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The single nucleotide polymorphism rs11643718 in *SLC12A3* is associated with the development of diabetic kidney disease in Chinese people with type 2 diabetes

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Abstract

Aims To examine the association between 24 literature-based single nucleotide polymorphisms and diabetic kidney disease in Chinese people with type 2 diabetes.

Methods and results Twenty-four candidate diabetic kidney disease-susceptible single nucleotide polymorphisms were genotyped in 208 participants with type 2 diabetes and diabetic kidney disease and 200 participants with type 2 diabetes without diabetic kidney disease (case and control groups, respectively), together with 206 healthy participants using MassARRAY. Rs11643718 in the *SLC12A3* gene was associated with diabetic kidney disease in the recessive model after adjusting for confounding factors, such as age and gender (adjusted odds ratio 2.056, 95% CI 1.120–3.776; $P = 0.020$). Meta-analyses further confirmed the association ($P = 0.002$). In addition, participants with the *GG* genotype had worse renal function and more albuminuria than those with the *AA+AG* genotype ($P < 0.05$). Renal section immunohistochemistry was conducted in participants with type 2 diabetes, diabetic kidney disease and *AA+AG* or *GG* genotypes and in participants with glomerular minor lesions. Together with data from the Nephroseq database, it was shown that the abundance of *SLC12A3* was reduced in patients with the *GG* genotype, while elevated expression of *SLC12A3* was associated with better renal function. In addition, rs10951509 and rs1345365 in *ELMO1*, which were determined to be in high linkage disequilibrium by SHEsis software, were also associated with diabetic kidney disease (adjusted $P = 0.010$ and 0.015, respectively).

Conclusions The *G* allele and *GG* genotype of *SLC12A3* rs11643718 are associated with the development of diabetic kidney disease in a Chinese population with type 2 diabetes.

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Introduction

Globally, nearly 425 million people are affected by diabetes mellitus, and the estimated population living with diabetes will be 629 million in 2045 [1]. With the rapid increase in type 2 diabetes incidence, the numbers of people with diabetic kidney disease (DKD) and end-stage renal disease are also increasing [2]. Although glycaemic control and blood pressure management have reduced the proportion of individuals with diabetes who progress to DKD and end-stage renal disease, these measures do not eliminate the risk of DKD [3–5]. In addition, it has been demonstrated that family aggregation exists in DKD [6], indicating that genetic factors may contribute to the development and progression of DKD.

In recent years, as a result of the development of genetic methods, numerous candidate gene association studies and genome-wide association studies have been conducted, and some of the single nucleotide polymorphisms (SNPs) that are related to DKD susceptibility have been identified [7]. The rs2268388 SNP in the *ACACB* gene is one of the most widely studied SNPs in DKD. The relationship of this SNP with DKD was first reported by a genome-wide association study in Japan [8] and was then validated by candidate gene

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What's new?

- Many diabetic kidney disease (DKD)-susceptible single-nucleotide polymorphims (SNPs) in type 2 diabetes have been identified; however, the relationship between some SNPs and DKD is still controversial. The effect of several SNPs on DKD in Chinese populations with type 2 diabetes is also unclear.
- This study found that the *G* allele and *GG* genotype in *SLC12A3* rs11643718, as well as the *G* allele and *GG+AG* genotype in *ELMO1* rs10951509 and rs1345365, may be risk factors for DKD in Chinese type 2 diabetes populations.
- Analysing these genotypes in people with type 2 diabetes will be helpful in identifying populations with high risk of DKD.

association studies in Asian Indian [9] and Chinese populations [10]. In addition, SNPs in genes such as *ELMO1* [11,12], *CNDP1* [13], *CNDP2* [13], *MTHFR* [14] and *SLC12A3* [15,16] were also recognized as DKD-related SNPs. Nevertheless, the effects of some SNPs are still controversial. Leak *et al*. [17] and Hanson *et al*. [18] found that SNPs in *ELMO1* (i.e. rs1345365, rs1981740 and rs10951509) were related to DKD. Unfortunately, the directions of association in these two studies were completely opposite. Moreover, due to racial differences and genetic backgrounds, whether these reported loci play a role in Chinese Han populations remains to be further confirmed.

In the present study, we selected 24 candidate SNPs mainly from studies in East Asian populations, the results of which mostly differed among studies or which had large reported odds ratios (ORs) suggesting deleteriousness, and aimed to verify the associations between these SNPs and DKD in a Chinese population with type 2 diabetes.

Participsants and methods

Participants

A total of 208 participants with type 2 diabetes and DKD (DKD group), 200 participants with type 2 diabetes without DKD (diabetes-only group) and 206 healthy participants (control group) were recruited from the National Clinical Research Centre for Metabolic Diseases Diabetes Centre in China, which includes the Department of Endocrinology and Nephrology, Second Xiangya Hospital of Central South University and the Department of Nephrology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, between November 2017 and October 2018. The inclusion criteria for participants in the diabetes-only group were as follows: (1) diagnosis of type 2 diabetes; (2) age 30–80 years; (3) diabetes duration >10 years; (4) not meeting the diagnostic criteria for DKD; and (5) no organic diseases such as tumour, hepatitis or autoimmune diseases that may cause additional burden on the kidney and impact the diagnosis of DKD. The participants in the DKD group had to meet the following additional criteria: (1) diagnosis of DKD and (2) no other renal diseases. To exclude primary and secondary glomerular lesions, participants with the following characteristics were excluded: (1) an estimated GFR (eGFR) that was declining rapidly; (2) manifested as nephrotic syndrome directly or progressing to macroalbuminuria directly without a microalbuminuria process; (3) proteinuria accompanied by massive erythrocyte sediment (variant erythrocytes >80%, erythrocyte tubular type or leukocyte tubular type); (4) proteinuria caused by other diseases; and (5) hypertension occurring before diabetes. Participants in the healthy control group were aged 30–80 years, and had no known diabetes or kidney disease.

SNPs and genotyping

Twenty-four SNPs located in 16 genes were selected from the literature. The selection of SNPs was mainly based on the following principles: 1) SNPs were preferred if they were reported in East Asian populations; 2) SNPs with larger OR values reported in the literature were preferred; 3) SNPs for which the OR values conflicted between studies were preferred; and 4) SNPs located in genes involved in the development of DKD from type 2 diabetes were preferred, such as those involved in glucose metabolism, lipid metabolism, angiogenesis, inflammation and oxidative stress. Detailed information about the selected SNPs and the representative sources of literature are summarized in Table S1. Peripheral blood of all participants was extracted and processed according to a standard procedure. A SpectroCHIPTM gene chip containing 24 selected SNPs was created. The PCR-MassARRAY method using an iPLEX Gold Reagent Kit (Sequenom, San Diego, CA, USA) was then used to genotype all the selected SNPs. The reaction products were then analysed by MassARRAY standard mass spectrometry. SNPs with >99% genotyping success rates were included in further analyses. The sequences of primers and probes are shown in Table S2.

Definitions

Diabetes mellitus was diagnosed according to the following criteria: symptoms of hyperglycaemia (polyuria, polydipsia, weight loss) and random plasma glucose level ≥ 11.1 mmol/l (200 mg/dl); fasting plasma glucose ≥7.0 mmol/l (126 mg/dl); 2-h plasma glucose level during an oral glucose tolerance test ≥11.1 mmol/l; or HbA_{1c} level ≥6.5% (47.54 mmol/mol). Diagnosis of DKD was made based on the presence of albuminuria (two of three specimens of urinary albumin/creatinine ratio collected within a 3- to 6-month period ≥30 mg/g creatinine) and/or reduced eGFR (<60 mL/min/1.73 m^2)

in the absence of signs or symptoms of other primary causes of kidney damage. Diagnosis of diabetic retinopathy relied on the presence of lesions such as microaneurysms, haemorrhages, retinal thickening or new vessel formation appearing on the fundus photographs. Diabetic neuropathy included diabetic peripheral neuropathy and autonomic neuropathy. The diagnosis was mainly confirmed by related clinical symptoms and electrophysiological investigations. Macrovascular complications consisted of coronary heart disease, atherosclerosis and cerebrovascular diseases. Diabetic foot was defined as infection, ulcer with or without deep tissue destruction of the lower extremities (ankle joint and below) caused by distal nerve abnormalities or peripheral vascular lesions. Hypertension was defined as more than two measurements of systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥80 mmHg, or current anti-hypertensive therapy. Alcohol or cigarette consumption was defined as a history of daily drinking or smoking for at least 1 year. Diabetes duration referred to the duration from the diagnosis of diabetes to the time when the participant was recruited into the study. Family history of diabetes was recorded if participants' parents, siblings or children were diagnosed with diabetes. BMI was obtained by dividing weight (kg) by height squared (m^2). Microalbuminuria was defined as an albuminuria level between 30 mg/24 h and 300 mg/24 h, while macroalbuminuria was defined as an albuminuria level exceeding 300 mg/24 h. An abnormal blood urea nitrogen level was defined as >7.14 mmol/l. An abnormal serum creatinine level was defined as > 133 μmol/l.

Meta-analyses

Relevant studies were searched in PubMed, Excerpta Medica Database (EMBASE) and the Cochrane database on 28 July 2019. Case–control studies were eligible for inclusion if the phenotypes of case and control groups were type 2 diabetes with and without DKD, if data on the association of selected SNPs and DKD were available, and if the frequencies of genotype in each group could be extracted. Duplicated studies, reviews, case reports and trials, non-clinical studies, studies that only evaluated a certain clinical stage of DKD, such as end-stage renal disease or advanced DKD, or other SNPs were excluded. Data extraction was conducted by two researchers independently. The extracted content for each SNP was as follows: mutation site, first author, publication year, language, country, ethnicity, case and control phenotype definition, sample sizes in case and control groups, gender and age of both groups, allele frequencies and genotype frequencies in both groups, and *P* value of Hardy–Weinberg equilibrium test in the control group.

Immunohistochemistry of renal tissues

Paraffin-embedded 3-μm-thick renal sections of eight type 2 diabetes participants with DKD and the *GG* genotype in rs11643718 and five with the *AA* or *AG* genotype were obtained for this study. In addition, renal sections of eight participants with glomerular minor lesions were collected as controls. The anti-*SLC12A3* antibody was purchased from Abcam (catalogue ab224762). These sections were deparaffinized, rehydrated and incubated with anti-*SLC12A3* antibody (1:200). After incubation with secondary antibodies, the sections were treated with diaminobiphenylamine and dyed with haematoxylin. Images were then collected using light microscopy, and Image-Pro Plus 6.0 software was used to analyse the results.

Statistical analyses

The data were analysed using SPSS 23.0. Quantitative and qualitative data are presented as mean ± SD values and number with percentages, respectively. The Hardy–Weinberg equilibrium test was performed using a chi-squared goodness-of-fit test with 1 degree of freedom. The differences in clinical variables between groups were compared using an unpaired Student's *t*-test or rank-sum test for quantitative data and a chi-squared test for qualitative data. The constituent ratios of genotypes and the distributions of alleles were compared between groups using the chi-squared test, and Bonferroni correction was used for multiple testing correction. Further comparison of genotype distributions between groups was conducted with dominant or recessive models using the chi-squared test. Confounding factors such as age, gender, BMI, diabetes duration, family history and HbA_{1c} were adjusted for using the multivariate logistic regression method. Data for the analyses of the correlation of *SLC12A3* expression level with eGFR, serum creatinine and proteinuria were obtained from the Nephroseq database (http://v5.nephroseq.org). The correlation analyses were carried out using Spearman's method and visualized using GraphPad Prism. The comparisons of *SLC12A3* expression levels among groups were conducted by analysis of variance (Tukey's honestly significant difference test) and were also visualized using GraphPad Prism. SHEsis online software was used for linkage disequilibrium [19]. Meta-analyses were performed using Review Manager version 5.3 (Nordic Cochrane Centre, Cochrane Collaboration, Copenhagen, 2014). Heterogeneity was measured using Q statistics and the I^2 test. A random-effects model was used to combine all the effects. *P* values, ORs and 95% CIs were calculated using Mantel–Haenszel statistics [20,21] for allele, dominant or recessive models. *P* values < 0.05 (two-tailed) were taken to indicate statistical significance.

Ethics

This study was approved by the Hunan Research Ethics Committees in the Second Xiangya Hospital of Central South University, China (XXF190105).

Results

Participant characteristics

The clinical characteristics of the participants in the diabetes-only and DKD groups are summarized in Table 1. There were no significant differences between the groups in age, gender, diabetes duration, BMI, fasting glucose levels, serum lipid levels, smoking frequency, drinking frequency or the incidence of macrovascular complications. However, when compared with participants in the diabetes-only group, uric acid, blood urea nitrogen and serum creatinine levels were significantly higher in the participants in the DKD group. In contrast, HbA_{1c}, haemoglobin and albumin were much lower in the DKD group. In addition, participants in the DKD group had higher incidences of albuminuria, hypertension, diabetic retinopathy, diabetic neuropathy and diabetic foot, as well as lower incidence of diabetes family history records.

Table 1 Clinical characteristics of type 2 diabetes participants with or without diabetic kidney disease

Variable	Diabetes-only group (*n*=200)	DKD group (*n*=208)	*P*
Age at study, years	60.6 ± 9.4	58.9 ± 10.5	0.094
Men, *n* (%)	110 (55)	129 (62)	0.150
Diabetes duration, years	14.7±4.8	14.1±5.6	0.540
BMI, kg/m^2	23.7±3.2	24.3±2.6	0.075
Family history, *n* (%)	89 (47)	66 (35)	0.016*
Cigarette history, *n* (%)	59 (31)	69 (34)	0.512
Alcohol history, *n* (%)	35 (18)	43 (21)	0.462
Fasting glucose, mmol/l	7.7 ± 2.6	8.5 ± 4.2	0.356
HbA_{1c}			<0.001*
mmol/mol	67 ± 19	63 ± 25	
%	8.3±1.7	7.9 ± 2.3	
Total cholesterol, mmol/l	4.4±1.0	4.5±1.4	0.726
HDL cholesterol, mmol/l	1.1±0.3	1.1±0.4	0.396
LDL cholesterol, mmol/l	2.7±0.9	2.8±1.1	0.313
Triglycerides, mmol/l	1.6±1.0	1.7±1.0	0.438
Haemoglobin, g/l	133±15	104±24	<0.001*
Albumin, g/l	39.2±3.6	31.2±6.1	<0.001*
Uric acid, μmol/l	287±74	335±101	<0.001*
Blood urea nitrogen, mmol/l	5.7±1.6	13.0±8.2	<0.001*
Serum creatinine, μmol/l	60±18	325±304	<0.001*
Urinary albumin level, *n* (%)			<0.001*
Normal	185 (95)	0 (0.0)	
Microalbuminuria	9 (4.6)	51 (32)	
Macroalbuminuria	0 (0.0)	107 (68)	
Hypertension, *n* (%)	82 (41)	166 (80)	<0.001*
Diabetic Retinopathy, *n* (%)	29 (15)	132 (67)	<0.001*
Diabetic Neuropathy, *n* (%)	96 (48)	133 (67)	<0.001*
Macrovascular complications, *n* (%)	107 (54)	122 (60)	0.222
Diabetic foot, *n* (%)	9 (4.5)	25 (13)	0.0039*

DKD, diabetic kidney disease.
Data are presented as the mean ± SD (quantitative characteristics), unless otherwise indicated. In statistical comparisons, Student's *t*-test or a rank-sum test was performed for quantitative characteristics, and a chi-squared test was used for qualitative characteristics.
*$P < 0.05$.

Associations of the *G* allele and *GG* genotype of rs11643718 in *SLC12A3* with diabetic kidney disease

The minor allele frequency values of rs11549465, rs1801282, rs2032487, rs4821480, rs4821481, rs6566810 and rs12604675 were <5% (Table S3). In addition, rs759853 was not in accordance with the Hardy–Weinberg equilibrium test in the healthy control group ($P = 0.017$; Table S3). Exclusion of the aforementioned eight SNPs led to 16 SNPs left for further association analyses.

To explore the relationship between these SNPs and DKD, comparisons of the genotype constituent ratio and allele distribution between the DKD and diabetes-only groups were conducted. As shown in Table 2, the constituent ratio of genotypes in *SLC12A3* rs11643718 was different between the DKD and diabetes-only groups ($P = 0.014$). A difference in allele distribution between the two groups was also observed. The *G* allele frequency was much higher in the DKD group than the diabetes-only group (95% vs 90%; $P = 0.015$). These results indicate that this SNP may be related to DKD in type 2 diabetes. However, the result was no longer significant after multiple testing correction (adjusted $P > 0.05$; Table 2). To further clarify the effect of rs11643718, comparison of genotypes of rs11643718 using the recessive model was conducted. As shown in Table 3, the frequency of the *GG* genotype was markedly higher in the DKD group (90% vs 81%, OR 2.089, 95% CI 1.178–3.704; $P = 0.011$). After correction for confounding factors such as age, gender, BMI, diabetes duration, family history and HbA_{1c} level, significant differences remained (adjusted OR 2.056, 95% CI 1.120–3.776; $P = 0.020$). These results suggest a potential risk role for the *GG* genotype of rs11643718 in DKD, which might be independent of such confounding factors. In addition, the OR for the *G* allele in rs11643718 was 1.935 (95% CI 1.126–3.326; Table 3). Furthermore, the Combined Annotation Dependent Depletion (CADD) score obtained using the online CADD tool indicated that rs11643718 may be functionally deleterious (Table S1). Overall, these results imply that the *G* allele and *GG* genotype of rs11643718 in *SLC12A3* were associated with DKD.

Association of the *G* allele and *GG*+*AG* genotype of rs10951509 and rs1345365 in *ELMO1* with diabetic kidney disease, and high linkage disequilibrium of these two SNPs

The *G* allele frequencies of rs10951509 and rs1345365 in *ELMO1* also differed between the DKD and diabetes-only groups [rs10951509: 32% vs 24% ($P = 0.014$); rs1345365:

Table 2 Comparisons of genotype constituent ratios and allele distributions of 16 eligible SNPs between participants with type 2 diabetes with and without diabetic kidney disease

	Diabetes-only group *n* (%)	DKD group *n* (%)	*P*	Adjusted *P* value†
rs1063537 *C>T*				
CC	105 (53)	112 (54)	0.691	
CT	84 (42)	81 (39)		
TT	11 (5.5)	15 (7.2)		
C	294 (74)	305 (73)	0.953	
T	106 (27)	111 (27)		
rs10795433 *A>C*				
AA	82 (41)	66 (32)	0.136	
AC	86 (43)	107 (51)		
CC	32 (16)	35 (17)		
A	250 (63)	239 (57)	0.141	
C	150 (38)	177 (43)		
rs10951509 *G>A*				
GG	16 (8.0)	25 (12)	0.059	
GA	64 (32)	82 (39)		
AA	120 (60)	101 (49)		
G	96 (24)	304 (52)	0.014*	0.224
A	304 (76)	284 (48)		
rs11643718 *G>A*				
GG	162 (81)	187 (90)	0.014*	0.224
GA	37 (19)	20 (10)		
AA	1 (0.5)	1 (0.5)		
G	361 (90)	394 (95)	0.015*	0.240
A	39 (10)	22 (5.0)		
rs1345365 *G>A*				
GG	18 (9.0)	28 (13)	0.086	
GA	64 (32)	79 (38)		
AA	118 (59)	101 (49)		
G	100 (25)	135 (32)	0.019*	0.304
A	300 (75)	281 (68)		
rs1617640 *C>A*				
CC	7 (3.5)	12 (5.8)	0.554	
CA	65 (33)	66 (32)		
AA	128 (64)	130 (63)		
C	79 (20)	90 (22)	0.507	
A	321 (80)	326 (78)		
rs1801133 *G>A*				
GG	88 (44)	82 (39)	0.488	
GA	89 (45)	95 (46)		
AA	23 (12)	31 (15)		
G	265 (66)	259 (62)	0.235	
A	135 (34)	157 (38)		
rs1917760 *G>T*				
GG	130 (65)	137 (66)	0.775	
GT	65 (33)	68 (33)		
TT	5 (2.5)	3 (1.4)		
G	325 (81)	342 (82)	0.722	
T	75 (19)	74 (18)		
rs2237897 *C>T*				
CC	97 (49)	123 (59)	0.084	
CT	89 (45)	71 (34)		
TT	14 (7.0)	14 (6.7)		
C	283 (71)	317 (76)	0.078	
T	117 (29)	99 (24)		
rs2241766 *T>G*				
TT	105 (53)	112 (54)	0.897	
TG	82 (41)	81 (39)		
GG	13 (6.5)	15 (7.2)		
T	292 (73)	305 (73)	0.919	
G	108 (27)	111 (26)		
rs2268388 *G>A*				
GG	122 (61)	108 (52)	0.109	
GA	60 (30)	83 (40)		
AA	18 (9.0)	17 (8.2)		
G	304 (76)	299 (72)	0.180	
A	96 (24)	117 (28)		
rs2283228 *A>C*				
AA	93 (47)	109 (52)	0.445	
AC	89 (45)	80 (38)		
CC	18 (9.0)	19 (9.1)		
A	275 (69)	298 (72)	0.368	
C	125 (31)	118 (28)		
rs429358 *T>C*				
TT	156 (78)	173 (83)	0.387	
TC	43 (22)	34 (16)		
CC	1 (0.5)	1 (0.5)		
T	355 (89)	380 (91)	0.215	
C	45 (11)	36 (9.0)		
rs4892247 *C>T*				
CC	7 (3.5)	4 (1.9)	0.252	
CT	56 (28)	72 (35)		
TT	137 (69)	132 (63)		
C	70 (18)	80 (19)	0.523	
T	330 (83)	336 (81)		
rs7412 *C>T*				
CC	166 (83)	173 (83)	0.476	
CT	33 (17)	31 (15)		
TT	1 (0.5)	4 (1.9)		
C	365 (91)	377 (91)	0.756	
T	35 (9.0)	39 (9.0)		
rs741301 *C>T*				
CC	18 (9.0)	23 (11)	0.706	
CT	91 (46)	97 (47)		
TT	91 (46)	88 (42)		
C	127 (32)	143 (34)	0.426	
T	273 (68)	273 (66)		

DKD, diabetic kidney disease.
Chi-squared tests were used for the comparisons of genotype constituent ratios and allele distributions between groups. Bonferroni correction was used for multiple testing correction. *$P < 0.05$. †*P* value after multiple testing correction.

32% vs 25% ($P = 0.019$), respectively (Table 2)], indicating the potential effect of rs10951509 and rs1345365 in DKD. The significance disappeared after multiple testing correction (adjusted $P > 0.05$). In the dominant model, the frequencies of the *GG+AG* genotype of rs10951509 and rs1345365 in *ELMO1* were higher in the DKD group [rs10951509: 51% vs 40%, OR 1.589, 95% CI 1.073–2.353 ($P = 0.020$); rs1345365: 51% vs 41%, OR 1.525, 95% CI 1.031–2.255 ($P = 0.034$)]. After adjustment for possible confounding factors, significant differences remained [rs10951509: adjusted OR 1.738, 95% CI 1.143–2.643 ($P = 0.010$); rs1345365: adjusted OR 1.681, 95% CI 1.106–2.555 ($P = 0.015$)]. These results suggest that the *GG+AG* genotypes of rs10951509 and rs1345365 in *ELMO1* might play an independent role in the development of DKD. In addition,

Table 3 Comparisons of genotype and allele of three critical SNPs between participants with type 2 diabetes with and without diabetic kidney disease

	Genotypes (%)		Alleles		OR (95% CI)		
rs11643718*	*AA+AG*	*GG*	*A*	*G*	*G*	*GG*	*GG*‡
Diabetes-only	38 (19)	162 (81)	0.10	0.90	1.935 (1.126, 3.326)	2.089 (1.178, 3.704)	2.056 (1.120, 3.776)
DKD	21 (10)	187 (90)	0.05	0.95	$P = 0.015$	$P = 0.011$	$P = 0.020$
rs10951509†	*AA*	*GG+AG*	*A*	*G*	*G*	*GG+AG*	*GG+AG*‡
Diabetes-only	120 (60)	80 (40)	0.76	0.24	1.472 (1.081, 2.004)	1.589 (1.073, 2.353)	1.738 (1.143, 2.643)
DKD	101 (49)	107 (51)	0.68	0.32	$P = 0.014$	$P = 0.020$	$P = 0.010$
rs1345365†	*AA*	*GG+AG*	*A*	*G*	*G*	*GG+AG*	*GG+AG*‡
Diabetes-only	118 (59)	82 (41)	0.75	0.25	1.441 (1.062, 1.956)	1.525 (1.031, 2.255)	1.681 (1.106, 2.555)
DKD	101 (49)	107 (51)	0.675	0.325	$P = 0.019$	$P = 0.034$	$P = 0.015$

DKD, diabetic kidney disease.
Chi-squared tests were used for the comparisons of frequencies of studied allele and genotype between diabetes-only and DKD groups.
*Recessive model studied.
†Dominant model studied.
‡Adjusted for age, gender, BMI, diabetes duration, family history and HbA_{1c}.

the ORs for the *G* allele in rs10951509 and rs1345365 were 1.472 (95% CI 1.081–2.004) and 1.441 (95% CI 1.062–1.956), respectively (Table 3). In summary, these results suggest that rs10951509 and rs1345365 in *ELMO1* were associated with DKD.

Additionally, the linkage disequilibrium analyses for rs741301, rs1345365 and rs10951509 in gene *ELMO1* showed that the D' values for rs1345365–rs10951509, rs741301–rs1345365 and rs741301–rs10951509 were 0.994, 0.038 and 0.044, respectively, and the r^2 values were 0.947, 0.001 and 0.002, indicating that rs1345365 and rs10951509 were in high linkage disequilibrium (Fig. S1).

Meta-analyses confirming the relationship between *SLC12A3* rs11643718 and diabetic kidney disease in people with type 2 diabetes

By screening the related literature based on the previously mentioned criteria, we obtained four, one and two articles for rs11643718, rs10951509 and rs1345365, respectively. Meta-analyses were only conducted for rs11643718, as the reports of rs10951509 and rs1345365 were limited in number. As shown in Fig. 1, a total of 1086 individuals with type 2 diabetes but without DKD and 1250 individuals with type 2 diabetes and DKD were collected in this meta-analysis. A moderate degree of heterogeneity existed in the recessive model ($I^2 = 45\%$, $P = 0.12$). The combined effect in the random-effects model showed that the *GG* genotype had a higher frequency in participants with DKD [91% vs 83%, OR 1.76, 95% CI 1.24–2.51; $P = 0.002$ (Fig. 1a)]. Additionally, in the allele model, moderate heterogeneity existed ($I^2 = 45\%$, $P = 0.12$), and the overall frequency of allele *G* was also higher in the DKD group (95% vs 91%), with an overall OR value of 1.68 [95% CI 1.21–2.34; $P = 0.002$ (Fig. 1b)]. The results of the meta-analyses confirmed the relationship between *SLC12A3* rs11643718 and DKD in people with type 2 diabetes.

Association of the *GG* genotype of *SLC12A3* rs11643718 with worse renal function and higher albuminuria in participants with type 2 diabetes and diabetic kidney disease

Comparisons of renal-related traits between the *AA+AG* and *GG* genotypes of *SLC12A3* rs11643718 were used to assess the clinical significance of this SNP. As shown in Table 4, uric acid levels and the frequencies of abnormal serum creatinine were not significantly different between the groups ($P = 0.607$ and $P = 0.076$). However, participants with the *GG* genotype had a higher incidence of abnormal blood urea nitrogen than participants with the *AA+AG* genotype (47% vs 32%; $P = 0.038$). In addition, when compared with the *AA+AG* genotype, participants with the *GG* genotype had a higher probability of albuminuria ($P = 0.0052$). Comparison of the renal-related traits for *ELMO1* rs10951509 and rs1345365 are shown in Tables S4 and S5. There was no difference in renal traits between individuals with the *GG+GA* genotype and those with the *AA* genotype.

Association of expression of *SLC12A3* with renal-related traits and diabetic kidney disease

Although gene *SLC12A3* is just biologically plausible but may not be directly linked to rs11643718, we conducted a preliminary assessment of the relationship between the expression of this gene and DKD, as well as its relationship with rs11643718.

Data from Nephroseq showed that *SLC12A3* was mainly expressed in the outer and inner renal cortex, and the abundance of *SLC12A3* in the tubulointerstitium was much higher than that in the glomeruli (Fig. 2a,b). Additionally,

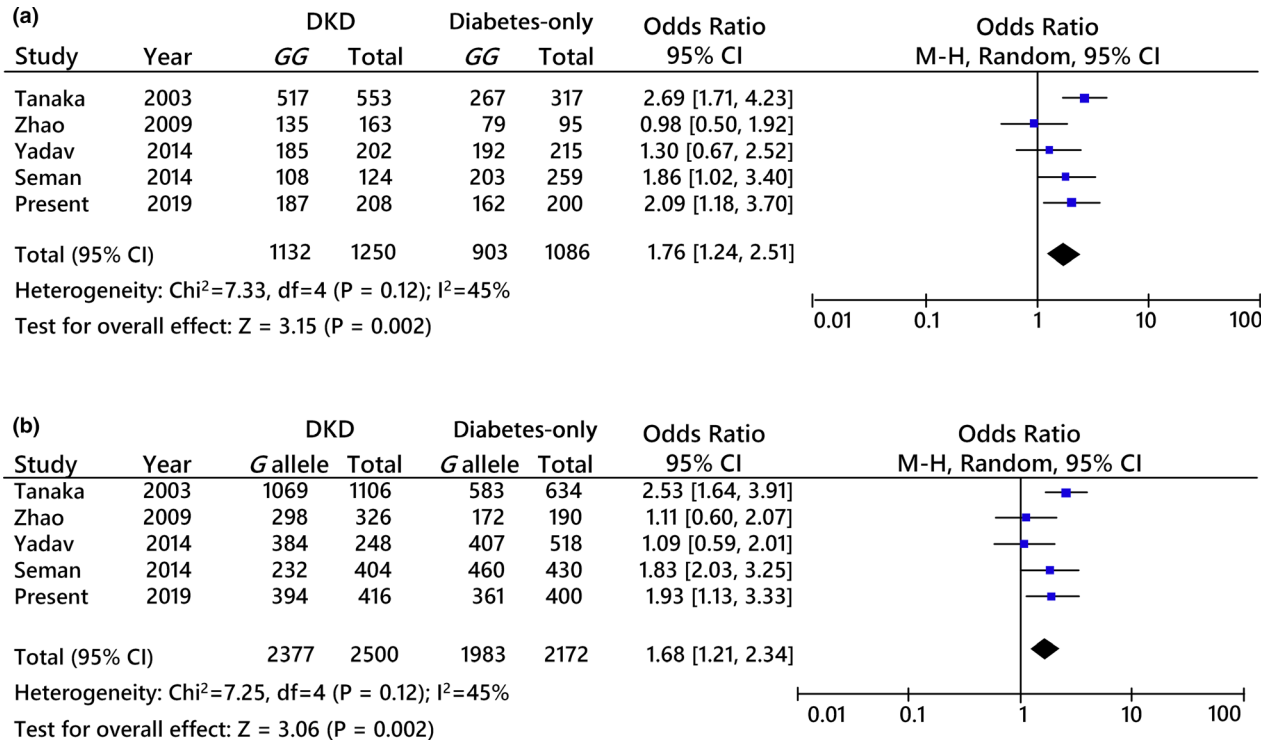

FIGURE 1 Forest plots of the association between the *SLC12A3* rs11643718 polymorphism and diabetic kidney disease (DKD) in people with type 2 diabetes. (a) Forest plot of genotypes in *SLC12A3* rs11643718 in the recessive model. (b) Forest plot of alleles in *SLC12A3* rs11643718 in the allele model. The position and area of the squares receptively reflect the odds ratio (OR) and weight of each study. The horizontal lines represent the 95% CI of the specific study. The diamond represents the overall OR and 95% CI. *P* values < 0.05 were considered statistically significant.

there was a lower expression of tubulointerstitium *SLC12A3* in DKD populations than in healthy populations (Fig. 2c). Furthermore, tubulointerstitial *SLC12A3* expression and eGFR were positively correlated in DKD (Fig. 2d).

Immunohistochemistry using anti-*SLC12A3* on renal biopsies was also conducted. As shown in Fig. 3a, the expression of *SLC12A3* was mainly localized in the distal tubules. The *SLC12A3* expression level of participants with type 2 diabetes and DKD was significantly lower when compared with the control group, especially in participants with the *GG* genotype in rs11643718 [DKD with *GG* vs control group 0.56 ± 0.14 vs 1.00 ± 0.09; $P < 0.0001$ (Fig. 3b)].

The results of immunohistochemistry and data from Nephroseq indicate that *SLC12A3* may be related to DKD, and the immunohistochemistry results also indicate the potential relationship between rs11643718 and *SLC12A3*.

Table 4 Comparisons of renal-related traits between different genotypes of *SLC12A3* rs11643718e

Variable	*AA+AG*	*GG*	*P*
Uric acid, μmol/l	306±87	313±93	0.607
Abnormal blood urea nitrogen, *n* (%)	19 (32)	163 (47)	0.038*
Abnormal Serum creatinine, *n* (%)	12 (20)	111 (32)	0.076
Urinary albumin level, *n* (%)			0.0052*
Normal	36 (69)	149 (50)	
Microalbuminuria	10 (19)	50 (17)	
Macroalbuminuria	6 (12)	101 (34)	

Data are presented as mean ± SD (quantitative characteristics) unless otherwise indicated.
Student's *t*-test or the rank-sum test was performed for quantitative variables, and chi-squared test for qualitative variables.
*$P < 0.05$.

Discussion

In the present study, the association between DKD and 24 SNPs was examined in Chinese individuals with type 2 diabetes. Three SNPs in *SLC12A3* (rs11643718) and *ELMO1* (rs10951509 and rs1345365) were found to be associated with DKD, especially rs11643718, the relationship between which and DKD was supported by more evidences, and was further confirmed by meta-analyses. Further analyses showed that the *GG* genotype in rs11643718 was associated with worse renal function. These results imply that the *G* allele and *GG* genotype in *SLC12A3* rs11643718 may be risk factors in the development of DKD in type 2 diabetes.

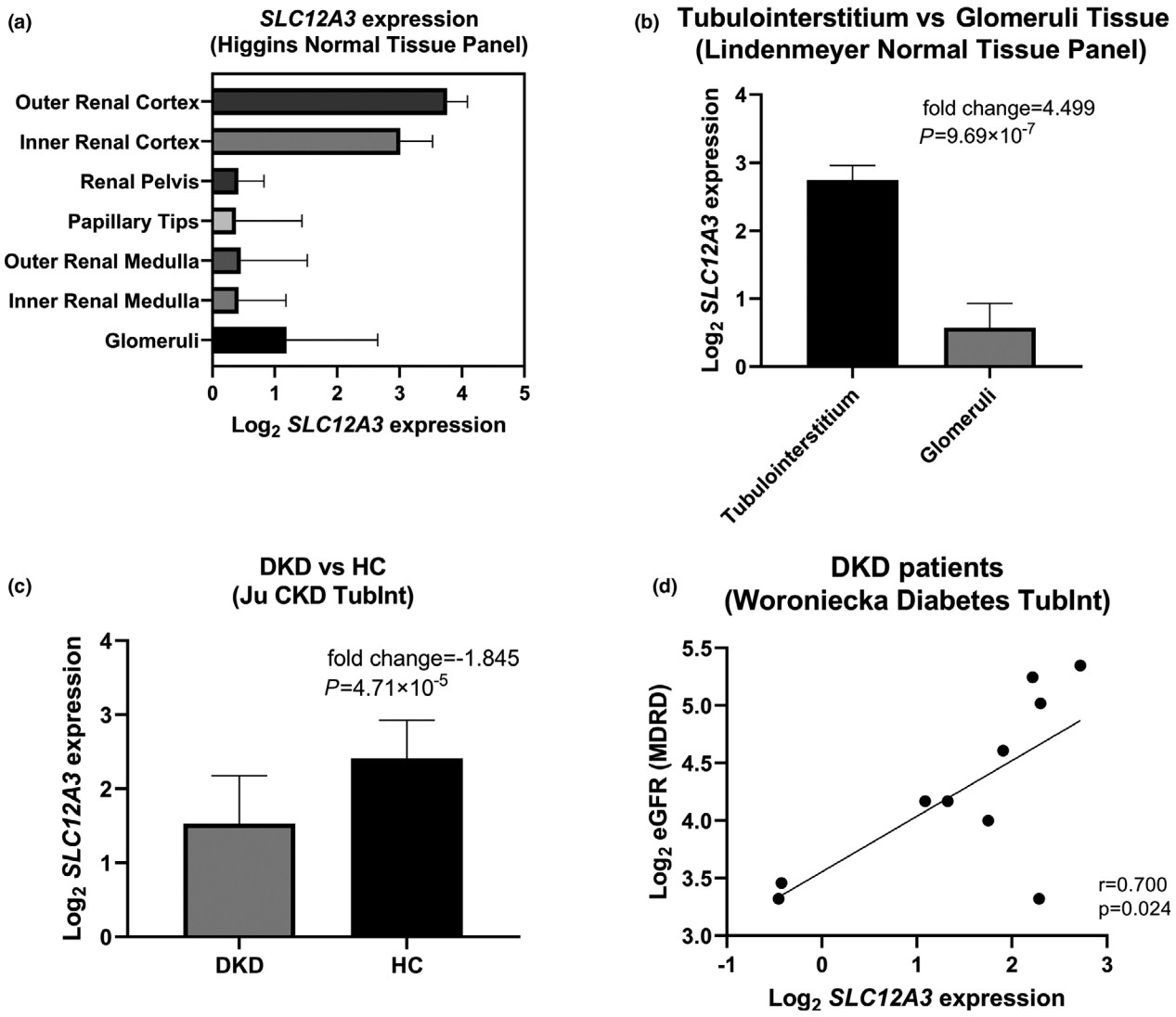

FIGURE 2 Nephroseq database-based analyses of the expression of *SLC12A3* in human renal tissue. (a) The tissue distribution of *SLC12A3* expression in normal human renal tissue. (b) Distribution difference of *SLC12A3* between renal tubulointerstitial and glomerulus. (c) The difference in tubulointerstitial *SLC12A3* expression between participants with diabetic kidney disease (DKD) and healthy controls (HC). (d) Tubulointerstitial *SLC12A3* expression was positively correlated with the estimated GFR (eGFR) in DKD populations. *P* values for (b) and (c) were obtained by Student's t-test; *P* values for (d) were obtained by Spearman's correlation analyses. *P* values < 0.05 were taken to indicate statistical significance.

SLC12A3 was first identified as a candidate for DKD by a Japanese genome-wide association study in 2003, which showed that the *A* allele in rs11643718 of *SLC12A3* might contribute to the reduction in DKD susceptibility [22]. A 10-year longitudinal study further confirmed the results in a Japanese population [23]. In the present study, we found that the *G* allele and *GG* genotype in *SLC12A3* rs11643718 was distributed more in the DKD group, which was consistent with the results of the above studies. However, contradictory results or results that showed no significant differences have also been reported. Research conducted in Korea led to the opposite conclusion that the *A* allele was associated with an increased risk for the development of DKD [24], while in a white [25] and an Indian [26] population, variations at this *SLC12A3* locus were found to be unlikely to explain the risk of advanced DKD among those with type 2 diabetes.

Several factors may explain these contradictory results. The 'winner's curse', whereby the initial study may find a larger effect size than subsequent replication studies, may have affected the first finding in the Japanese population [25]. However, this would not explain the conflicting results of later studies. Ethnic differences may be an important contributor to these contradictory results, which may thus indicate that such differences and differences in underlying biology exist in DKD. In Japanese [22,23] and Malaysian populations [16], it was found that the *G* allele in rs11643718 might be a risk allele, while in a Korean population [24], the conclusion was the opposite. Studies

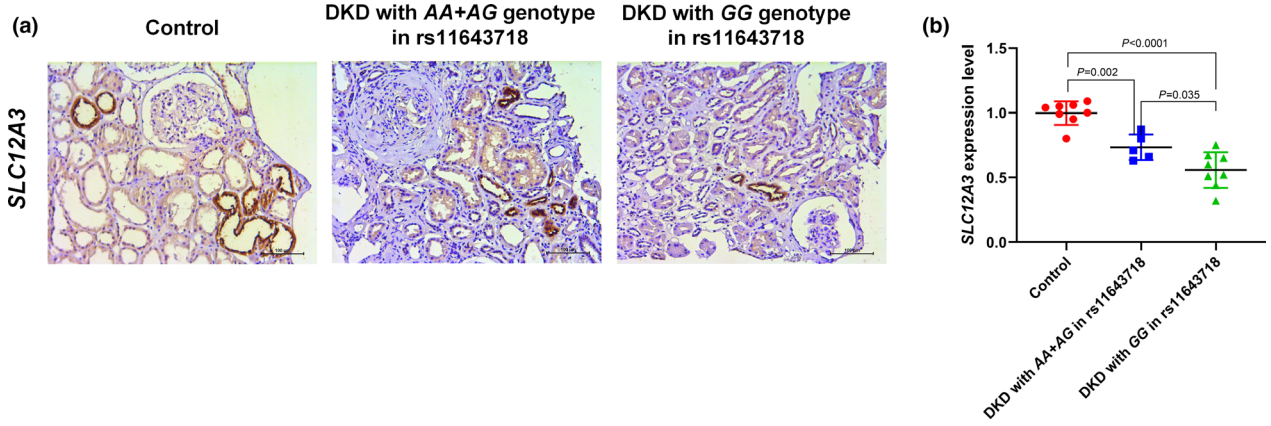

FIGURE 3 The expression level of *SLC12A3* in renal tubules was decreased in participants with type 2 diabetes and diabetic kidney disease (DKD), especially those with the *GG* genotype in rs11643718. (a) Immunohistochemistry staining of renal biopsy tissues with *SLC12A3* antibody in participants with glomerular minor lesions, participants with type 2 diabetes, DKD and the *AA+AG* genotype in rs11643718, and participants with type 2 diabetes, DKD and the *GG* genotype in rs11643718. Control, $n = 8$; DKD with the *AA+AG* genotype in rs11643718, $n = 5$; DKD with the *GG* genotype in rs11643718, $n = 8$. Bar = 100 μm. (b) The difference in renal tubule *SLC12A3* abundance between groups. Values are presented as mean ± SD. The *P* value was obtained by the Tukey honestly significant difference test. *P* values < 0.05 were taken to indicate statistical significance.

in white [25] and Indian populations [26] showed that this SNP was not significant. However, the results of the present study in a Chinese population differ from those found in a Chinese population by Zhao *et al.* [27]. The limited sample size might have led to the non-significant results shown by Zhao *et al.* and to the differences in results among studies. Differing inclusion criteria for cases may be a more important contributor to the variation. Tanaka *et al.* [22] selected individuals with diabetic retinopathy as well as overt nephropathy or who were receiving chronic renal replacement therapy as their kidney disease cases, while Kim *et al.* [24] focused on individuals with end-stage renal disease, and Daniel *et al.* [25] focused on those with advanced DKD (proteinuria or chronic renal failure or end-stage renal disease). By combining similarly designed studies, our meta-analyses expanded the sample size and found that the effect of the *G* allele and *GG* genotype on DKD might be universal.

Rs11643718 is located in exon 23 of the gene *SLC12A3* that encodes a renal thiazide-sensitive Na^+/Cl^- cotransporter, which plays an important role in maintaining electrolyte homeostasis and regulating blood pressure [28]. This SNP causes Arg/Gln amino acid change of the *SLC12A3* protein, and it was reported that the protein structures of *SLC12A3* changed dramatically when the mutant allele 913Gln substituted the wild allele Arg913 in amino acid sequences, suggesting that rs11643718 is functionally relevant [16]. Although there is no evidence strongly linking rs11643718 to the expression level of gene *SLC12A3*, our results suggest that this SNP and the expression level of *SLC12A3* might be related. However, more in-depth and comprehensive research is needed to further explore the relationship between rs11643718 and *SLC12A3*, and to explore the relationship between this SNP and other nearbygenes.

For *ELMO1*, first reported by Shimazaki *et al.* [11], 10 SNPs of the *ELMO1* gene were found to be related to DKD. Then, Leak *et al.* [17] found that variants in intron 13 of the *ELMO1* gene appear to confer risk of type 2 diabetes-associated end-stage renal disease in a black population, including *G* alleles of rs10951509 and rs1345365 [17]. However, contrasting results were found in Pima Indians and in China. The family study by Hanson *et al.* [18] indicated that *G* alleles in rs10951509 and rs1345365 might be protective against DKD, and the results of the study by Wu *et al.* [12] showed that the *G* allele of rs10951509 may be a protective allele of DKD in a Chinese population, while rs1345365 was not related to the disease [12]. Furthermore, no significant association was also reported in Mexican American populations [29] and Iranian populations [30]. Our results showed that the *G* alleles and *GG* genotypes of *ELMO1* rs10951509 and rs1345365 were more frequent in the DKD group, which was consistent with the results of Leak *et al.* [17]. In addition to the differences in race and genetic background, limited sample size and the distinct definitions of cases might be more important contributors to the contradictory results of SNPs in *ELMO1*.

The present study also has some potential limitations. The effect estimates were based on a hospital cross-sectional study, therefore, selective bias and information bias could not be avoided. In addition, unlike prospective studies, case–control studies cannot ascertain causality. The limited sample size may also have affected the test power. Hence, a large, multicentre, prospective study is necessary in the future. The definition of DKD also presents a challenge. Differences in the definition of disease and the selection of research populations have led to large heterogeneity among the research results and are not conducive to accurate analyses of the sources of heterogeneity. More international

cooperation may help to replicate the same research design in different ethnic groups and solve this problem. Although our results indicate that there may be a correlation between rs11643718 and the expression level of *SLC12A3*, the relationship is still unclear, and further verification by expanding the sample size is required, while the specific mechanism of rs11643718 in the development of DKD remains to be further studied.

In summary, this study mainly identified the *G* allele and *GG* genotype of rs11643718, as well as the *G* allele and *GG+AG* genotype of rs10951509 and rs1345365 as possible risk factors for the progression from type 2 diabetes to DKD in Chinese individuals. Analysing these genotype in Chinese populations with diabetes might contribute to the identification of people who are at high risk of developing DKD, thus influencing the selection of further treatment strategies.

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Competing interests

None declared.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Information and representative literature sources for the 24 selected SNPs.
Table S2 Sequences of primers and probes for genotyping of the 24 SNPs.
Table S3 Minor allele frequencies and Hardy–Weinberg equilibrium test of the 24 SNPs.
Table S4 Comparisons of renal related traits between different genotypes of ELMO1 rs10951509.
Table S5 Comparisons of renal related traits between different genotypes of ELMO1 rs1345365.
Figure S1 Pairwise linkage disequilibrium plot between SNPs (rs741301, rs1345365 and rs10951509) in gene ELMO1.