with 5 ng/ml insulin. All data are representative of three independent experiments.
Ac-SOD2, acetyl k68 SOD2; Ins, insulin; qPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; SIRT3, sirtuin 3; SOD2, super oxide dismutase.

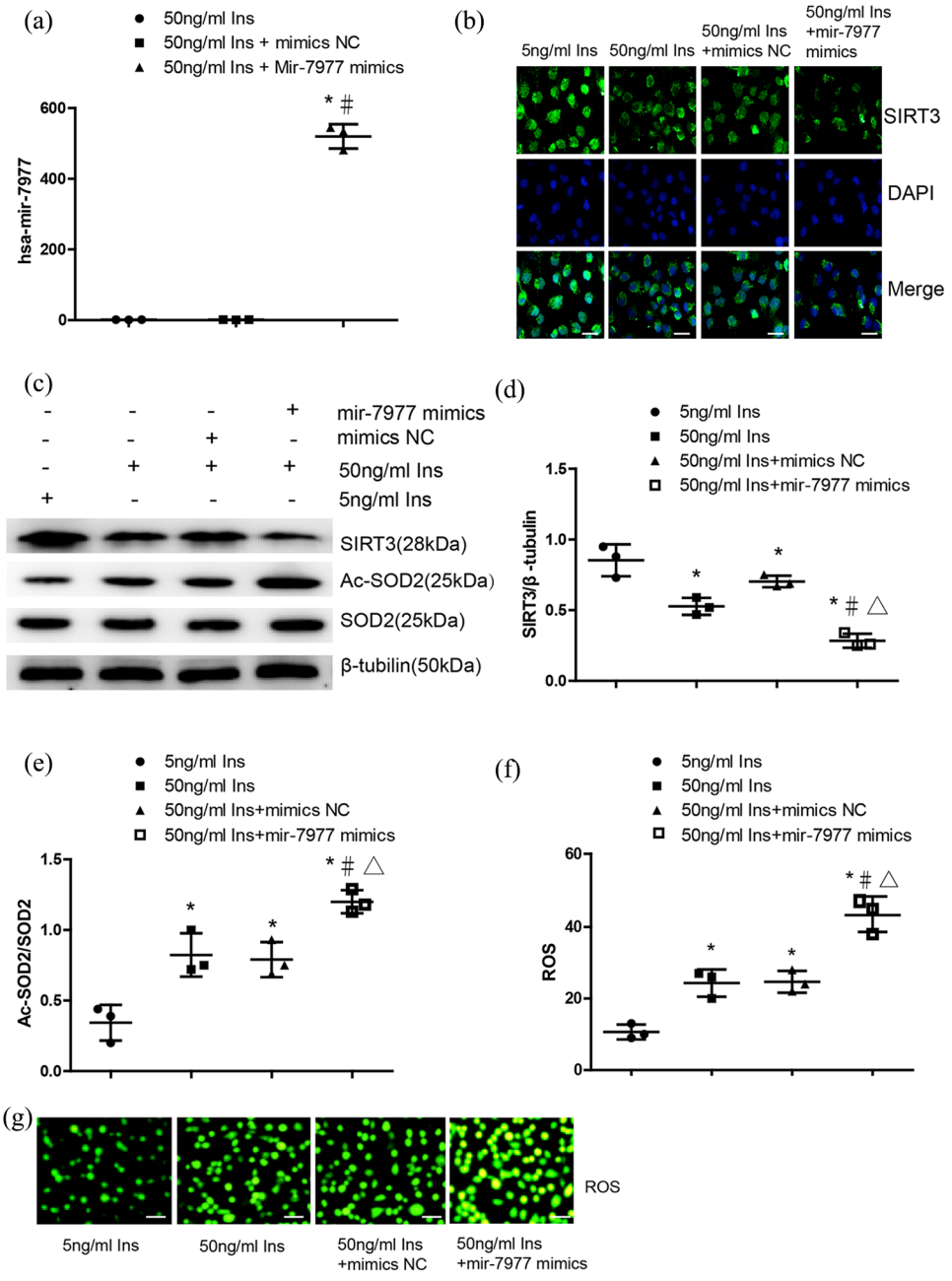

Figure 4. MiR-7977 decreases SIRT3 and increases Ac-SOD2/SOD2 and ROS in HK-2 cells. (a) quantitative polymerase chain reaction analysis of mir-7977 in HK-2 cells treated with mir-7977 mimics. (b) Immunofluorescence staining of SIRT3 in HK-2 cells. (c) Western blot analysis of SIRT3, Ac-SOD2, and SOD2 in HK-2 cells. (d, e) Quantification of protein expression of SIRT3 and Ac-SOD2/SOD2, respectively. (f, g) Level of ROS in HK-2 cells after transfection with mir-7977 mimics (g) and quantification (f). The scale bar represents 20 μm. Data are expressed as mean ± SD.

In a: $^{*}p < 0.05$ compared with the 50 ng/ml insulin group, $^{\#}p < 0.05$ compared with the 50 ng/ml insulin + mir-7977 mimics control group. In b–g: $^{*}p < 0.05$ compared with the 5 ng/ml insulin group, $^{\#}p < 0.05$ compared with the 50 ng/ml insulin group, $^{\triangle}p < 0.05$ compared with 50 ng/ml insulin + mir-7977 mimics control group. All data are representative of three independent experiments.

Ins, insulin; NC, normal control.

Figure 6a–d). In addition, endocytosis of TRITC-albumin was decreased in the 50 ng/ml group compared with the 5 ng/ml and 0 ng/ml groups ($p < 0.05$; Figure 6e, f).

SIRT3 regulates cubilin expression and endocytosis of albumin in HK-2 cells

To determine whether SIRT3 regulates the expression of cubilin and endocytosis of albumin, HK-2 cells were transfected with SIRT3 siRNA to suppress SIRT3 expression. The data showed that cubilin expression (Figure 7a–g) was further reduced in cells transfected with SIRT3 siRNA than in cells transfected with siRNA NC after treatment with 5 ng/ml or 50 ng/ml insulin ($p < 0.05$). Furthermore, consistent with the expression of cubilin, endocytosis of albumin was inhibited in cells transfected with SIRT3 siRNA (Figure 7h, i).

Taken toghther, our findings indicate that a high level of insulin can activate mir-7977/SIRT3 signaling, resulting in tubular injury by inducing oxidative stress as well as reabsorption dysfunction by inhibiting cubilin expression, ultimately contributing to IGT-RI (Figure 8).

Discussion

In this study, it was found that 12.1% of IGT patients had MAU. We termed this condition "IGT-RI." Patients with IGT-RI not only had MAU, but also obvious tubular injury, especially, reabsorption dysfunction. Compensatory hyperinsulinemia may be the underlying mechanism of IGT-RI. MicroRNA profiling revealed that the expression of numerous microRNAs, including mir-7977, was altered in IGT-RI patients. According to KEGG and GO analysis, differentially expressed microRNAs were involved in insulin signaling and the cellular response to oxidative stress. *In vitro*, it was concluded that a high level of insulin can activate mir-7977/SIRT3 signaling, resulting in tubular injury by inducing oxidative stress as well as reabsorption dysfunction by inhibiting cubilin expression.

Recent studies have indicated that there are no structural alterations in the glomerular filtration barrier in early diabetic nephropathy.[22,23] Furthermore, the onset of albuminuria is not associated with glomerular permeability changes, but rather is due to changes in the proximal tubules handling albumin.[22,23] In previous animal experiments using OLETF rats, it was found that by electron microscopy that the main presentation of RI was tubular injury, and the glomerular filtration barrier was nearly normal in the IGT stage.[10] In addition, in this study, there was no significant difference in the eGFR between IGT and IGT-RI patients. Thus, we consider that MAU in the IGT stage may be mainly due to tubular injury, especially reabsorption dysfunction, rather than glomerular filtration barrier damage or glomerular hemodynamics. NAG and GAL, lysosomal enzymes, are sensitive biomarkers that reflect structural injury in renal tubular epithelial cells in DKD.[24,25] RBP and β2-MG are low-molecular-weight proteins that are easily filtered by glomeruli and reabsorbed in proximal tubules, thus, elevation of RBP[26] and β2-MG[27] in the urine can reflect reabsorption dysfunction in renal tubular epithelial cells.[28,29] We have also confirmed that RBP could be used as reliable predictors of DKD.[30] In this study, it was found that 100% of patients with IGT-RI had reabsorption dysfunction, and 58.3% had structural damage in renal tubules. Furthermore, RBP and NAG levels were significantly associated with UMA, and they are independent risk factors of IGT-RI. Therefore, we concluded that the presence of MAU in IGT patients may be due to tubular injury, especially reabsorption dysfunction. Further, 54.0% of IGT patients had reabsorption dysfunction and 25.3% had structural damage. Thus, tubular injury may occur in the early stage of IGT-RI.

It is well known that insulin resistance and compensatory hyperinsulinemia are associated with the occurrence of chronic kidney disease.[31] Moreover, insulin resistance[32] and hyperinsulinemia are important causes of albuminuria in T2DM.[33] In this study, notably in the IGT-RI group, 2-h postprandial glucose did not differ from that in the IGT group, whereas 2-h postprandial insulin was nearly 47% higher than that in the IGT group. No significant differences in general characteristic indicators, such as age, gender, BMI, blood pressure, serum lipids, uric acid, and creatinine were observed between the two groups. In addition, 2-h postprandial insulin was significantly associated with UMA and was an independent risk factor for IGT-RI. Furthermore, 2-h postprandial insulin was significantly associated with RBP and NAG; thus, we believe that postprandial hyperinsulinemia may cause tubular injury and especially, reabsorption dysfunction, ultimately contributing to

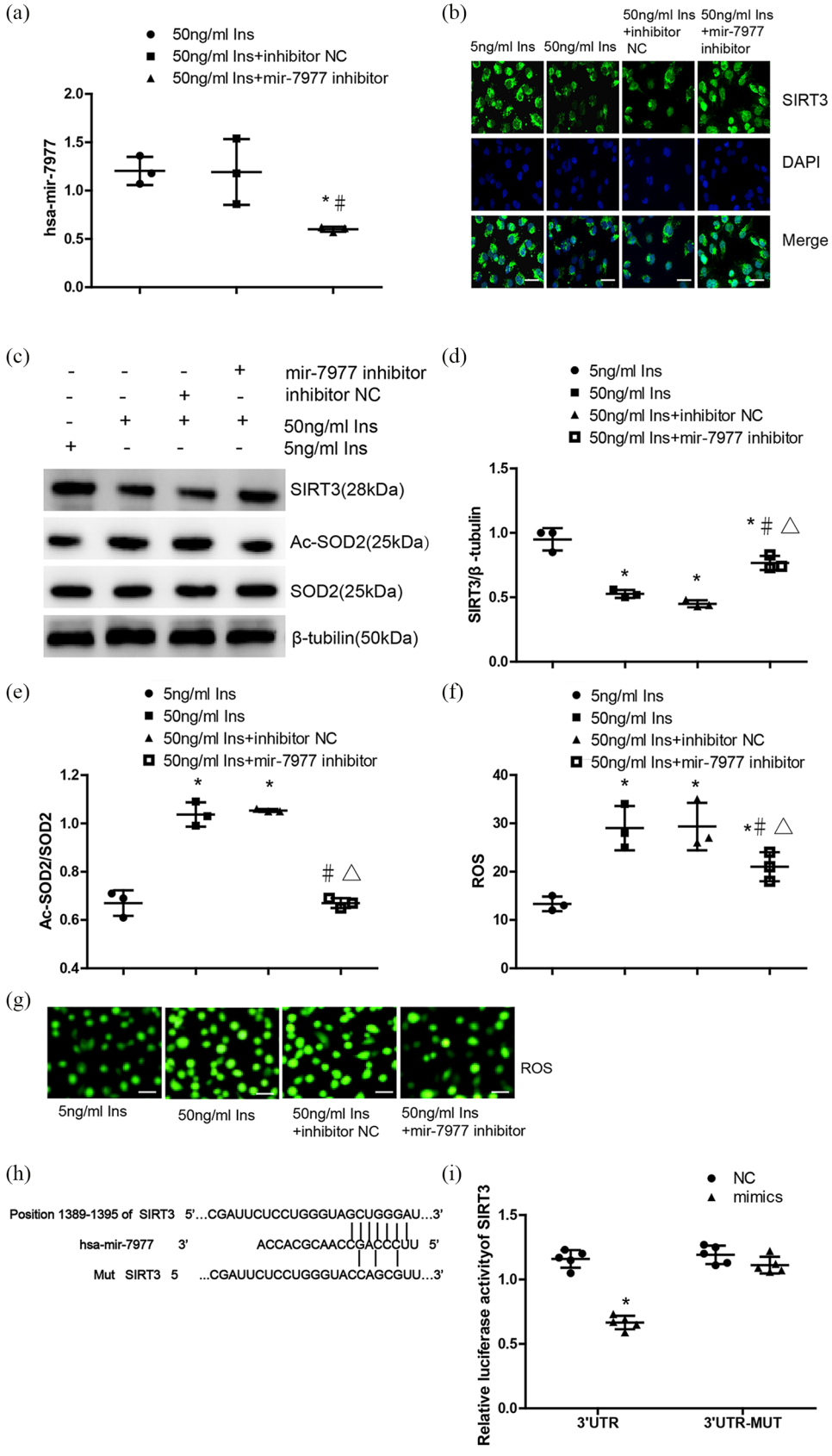

Figure 5. *(Continued)*

Figure 5. MiR-7977 inhibitor reverses SIRT3 and decreases Ac-SOD2/SOD2 and ROS in HK-2 cells. (a) quantitative polymerase chain reaction analysis of mir-7977 in HK-2 cells treated with mir-7977 inhibitor. (b) Immunofluorescence staining of SIRT3 in HK-2 cells; (c) Western blot analysis of SIRT3, Ac-SOD2, and SOD2 in HK-2 cells. (d, e) Quantification of protein expression of SIRT3 and Ac-SOD2/SOD2, respectively. (f, g) Level of ROS in HK-2 cells after transfection with mir-7977 inhibitor (g) and quantification (f). (h) Sequence alignment of miR-7977 and human SIRT3 3′-UTR and mutant (mut). (i) Luciferase assays results. The scale bar represents 20 μm. Data are expressed as mean ± SD.
In a: $^{*}p < 0.05$ compared with the 50 ng/ml insulin group, $^{\#}p < 0.05$ compared with the 50 ng/ml insulin + inhibitor control group. In b–g: $^{*}p < 0.05$ compared with the 5 ng/ml insulin group, $^{\#}p < 0.05$ compared with the 50 ng/ml insulin group, $^{\triangle}p < 0.05$ compared with the 50 ng/ml insulin + inhibitor control group. In i: $^{*}p < 0.05$ compared with the control group. All data are representative of three independent experiments.
Ins, insulin; NC, normal control.

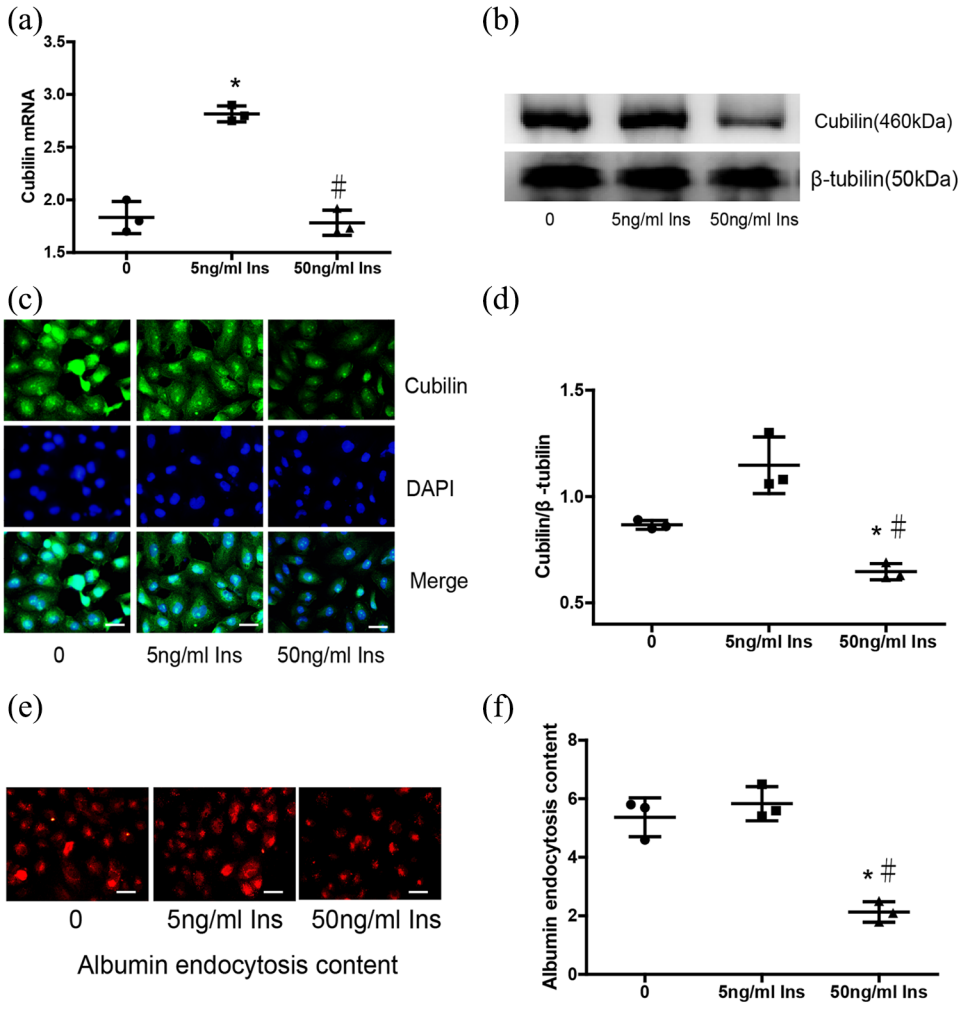

Figure 6. High-level insulin decreases cubilin expression and albumin endocytosis. (a) quantitative polymerase chain reaction analysis of cubilin mRNA expression in HK-2 cells after treatment with the indicated concentrations of insulin for 24 h. (b) Western blot analysis of cubilin in HK-2 cells. (c) Immunofluorescence staining of cubilin in HK-2 cells. (d) Quantification of protein expression of cubilin in B. (e, f) Effect on albumin endocytosis of treatment with insulin at the indicated concentrations for 24 h as revealed by confocal microscopy and quantification (f). The scale bar represents 20 μm. Data are expressed as mean ± SD.
$^{*}p < 0.05$ compared with blank control group, $^{\#}p < 0.05$ compared with the 5 ng/ml insulin group. All data are representative of three independent experiments.
Ins, Insulin.

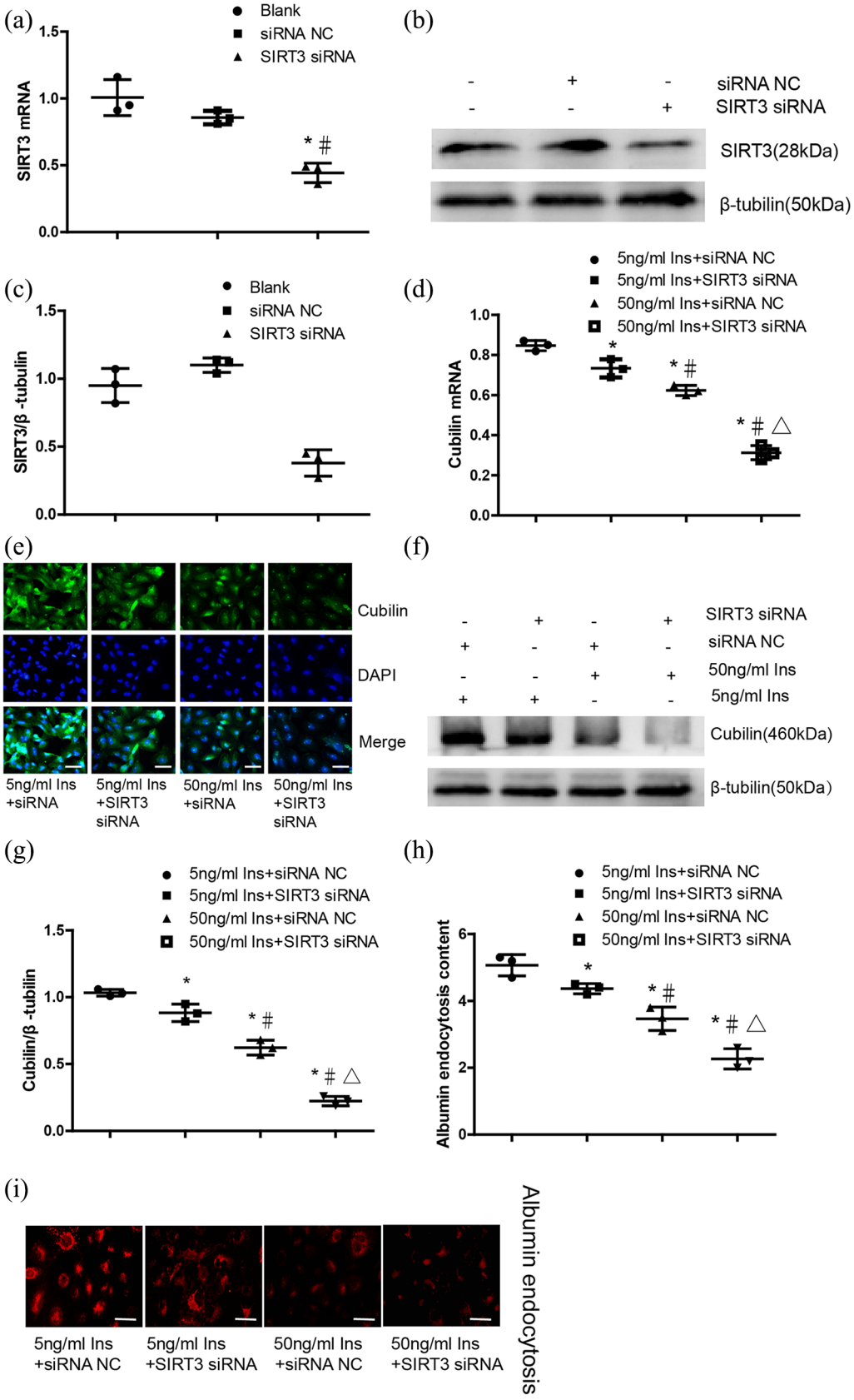

Figure 7. *(Continued)*

Figure 7. SIRT3 regulates cubilin expression and albumin endocytosis. (a) qPCR analysis of SIRT3 siRNA on the expression of SIRT3 mRNA in HK-2 cells. (b) Western blot analysis of SIRT3 in HK-2 cells transfected with SIRT3 siRNA. (c) Quantification of protein expression of SIRT3 in b. (d) qPCR analysis of the effect of SIRT3 siRNA on cubilin mRNA expression in HK-2 cells. (e) Immunofluorescence staining of cubilin in HK-2 cells. (f) Western blot analysis of cubilin in HK-2 cells transfected with SIRT3 siRNA. (g) Quantification of protein expression of cubilin in f. (h, i) Effect on albumin endocytosis in HK-2 cells transfected with SIRT3 siRNA as revealed by confocal microscopy (i) and quantification (h). The scale bar represents 20 μm. Data are expressed as mean ± SD.
In a–c: $^{*}p < 0.05$ compared with the blank control group; $^{\#}p < 0.05$ compared with the siRNA control group. In d–i: $^{*}p < 0.05$ compared with 5 ng/ml insulin + siRNA NC group, $^{\#}p < 0.05$ compared with 5 ng/ml insulin + SIRT3 siRNA group; $^{\triangle}p < 0.05$ compared with 50 ng/ml insulin + siRNA control group. All data are representative of three independent experiments.
qPCR, quantitative polymerase chain reaction; Ins, Insulin; NC, normal control.

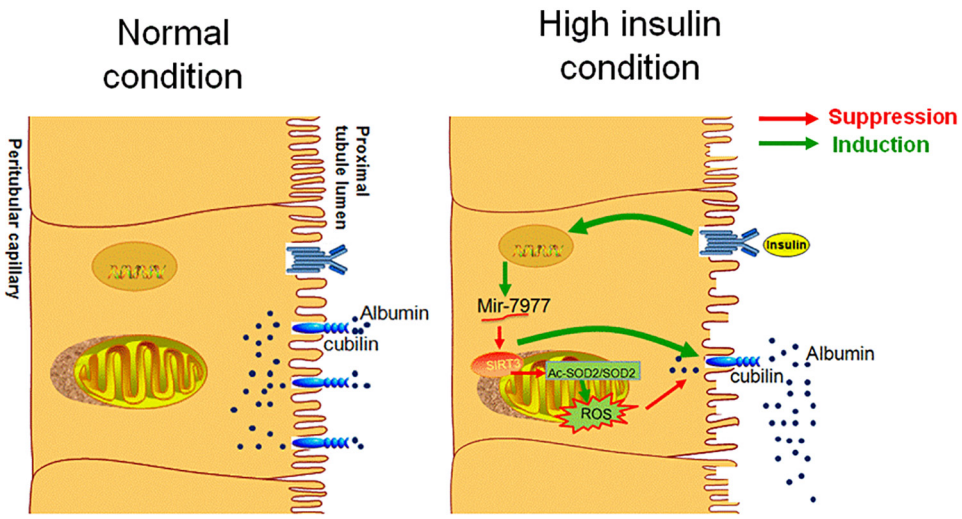

Figure 8. Mechanism of hyperinsulinemia on endocytosis function of proximal tubular epithelial cells in IGT-RI. Under normal condition, albumin which filtered out by the glomerular filtration barrier is mainly absorbed by the proximal tubular epithelial cells. Under high level of insulin, the expression of mir-7977 is increased, which inhibits its target gene SIRT3. The inhibition of SIRT3 increases the acetylation of SOD2 and thus leads to oxidative stress. The inhibition of SIRT3 also decreases the level of cubilin, a receptor mediating albumin reabsorption in the membrane of proximal tubule epithelial cells, and reduces albumin endocytosis by tubular epithelial cells.
IGT-RI, impaired glucose tolerance-renal injury

IGT-RI. Further studies are needed to verify the causative link between these phenomenon.

In previous animal experiments, we demonstrated that a high level of insulin inhibits the IRS-1/PI3-K/Akt insulin signaling pathway, resulting in tubular injury.[10] Consistent herewith, KEGG pathway-enrichment analysis revealed that differentially expressed microRNAs in the urine of IGT-renal patients were involved in insulin signaling. This adds to the evidence that hyperinsulinemia may be involved in RI by regulating microRNAs. Therefore, we further studied whether a high level of insulin induces a microRNA-associated mechanism in tubular injury.

MiRNAs fine-tune protein expression through direct targeting of mRNAs encoding diverse signaling molecules and pathways, and exert important roles in DKD.[34] Microarray results in this study revealed that mir-7977 was the most abundant and exhibited the strongest increase in IGT-renal patients. Thus, we studied mir-7977-associated mechanisms in *in-vitro* experiment. Roles of mir-7977 have only been reported in lupus nephropathy[35] and hematopoietic diseases[36] to date. SIRT3, a nicotinamide adenine dinucleotide-dependent histone deacetylase, is primarily located in the mitochondria. Some studies have indicated that SIRT3 is closely related to insulin resistance.[14,15] In addition, SIRT3 reduces ROS

by directly deacetylating SOD2, a key mitochondrial antioxidant enzyme, thereby increasing the activity of SOD2 and protecting the cells against oxidative stress.[37,38] SIRT3 has protective effects on acute kidney injury,[39] hypertensive kidney injury,[40] and DKD,[16] and it can protect renal tubules from oxidative stress, and inflammatory,[41] and high glucose-induced apoptosis.[17] Based on a TargetScan database search, we identified that SIRT3 may be a target gene of mir-7977. In addition, GO enrichment analysis showed that differentially expressed microRNAs may regulate the cellular response to oxidative stress. Therefore, we determined whether mir-7977/SIRT3/SOD2/ROS are involved in RI induced by a high level of insulin by conducting cell experiments. The results demonstrated that a high level of insulin significantly increased mir-7977 expression and decreased SIRT3 expression, resulting in oxidative stress. To confirm whether mir-7977 can affect SIRT3, we upregulated and downregulated miR-7977 in HK-2 cells by transfecting them with miR-7977 mimics or treating them with an inhibitor. Upon upregulation of miR-7977, SIRT3 expression was suppressed, whereas downregulation of mir-7977 resulted in upregulation of SIRT3 expression. Using a luciferase reporter assay, we confirmed that mir-7977 can bind to the 3′-UTR of *SIRT3* mRNA and suppress its mRNA and protein expression. Thus, mir-7977/SIRT3 signaling pathway-associated oxidative stress may cause injury in tubular epithelial cells.

As mentioned above, the presence of MAU in patients with IGT-RI may be caused by reabsorption dysfunction of renal tubular epithelial cells. Cubilin, a highly expressed receptor in the membrane of proximal tubule epithelial cells, plays a key role in mediating albumin reabsorption.[42] Some studies have indicated that gene mutations[43] or any other factors affecting the expression and function of cubilin[44] can increase albuminuria. In cubilin-deficient mice, albumin absorption by proximal tubule cells is decreased, resulting in albuminuria.[45] In previous experiments, we have demonstrated that in IGT stage of OLETF rats, cubilin was inhibited in the kidney by immunohistochemical method. However, whether hyperinsulinemia affects the expression of cubilin remained unknown. Our study showed that at a high level, insulin inhibits cubilin expression. Consistent herewith, the endocytosis of albumin in HK-2 cells was also reduced after treatment with a high level of insulin. As mentioned above, we confirmed that at a high level, insulin can decrease SIRT3 expression. To further test whether SIRT3 can regulate the expression of cubilin, we knocked down SIRT3 expression using siRNA. The results indicated that downregulation of SIRT3 resulted in inhibition of cubilin expression. Consistent herewith, it reduced endocytosis of albumin in HK-2 cells. Thus, SIRT3 regulates cubilin expression, which affects reabsorption function in HK-2 cells. Therefore, we concluded that a high level of insulin may reduce the reabsorption of albumin in renal tubular epithelial cells by activating mir-7977/SIRT3/cubilin signaling.

In conclusion, our study provides strong evidence for the existence, characteristics, and underlying mechanisms of renal injury in IGT patients. In addition, mir-7977/SIRT3 signaling involved in tubular injury and reabsorption dysfunction may serve as a new therapeutic target for preventing IGT -RI.

Limitation

A multicenter study with large sample size is needed to verify the prevalence, characteristics, and risk factors of RI in IGT patients. In addition, a prospective study is needed to determine the causal relationship between hyperinsulinemia and IGT-RI. Further, we only observed the effect of SIRT3 on the expression of cubilin and endocytosis of albumin; further studies are needed to explore specific regulatory mechanisms of the two proteins.

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Author contributions

ZG performed the research, analyzed the data, and wrote the manuscript. BC and JY designed the experiments and revised the manuscript. ZW, XY, MS, JW, MZ, XC, YZ, SY, YQ and JX analyzed and interpreted the data. HZ contributed to statistical analyses. YH collected the experimental data. JY and BC are co-corresponding authors and contributed equally to this study. All authors reviewed and approved the final version of the

manuscript. BC and JY take full responsibility for the work and approved the final version to be published.

Conflict of interest statement

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Supplemental material

Supplemental material for this article is available online.

References

1. Collins AJ, Foley RN, Chavers B, *et al.* United States Renal Data System 2011 Annual Data Report: Atlas of chronic kidney disease & end-stage renal disease in the United States. *Am J Kidney Dis* 2012; 59: A7.
2. Fioretto P, Caramori ML and Mauer M. The kidney in diabetes: dynamic pathways of injury and repair. The Camillo Golgi Lecture. *Diabetologia* 2008; 51: 1347–1355.
3. Zhang Y, Yang J, Zheng M, *et al.* Clinical characteristics and predictive factors of subclinical diabetic nephropathy. *Exp Clin Endocrinol Diabetes* 2015; 123: 132–138.
4. Singleton JR, Smith AG, Russell JW, *et al.* Microvascular complications of impaired glucose tolerance. *Diabetes* 2003; 52: 2867–2873.
5. Carlsson AC, Calamia M, Riserus U, *et al.* Kidney injury molecule (KIM)-1 is associated with insulin resistance: results from two community-based studies of elderly individuals. *Diabetes Res Clin Pract* 2014; 103: 516–521.
6. Markus MRP, Ittermann T, Baumeister SE, *et al.* Prediabetes is associated with microalbuminuria, reduced kidney function and chronic kidney disease in the general population: The KORA (Cooperative Health Research in the Augsburg Region) F4-Study. *Nutr Metab Cardiovasc Dis* 2018; 28: 234–242.
7. Jadhakhan F, Marshall T, Ryan R, *et al.* Risk of chronic kidney disease in young adults with impaired glucose tolerance/impaired fasting glucose: a retrospective cohort study using electronic primary care records. *BMC Nephrol* 2018; 19: 42.
8. Wang XL, Lu JM, Pan CY, *et al.* A comparison of urinary albumin excretion rate and microalbuminuria in various glucose tolerance subjects. *Diabet Med* 2005; 22: 332–335.
9. Tapp RJ, Shaw JE, Zimmet PZ, *et al.* Albuminuria is evident in the early stages of diabetes onset: results from the Australian Diabetes, Obesity, and Lifestyle Study (AusDiab). *Am J Kidney Dis* 2004; 44: 792–798.
10. Zhang Y, Yang SH, Cui X, *et al.* Hyperinsulinemia can cause kidney disease in the IGT stage of OLETF rats via the INS/IRS-1/PI3-K/Akt signaling pathway. *J Diabetes Res* 2019; 4709715.
11. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009; 136: 215–233.
12. Krol J, Loedige I and Filipowicz W. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet* 2010; 11: 597–610.
13. Simpson K, Wonnacott A, Fraser DJ, *et al.* MicroRNAs in diabetic nephropathy: from biomarkers to therapy. *Curr Diab Rep* 2016; 16: 35.
14. Hirschey MD, Shimazu T, Jing E, *et al.* SIRT3 deficiency and mitochondrial protein hyperacetylation accelerate the development of the metabolic syndrome. *Mol Cell* 2011; 44: 177–190.
15. Lantier L, Williams AS, Williams IM, *et al.* SIRT3 is crucial for maintaining skeletal muscle insulin action and protects against severe insulin resistance in high-fat-fed mice. *Diabetes* 2015; 64: 3081–3092.

16. Liu Z, Liu H, Xiao L, *et al*. STC-1 ameliorates renal injury in diabetic nephropathy by inhibiting the expression of BNIP3 through the AMPK/SIRT3 pathway. *Lab Invest* 2019; 99: 684–697.

17. Jiao X, Li Y, Zhang T, *et al*. Role of Sirtuin3 in high glucose-induced apoptosis in renal tubular epithelial cells. *Biochem Biophys Res Commun* 2016; 480: 387–393.

18. American Diabetes Association. Introduction: Standards of Medical Care in Diabetes-2018. *Diabetes Care* 2018; 41(Suppl. 1): S1–S2.

19. Stevens PE, Levin A, Kidney Disease: Improving Global Outcomes Chronic Kidney Disease Guideline Development Work Group Members. Evaluation and management of chronic kidney disease: synopsis of the kidney disease: improving global outcomes clinical practice guideline. *Ann Intern Med* 2012; 158: 825–830.

20. Levey AS, Stevens LA, Schmid CH, *et al*. A new equation to estimate glomerular filtration rate. *Ann Intern Med* 2009; 150: 604–612.

21. Nakamura N, Matsui T, Ishibashi Y, *et al*. Insulin stimulates SGLT2-mediated tubular glucose absorption via oxidative stress generation. *Diabetol Metab Syndr* 2015; 24: 48.

22. Dickson LE, Wagner MC, Sandoval RM, *et al*. The proximal tubule and albuminuria: really! *J Am Soc Nephrol* 2014; 25: 443–453.

23. Russo LM, Sandoval RM, Campos SB, *et al*. Impaired tubular uptake explains albuminuria in early diabetic nephropathy. *J Am Soc Nephrol* 2009; 20: 489–494.

24. Vaidya VS, Niewczas MA, Ficociello LH, *et al*. Regression of microalbuminuria in type 1 diabetes is associated with lower levels of urinary tubular injury biomarkers, kidney injury molecule-1, and N-acetyl-beta-d-glucosaminidase. *Kidney Int* 2011; 79: 464–470.

25. Kern EF, Erhard P, Sun W, *et al*. Early urinary markers of diabetic kidney disease: a nested case-control study from the Diabetes Control and Complications Trial (DCCT). *Am J Kidney Dis* 2010; 55: 824–834.

26. Hong CY, Chia KS and Ling SL. Urinary protein excretion in type 2 diabetes with complications. *J Diabetes Complication* 2000; 14: 259–265.

27. Aksun SA, Ozmen D, Ozmen B, *et al*. Beta2-microglobulin and cystatin C in type 2 diabetes: assessment of diabetic nephropathy. *Exp Clin Endocrinol Diabetes* 2004; 112: 195–200.

28. Colombo M, Looker HC, Farran B, *et al*. Serum kidney injury molecule 1 and beta2-microglobulin perform as well as larger biomarker panels for prediction of rapid decline in renal function in type 2 diabetes. *Diabetologia* 2019; 62: 156–168.

29. Gluhovschi C, Gluhovschi G, Petrica L, *et al*. Urinary biomarkers in the assessment of early diabetic nephropathy. *J Diabetes Res* 2016; 4626125.

30. Qin Y, Zhang S, Shen X, *et al*. Evaluation of urinary biomarkers for prediction of diabetic kidney disease: a propensity score matching analysis. *Ther Adv Endocrinol Metab* 2019; 10: 1–11.

31. Leyking S and Fliser D. Insulin resistance in CKD. *Clin J Am Soc Nephrol* 2014; 9: 638–640.

32. Parvanova AI, Trevisan R, Iliev IP, *et al*. Insulin resistance and microalbuminuria: a cross-sectional, case-control study of 158 patients with type 2 diabetes and different degrees of urinary albumin excretion. *Diabetes* 2006; 55: 1456–1462.

33. Kohler KA, McClellan WM, Ziemer DC, *et al*. Risk factors for microalbuminuria in black americans with newly diagnosed type 2 diabetes. *Am J Kidney Dis* 2000; 36: 903–913.

34. Wang B, Komers R, Carew R, *et al*. Suppression of microRNA-29 expression by TGF-beta1 promotes collagen expression and renal fibrosis. *J Am Soc Nephrol* 2012; 23: 252–265.

35. Navarro-Quiroz E, Pacheco-Lugo L, Navarro-Quiroz R, *et al*. Profiling analysis of circulating microRNA in peripheral blood of patients with class IV lupus nephritis. *PloS One* 2017; 12: e0187973.

36. Horiguchi H, Kobune M, Kikuchi S, *et al*. Extracellular vesicle miR-7977 is involved in hematopoietic dysfunction of mesenchymal stromal cells via poly(rC) binding protein 1 reduction in myeloid neoplasms. *Haematologica* 2016; 101: 437–447.

37. Qiu X, Brown K, Hirschey MD, *et al.* Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. *Cell Metab* 2010; 12: 662–667.

38. Tao R, Coleman MC, Pennington JD, *et al.* Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress. *Mol Cell* 2010; 40: 893–904.

39. Morigi M, Perico L, Rota C, *et al.* Sirtuin 3-dependent mitochondrial dynamic improvements protect against acute kidney injury. *J Clin Invest* 2015; 125: 715–726.

40. Lin JR, Zheng YJ, Zhang ZB, *et al.* Suppression of endothelial-to-mesenchymal transition by SIRT (Sirtuin) 3 alleviated the development of hypertensive renal injury. *Hypertension* 2018; 72: 350–760.

41. Kitada M, Kume S and Koya D. Role of sirtuins in kidney disease. *Curr Opin Nephrol Hypertens* 2014; 23: 75–79.

42. Aseem O, Smith BT, Cooley MA, *et al.* Cubilin maintains blood levels of HDL and albumin. *J Am Soc Nephrol* 2014; 25: 1028–1036.

43. Boger CA, Chen MH, Tin A, *et al.* CUBN is a gene locus for albuminuria. *J Am Soc Nephrol* 2011; 22: 555–570.

44. Schreiber A, Theilig F, Schweda F, *et al.* Acute endotoxemia in mice induces downregulation of megalin and cubilin in the kidney. *Kidney Int* 2012; 82: 53–59.

45. Amsellem S, Gburek J, Hamard G, *et al.* Cubilin is essential for albumin reabsorption in the renal proximal tubule. *J Am Soc Nephrol* 2010; 21: 1859–1867.