Genetic and Biological Effects of Sodium-Chloride Cotransporter (*SLC12A3*) in Diabetic Nephropathy

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Key Words

SLC12A3 · Diabetic nephropathy · Type 2 diabetes

Abstract

Background/Aims: Solute carrier family 12 member 3 (*SLC12A3*) encodes a sodium/chloride transporter in kidneys. Previous reports suggest that Arg913Gln polymorphism in this gene is associated with diabetic nephropathy (DN), but the data appear to be inconsistent. Up to now, there is no biological evidence concerning the effects of *SLC12A3* in DN. In this study, we aim to evaluate the genetic effects of the *SLC12A3* gene and its Arg913Gln polymorphism with genetic and functional analyses. **Methods:** We genotyped *SLC12A3* genetic polymorphisms including Arg913Gln in 784 non-diabetes controls and 633 type 2 diabetes (T2D) subjects with or without DN in a Malaysian population and performed a meta-analysis of the present and previous studies. We further analyzed the role of *slc12a3* in kidney development and progress of DN in zebrafish and db/db mice. **Results:** We found that *SLC12A3* Arg913Gln polymorphism was associated with T2D ($p = 0.028$, OR = 0.772, 95% CI = 0.612–0.973) and DN ($p = 0.038$, OR = 0.547, 95% CI = 0.308–0.973) in the Malaysian cohort. The meta-analysis confirmed the

protective effects of *SLC12A3* 913Gln allele in DN (Z-value = -1.992 , $p = 0.046$, OR = 0.792). Furthermore, with knockdown of zebrafish ortholog, *slc12a3* led to structural abnormality of kidney pronephric distal duct at 1-cell stage. *Slc12a3* mRNA and protein expression levels were upregulated in kidneys of db/db mice from 6, 12, and 26 weeks at the age. **Conclusion:** The present study provided the first biological and further genetic evidence that *SLC12A3* has genetic susceptibility in the development of DN, while the minor 913Gln allele in this gene confers a protective effect in the disease.

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Introduction

There is an alarmingly high prevalence of type 2 diabetes (T2D) in Malaysia with a latest prevalence rate of 14.9% for adults aged 30 and above [1]. Diabetic nephropathy (DN) is a clinical syndrome characterized by persistent albuminuria and progressive decline in the glomerular filtration rate (GFR). This syndrome is the leading cause of

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chronic kidney disease and end-stage renal disease (ESRD). In Malaysia, ESRD caused by diabetes has increased and accounted for more than 50% of incident dialysis patients [2–5]. The public burden due to DN in Malaysia is enormous. T2D and DN are complex diseases reflecting the interplay of genetic and environmental factors. Therefore, we recently conducted genetic studies among the Malaysian population to investigate the genetic effects in the diseases.

Solute carrier family 12 member 3 (*SLC12A3*) encodes a sodium/chloride transporter in kidneys, and this transporter mediates reabsorption of Na⁺ and of Cl[−] in the distal convoluted tubule [6]. In 2003, Tanaka et al. performed a genome-wide analysis of gene-based single nucleotide polymorphisms (SNPs) in a Japanese population and found that Arg913Gln polymorphism in the *SLC12A3* gene was associated with reduced risk to DN in T2D [7]. Another 10-year longitudinal study in the same population confirmed that the 913Gln allele of this polymorphism conferred a protective effect in DN [8]. In 2006, Tim et al. reported the genetic association of the *SLC12A3* Arg913Gln polymorphism with ESRD in a Korean population. But the data implicated that the 913Gln allele might confer the risk for DN [9]. In 2008, Ng et al. demonstrated that the *SLC12A3* genetic polymorphisms including Arg913Gln were not associated with T2D and DN in an American Caucasians population [10]. Obviously, there are confusions among these previous genetic studies. Until now, no biological study of *SLC12A3* in DN has been reported. Therefore, it is necessary to evaluate the genetic effects of *SLC12A3* in T2D and DN with genetic and functional analyses.

In this study, we initially performed a genetic association study of eight Tag *SLC12A3* polymorphisms in a Malaysian population, including non-diabetic control subjects, T2D patients with or without DN, and performed a meta-analysis of the association between the *SLC12A3* Arg913Gln polymorphism and DN from the present and previous studies [7, 9, 10]. We further investigated the role of *slc12a3* in kidney development with zebrafish embryos and in the progress of DN with db/db mice. Based upon the data from both genetic and functional analyses in the patients with DN and animal models, we thus enabled to evaluate the genetic effects of *SLC12A3* in DN.

Research Design and Methods

Subjects

Malaysia is a country with multi-cultures and multi-ethnic populations. We collected the samples of subjects with normal glucose tolerance (NGT) and patients with T2D from the collaborat-

ing centers all over Malaysia. The ethnic distribution of our study subjects was 65.8% Malays, 17.3% Chinese, and 16.9% Indians. In the present study, there was no difference of allelic frequencies of the *SLC12A3* polymorphisms among these three ethnic groups. We thus combined all subjects together for the analyses. In all, 784 individuals with NGT (controls) and 633 patients with T2D (cases) were included into this study. Diagnoses of T2D were done based on World Health Organization (WHO) criteria [11]. The diagnoses of DN were based on the urine albumin-to-creatinine ratio (ACR) suggested by the American Diabetes Association (ADA) [12]. The patients with T2D and normoalbuminuria (ACR <3.5 mg/mmol) were considered controls for DN, while the patients with macroalbuminuria (ACR ≥35 mg/mmol) and ESRD who needed dialysis were included as the cases for DN. Creatinine in serum and urine were measured using Randox Assayed Multisera (Randox Laboratories Ltd., Crumlin, UK). Urine or serum was mixed with sodium hydroxide in a biochemistry analyzer (Selectra E, MEDEX, Gyeonggi-do, South Korea). Creatinine in the alkaline solution reacted with picric acid to form a colored complex. The amount of the complex formed was measured at a wavelength of 490 to 510 nm. The reading of the complex measured is directly proportional to the creatinine concentration. The estimate of glomerular filtration rate (GFR) in the dialysis patient was calculated by the mean of renal urea and creatinine clearance from a 24-hour urine correction [13]. Clinical characteristics of all subjects with NGT and T2D with and without DN are summarized in table 1. In this table, 250 patients with microalbuminuria (ACR 3.5–35 mg/mmol) were included into the group of T2D but excluded from the groups of T2D with or without DN.

All subjects answered a set of questionnaires and underwent clinical and physical examinations. Informed consent was obtained from all subjects, and the study was approved by the local ethical committees. Data and materials transfer agreement from Institute for Medical Research, Malaysia to Karolinska Institutet, Sweden was signed prior to the study.

SNP Selection and Genotyping

Eight SNPs in the *SLC12A3* gene were selected based on the information from the dbSNP and HapMap. The genetic location of these SNPs in gene map is summarized in table 2. They are tag-SNPs predicted by HapMap or non-synonymous SNPs. Validation and genotyping experiments were done using the TaqMan allelic discrimination technique. All SNP genotyping assays were designed and synthesized by Life Technologies (Grand Island, USA). ID numbers of the SNP genotyping assays were available upon request. PCR and post genotype scoring were performed with the ABI7300 real-time PCR system (Applied Biosystems, Foster City, USA). For genotyping quality control, the subjects were distributed randomly across PCR plates with the cases and controls. Negative controls (universal-mixture and distilled water blanks, at least three) were included on each plate.

Meta-Analysis

Meta-analysis is the statistical procedure for combining data from multiple studies. Recently, a team of experts in the United States and United Kingdom have developed a comprehensive meta-analysis software (CMA version 2), which is widely recognized for its clarity, ease of use, and accuracy [14]. We thus applied this program for meta-analysis. Three previous [7, 9, 10] and present studies were included into our meta-analysis because they met the

Table 1. Clinical characteristics of Malaysian subjects

Group	NGT	All T2D	T2D – DN	T2D + DN	p values ^{a/b}
N (men/women)	784 (368/416)	633 (320/313)	259 (111/148)	124 (68/56)	
Age, years	44±15	56±11	56±10	57±10	<0.001/<0.001
Diabetic duration, years	–	13±8	13±8	13±8	–/NS
BMI, kg/m ²	25.8±6.1	27.9±5.3	28.6±85.3	27.2±6.0	<0.001/<0.001
SBP, mm Hg	127±18	142±24	138±20	154±29	<0.001/<0.001
DBP, mm Hg	79±11	80±12	80±12	82±14	NS/NS
FPG, mmol/l	4.9±1.0	5.3±2.1	5.3±2.1	6.4±0.7	<0.049/<0.001
HbA1c, mmol/mol	5.4±0.8	8.0±2.2	8.0±1.9	8.8±2.2	<0.001/<0.001
eGFR, ml/min/1.73 m ²	115.4±58.1	91.6±50.4	94.7±35.1	59.8±35.5	<0.001/<0.001
Serum creatinine, µmol/l	68.0±38.6	115.9±105.9	90.8±71.1	202.3±171.2	<0.001/<0.001
ACR	0.8±0.7	54.7±179.5	1.3±1.7	269.8±341.2	<0.001/<0.001
Sodium, mmol/l	–	138.6±2.8	138.7±2.8	139.1±2.7	–/NS
Potassium, mmol/l	–	4.8±2.9	5.1±4.1	4.4±0.9	–/NS

Data are expressed as means ± SD. NGT = Normal glucose tolerance; T2D = type 2 diabetes; DN = diabetic nephropathy; BMI = body mass index; SBP and DBP = systolic and diastolic blood pressures; FPG = fasting plasma glucose; HbA1c = glycosylated hemoglobin; eGFR = estimated glomerular filtration rate; ACR = albumin creatinine ratio; p values were from tests of NGT vs. all ^a T2D and T2D – DN vs. ^b T2D + DN.

Table 2. Allelic frequencies of *SLC12A3* genetic polymorphisms in Malaysian population

SNP ID	SNP type	Location	Contig position*	MAF
rs2304480	W = T/A	Intron 2	10515487	0.133
rs1529927	S = G/C Gly263Ala	Exon 6	10518786	0.006
rs2304483	R = G/A	Intron 13	10532152	0.004
rs11643718	R = G/A Arg913Gln	Exon 23	10547718	0.123
rs6499858	Y = C/T	Intron 23	10549319	0.484
rs12708965	Y = C/T Arg928Cys	Exon 24	10550518	0.038
rs11640308	R = G/A	Intron 25	10554168	0.084
rs2010501	Y = C/T	Intron 25	10559738	0.262

Nucleotide and amino acid codes are provided by International Union of Pure and Applied Chemistry (IUPAC). * Contig accession number is NT_010498.15 in GRCh37.p10; MAF = Minor allele frequency.

following criteria: (1) the case-control population genetic association studies were considered; (2) the association between the *SLC12A3* Arg913Gln polymorphism and DN in T2D were evaluated; (3) the genotype distribution of the *SLC12A3* Arg913Gln polymorphism was described and the results were expressed as OR and 95% CI. We thus included all the three previous and present studies into the meta-analysis.

Bioinformatics and Clinical Analyses

Transcription Element Search System (TESS) and Protein Structure Prediction Server (PS²) are automated homology modeling servers used for conducting analyses of protein structures [15]. The methods were used in both template selection and target-template alignment [16]. A final three-dimensional structure of protein is built using the modeling package. To predict the possi-

bly functional differences of *SLC12A3* protein caused by the Arg913Gln polymorphism, we adapted these two programs for analysis of *SLC12A3* protein structures by adding amino acid sequences with either Arg or Gln at the position of 913 into the program. Further analyses of clinical parameters including eGFR and serum creatinine according to the genotypes of *SLC12A3* Arg913Gln polymorphism in NGT, T2D with and without DN were performed.

Analysis of the *slc12a3* Gene Expression in the Progress of Diabetic Nephropathy with db/db Mice

We used diabetic db/db mice at the ages of 6-, 12-, and 26-weeks to investigate the changes of *slc12a3* gene expression in the progress of DN because there was no suitable transfection study approach available. A total of 70 male homozygous db/db mice and

non-diabetic control littermates of the C57BL/KsJ strain were obtained from colonies at Karolinska University Hospital, Stockholm. All experimental procedures were approved by the North Stockholm's Ethical Committee for Care and Use of Laboratory Animals. Briefly, the mice were housed 2–4 per cage in a 12/12 hour light/dark cycle. Water and standard diet were provided ad libitum. The mice were tested for blood glucose levels and body weight. Kidney tissues from 6-, 12- and 26-week old db/db and control mice were harvested and stored with RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) at -80°C until use. Freshly collected kidney tissues were used in immunohistochemistry experiments.

To extract total mRNAs, homogenization of kidney tissues was prepared with a Mini Beadbeater according to the protocol that we developed in our laboratory [17]. Total mRNAs from kidney tissues in each animal were extracted by using an RNeasy Mini Kit (Qiagen). The integrity and concentration of mRNA samples were assessed with 1.2% agarose gel electrophoresis and determined spectrophotometrically in NanoDrop ND2000 (Thermo, Uckfield, UK). Based on the templates of mRNA samples, cDNAs were synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). To detect the *slc12a3* gene mRNA expression levels, a specific TaqMan gene expression assay (ID No. Mm00490213_m1, Life Technologies, Grand Island, USA) was designed according to the mRNA sequence of the gene in mouse (NM_019415.2). The primers covered 19–20 exon boundary of the mouse *slc12a3* gene. For control, the assays of Gapdh (Mm99999915_m1) and 18s (Mm03928990_g1) were used. TaqMan real time RT-PCR experiments were performed with a standard protocol and in an ABI7300 real-time PCR system (Applied Biosystems). The experiments were replicated on two or three occasions.

To detect *slc12a3* protein expression levels in kidneys of db/db and control mice, we used an immunohistochemistry protocol. Freshly collected tissues/kidneys were embedded into OCT solution and stored at -80°C until sectioning. One hour prior to sectioning, the tissue blocks were brought to -20°C and cut into (5–15 μm) cryosections placed on a frosted glass. After drying the sections for 30 min at room temperature they were fixed with ice-cold acetone for 20 min. Acetone was evaporated and the sections were hydrated for 10 min with PBS after which they were blocked with 5% goat serum in PBS for 30 min at room temperature. The sections were stained with rabbit anti-SLC12A3 from Abcam (Cambridge, UK) (1:200) poly-clonal antibodies for 1 h at 37°C in the blocking buffer. Goat anti-rabbit Alexa Fluor 488-conjugates (Life Technologies, Foster City, USA) were used as secondary antibodies (1:1,000), and incubated with the sections together with a nuclear DAPI (1:2,000) staining for 30 min at 37°C . The sections were mounted with (Dako, Glostrup, Denmark) fluorescent mounting medium and analyzed with conventional fluorescence microscopy (Leica, Wetzlar, Germany).

Analysis of slc12a3 in the Kidney Development with Zebrafish

To further understand whether *slc12a3* plays a role in the development of kidneys, we applied zebrafish as an animal model to examine the pathological changes when the gene was knocked down. Zebrafish was maintained by the core facility at Karolinska Institutet. The morpholino antisense oligonucleotide (MO) was used to knock down the *slc12a3* gene in zebrafish. A splicing blocking MO (E313-*slc12a3*-MO) targeting the exon 3 of the gene (5'-CAACAGAGATTGACATACCTATCCC-3') was purchased

from Gene Tools (Philomath Oregon USA). The MO with 350 μm was injected into the yolk of one- to two-cell embryos from *Tg(cdh17:mCherry)* line. In vivo analyses of injected zebrafish were done with a fluorescence microscopy (Leica TCS SP8, Germany).

Statistical Analyses

In human genetic study, statistical power was calculated using PowerSampleSize (PS version 2.1.31). The sample sizes of cases and controls were sufficient to detect the association with ~85% power (5% level) if allele frequency 0.15. Hardy-Weinberg equilibrium (HWE) for genotype distribution in each SNP was assessed using the Pearson Chi-square (χ^2) statistic. An additive model was used to identify the difference in genotype distribution between cases and controls. A 2×2 contingency table was used to test the difference of minor allele frequencies between cases and controls. Odds ratios (OR) and 95% confidence intervals (CI) were calculated to test the relative risk for association. Tests for association between genotypes and clinical parameters were performed by using ANOVA for normally distributed traits, or alternatively Kruskal-Wallis analysis of ranks for traits with non-normal distribution. Normal probability plots were created and parameter distributions were transformed to natural logarithm when needed for obtaining a normal distribution before performing statistical analysis of the relevant phenotypes. Data presented in the table and figures are means $\pm$ SD. *p* value less than 0.05 were considered significant. In animal study, tests for comparison of continuous variables between two groups were assessed by the unpaired *t* test. All data were analyzed using the PASW statistic program (SPSS 22.0, Chicago, Ill., USA).

Results

Association of SLC12A3 Arg913Gln Polymorphism with Diabetic Nephropathy

We performed the genetic association study of the *SLC12A3* gene in Malaysian subjects with NGT and T2D patients with or without DN. The genotype distributions of 8 SNPs in the *SLC12A3* gene were kept in Hardy-Weinberg equilibrium. Minor allele frequencies (MAFs) of all studied SNPs in Malaysian population were summarized in table 2. In SNP rs11643718, the frequency of 913Gln allele in this population was 0.123. Single marker association analyses indicated that the *SLC12A3* Arg913Gln polymorphism was associated with reduced risk in T2D ($p = 0.028$, OR = 0.772, 95% CI = 0.612–0.973) and DN ($p = 0.038$, OR = 0.547, 95% CI = 0.308–0.973) (see table 3).

We carried out a meta-analysis based upon the data from the present and previous genetic studies [7–9]. The genotype distributions of the *SLC12A3* Arg913Gln polymorphism in Malaysians, Japanese, Koreans, and American Caucasians population are summarized in table 3. The present study and the report from Tanaka et al. [7] implicated that the *SLC12A3* 913Gln allele had the

Table 3. Association of *SLC12A3* Arg913Gln polymorphism with type 2 diabetes and diabetic nephropathy

Population	Group	N	Genotypes			MAF	p value	OR (95% CI)	Reference
			Arg913Arg	Arg913Gln	Gln913Gln				
Malaysians	NGT	784	604	153	27	0.132			Present study
	All T2D	633	507	119	7	0.105	0.028	0.772 (0.612–0.973)	
	T2D – DN	259	203	54	2	0.112			
	T2D + DN	124	108	16	0	0.065	0.038	0.547 (0.308–0.973)	
Japanese	T2D – DN	543	463	78	2	0.076			Tanaka et al., 2003
	T2D + DN	716	668	46	2	0.035	<0.001	0.443 (0.309–0.636)	
Koreans	T2D – DN	183	164	18	1	0.055			Kim et al., 2006
	T2D + ESRD	175	136	37	2	0.117	0.003	2.295 (1.573–3.239)	
American Caucasians	T2D – DN	164	130	32	0	0.098	0.397		Ng et al., 2008
	T2D + DN	277	217	55	5	0.117		1.213 (0.775–1.897)	

NGT = Normal glucose tolerance; T2D = type 2 diabetes; DN = diabetic nephropathy; ESRD = end-stage renal disease; MAF = minor allele frequency.

protective genetic effect in DN, while the study from Kim et al. [8] showed that this allele had the risk susceptibility in ESRD. Ng et al. [9] found that there was no association between *SLC12A3* Arg913Gln polymorphism and DN in American Caucasians with T2D. All data from the present and three previous studies were included into the meta-analysis. We found that the *SLC12A3* Arg913Gln polymorphism was associated with the reduced risk for DN in T2D (Z-value = –1.992, $p = 0.046$, OR = 0.792, 95% CI = 0.629–0.996) (fig. 1).

Clinical Parameters in the Patients According to the Genotypes of *SLC12A3* Arg913Gln Polymorphism

Up to now, there is no direct biological evidence for the role of *SLC12A3* in DN mainly due to no suitable reporting system available for transfection experiment. In order to understand the functional consequence caused by *SLC12A3* Arg913Gln polymorphism, we first analyzed the changes of *SLC12A3* protein structures with bioinformatics. The images implicated that the protein structure of *SLC12A3* was dramatically changed when the mutant allele 913Gln substituted the wild allele Arg913 in amino acid sequences, suggesting that this polymorphism might have a functional relevance. We thus analyzed clinical parameters according to the genotypes of *SLC12A3* Arg913Gln polymorphism. Data demonstrated that among T2D patients, the carriers with Gln913Gln genotype had relatively low serum creatinine and high eGFR levels compared with the patients carrying the Arg913Arg genotype (online supporting information

Fig. 1. Meta-analysis of *SLC12A3* Arg913Gln polymorphism.

fig. 1, www.karger.com/doi/10.1159/000368916). However, the differences were not statistically significant mainly due to high standard deviations.

Upregulation of the *slc12a3* Gene Expression in Kidneys of *db/db* Mice

The genetic homology of *SLC12A3* between mice and humans was 90%. To understand the functional role of *SLC12A3* in DN, we further investigated the *slc12a3* gene expression at both the mRNA and protein levels in *db/db* mice at the different ages. Figure 2a, b and c showed that the *slc12a3* gene in kidneys of *db/db* mice at the ages of 6, 12, and 26 weeks was significantly over-expressed at mRNA levels compared with the control mice at the same ages. Figure 3a and b demonstrated that *slc12a3* protein with the stained antibody was distributed in the kidney distal con-

Fig. 2. *Slc12a3* mRNA expression levels in kidneys of db/db and control mice at the ages of 6, 14, and 26 weeks.

Fig. 3. *Slc12a3* in distal convoluted tubule of kidneys in db/db mice at the age of 6 weeks.

volute tubule of db/db and control mice at the age of 6-weeks. The signal intensity in kidneys of db/db mice was significantly higher than that in the control mice (fig. 3c).

Role of slc12a3 in Zebrafish Pronephric Duct Epithelium

Because the *slc12a3* gene expression at mRNA and protein levels in the kidneys of db/db mice at the age of 6-weeks were found to be significantly increased compared with the controls, we were interested to explore whether *slc12a3* plays a role in kidney tubular epithelium. The zebrafish *slc12a3* gene is conserved with 62% of amino acid identity compared with the human. We applied a specific MO-mediated antisense knockdown approach in zebrafish and found that the knockdown of zebrafish *slc12a3* did not lead to global alteration of embryonic de-

velopment compared to the wild-type embryos. Under the fluorescence microscopic analysis, however, pronephric duct epithelial structure defined by red signal (mCherry) in the cloacal portion was significantly altered at 4 dpf (fig. 4). The penetrance of this abnormal morphology was 35%. This implicated the importance of *slc12a3* in zebrafish pronephric distal duct, particularly in the cloacal development.

Discussion

In this study, we replicated the association of the *SLC12A3* genetic polymorphism with DN in Malaysian T2D patients and demonstrated that the minor allele 913Gln in the *SLC12A3* Arg913Gln polymorphism con-

Fig. 4. Phenotypes of zebrafish at 4 dpf with morpholino knock-down of the *slc12a3* gene.

ferred a protective effect in DN. Furthermore, we performed functional analyses with zebrafish and db/db mice and provided the first evidence that *slc12a3* plays the important roles in kidney cloacal development and in the progress of DN.

Genetic studies in T2D have successfully identified a list of 60 loci that are associated with the disease or glyce-mic traits. However, the progress of genetic study in DN has been hampered mainly due to the complexity of the disease and difficulty of collection and characterization of the subjects [18, 19]. Contradiction of the data from genetic studies of the same gene in DN is sometime seen [20]. The study of the *SLC12A3* gene in DN is such a typical example. Eleven years ago, Tanaka et al. published the first genetic study of *SLC12A3* and demonstrated that the *SLC12A3* Arg913Gln polymorphism was associated with DN by a protective effect in a Japanese population [7]. Five years later, Ng et al. found the negative association of the *SLC12A3* genetic polymorphisms with DN in a Caucasian population and they explained two possibilities [10]. First, there was a publication bias caused by ‘winner’s curse’ in the report of Tanaka et al. because there was no direct biological evidence in the literature. Second, the genetic effect of *SLC12A3* may be population specific. In the present study, we found that the *SLC12A3*

Arg913Gln polymorphism was associated with DN in a Malaysian population with a protective effect. Thus, our findings support the report from Tanaka et al. [7] and disagree with the so called ‘winner’s curse’. In the recent years, several genome wide association studies (GWAS) of DN have been performed in Caucasian populations. Although the results from these GWAS were inconsistent, no association of *SLC12A3* with DN was reported. Most likely, *SLC12A3* genetic effects are population specific to Asian but not Caucasian populations. In addition, Kim et al. reported that the *SLC12A3* Arg913Gln polymorphism was significantly associated with ESRD in a Korean population. However, in contrast to the report from Tanaka et al. [7], the 913Gln allele was associated with an increased risk of DN [9]. Kim et al. did not sufficiently explain how to understand the contradiction although they stated that the use of prevalent ESRD patients as cases might lead to strong survival bias. It seems that the reason behind the opposite genetic effects of *SLC12A3* in DN still needs to be clarified. Furthermore, data from the present study suggest that *SLC12A3* has genetic effects in T2D. In the previous studies [7–10], only T2D patients with and without DN were included but not non-diabetic control subjects. Therefore, the genetic association of this gene with T2D was ignored.

From the first genetic report of *SLC12A3* in DN [7] until now, there is lack of biological evidence to support the genetic study mainly due to no transfection approach available. In general, the animal model of db/db mouse appears to exhibit the most consistent and robust increase in albuminuria and mesangial matrix expansion. This animal has been used as a model of progressive diabetic renal disease. The findings from various studies on the renal pathology of db/db mouse have stated that this is a suitable model in the context of human DN [21]. We therefore took the opportunity to use db/db mice as animal model for analysis of the *slc12a3* gene expression at both mRNA and protein levels. Based upon the data from quantitative analyses with real time RT-PCR and morphological observations with specific antibody, we found that the *slc12a3* gene expression in the kidneys of db/db mice at the ages of 6, 14, and 26 weeks were upregulated compared with the controls. Interestingly, the *slc12a3* gene expression was found to be upregulated not only in elder db/db mice but also in young mice at the age of 6 weeks. It may be necessary to understand whether this gene plays a role in the kidney development. Zebrafish has been widely used to study principle pathways of kidney development and function [22–24]. We thus knocked down the *slc12a3* gene activity with morpholino tech-

nique and found that *slc12a3* may affect the pronephric distal duct structure. Based on the experiments with two animal models, the present study for the first time provides the biological evidence suggesting that SLC12A3 plays an important role in the kidney cloacal development and in the progress of DN.

Protein structure prediction with comparative modeling is one of the most reliable methods to predict functional consequences directly from amino acid sequences [15, 16]. The *SLC12A3* Arg913Gln polymorphism is non-synonymous. To investigate the biological consequence of the *SLC12A3* Arg913Gln polymorphism in humans, we adapted bioinformatics programs to understand the changes of 3D structures of SLC12A3 protein. We found that the structural morphology of SLC12A3 protein was changed with the mutant allele 913Gln compared to the wild type. We further comparatively analyzed the clinical parameters in T2D and DN patients according to the genotypes of the *SLC12A3* Arg913Gln polymorphism and found that the carriers with Gln913Gln genotype had relatively low serum creatinine and high eGFR levels compared with the patients carrying the Arg913Arg genotype. This observation is consistent with the genetic association study, which indicates that the minor allele 913Gln has protective effects in DN. SLC12A3 regulates the uptake of Na together with the Cl from the lumen into the kidney endothelial cell in the distal convoluted tubule (DCT). Sodium then returns to the blood system via the ATP-dependent sodium-potassium pump. Accumulated documents have demonstrated that the mutations in the *SLC12A3* gene caused Gitelman's syndrome [25–27]. This is a monogenic autosomal recessive disorder characterized by metabolic alkalosis, hypokalemia, hypomagnesemia, and hypocalciuria, while DN is a polygenic complex disease. Considering that the minor allele 913Gln in the *SLC12A3* gene has a protective effect, we have a hypothesis that the roles of SLC12A3 in DN and Gitelman's syndrome may be opposite. SLC12A3 had been used as targets for thiazide, an anti-hypertensive drug by inhibiting the binding of chloride ion to *SLC12A3*, resulting in the loss of function of the SLC12A3 protein [27–29]. SLC12A3 is involved in the RAAS pathway in the lumen of a kidney cell. The role of this molecule in this pathway still needs to be explored. Based on the findings in the present study that *slc12a3* is upregulated in DN, while the mutant allele 913Gln allele has protective effects in DN, we have deduced a hypothesis that inhibition of SLC12A3 activity as the target may result in the reduction of susceptibility to DN. Further investigation to understand the cellular mechanism will be considered.

In summary, the present study provided the first biological evidence that SLC12A3 plays an important role in kidney cloacal development and in the progress of DN. Furthermore, based upon the genetic study of the *SLC12A3* gene, we enabled to conclude that this gene has the susceptibility to DN in T2D, while the minor 913Gln allele of the *SLC12A3* Arg913Gln polymorphism confers a protective effect in DN.

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Disclosure Statement

None declared.

Author Contributions

HFG, KB, WNW and CGÖ proposed the project; HFG and NAS designed the study; WNW and NAS collected clinical data; NAS, BH, JO and HFG collected experimental data; NAS and HFG analyzed the data and wrote the manuscript; all contributed to data interpretation, discussion, and revision of the manuscript. HFG is the guarantor of this study and as such has full access to all the data and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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