Original Paper

Polymorphisms in the *SLC12A3* Gene Encoding Sodium-Chloride Cotransporter are Associated with Hypertension: A Family-Based Study in the Mongolian Population

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Key Words

Hypertension • *SLC12A3* • Family-based association test (FBAT) • Mongolian • Haplotype

Abstract

Background/Aims: Hypertension or persistent high blood pressure (BP) is a leading cause of death worldwide. Extensive evidence indicates that the thiazide-sensitive Na⁺-Cl⁻ cotransporter (NCC) affects BP via regulation of renal sodium reabsorption. However, the relationship between genetic variants of the NCC-encoding *SLC12A3* gene and hypertension in the Mongolian population is still ambiguous. In this study, we aimed to genotype an extended cohort of hypertensive Mongolian families for polymorphisms in the *SLC12A3* locus. **Methods:** Eighty-eight families with a history of hypertension, including parents, offspring, and relatives underwent clinical testing. Family-based association tests and haplotype analysis were used to evaluate the association between hypertension and polymorphisms in the *SLC12A3* locus. **Results:** We identified three single nucleotide polymorphisms (SNPs), one in the *SLC12A3* coding region ($p = 0.05$) and two in the intron ($p = 0.02$ and $p = 0.07$), which were significantly associated with the hypertension phenotype. Haplotype-specific association tests confirmed the correlation of these SNPs with hypertension ($p < 0.05$). **Conclusion:** These results suggest that SNPs in the *SLC12A3* gene confer susceptibility to hypertension in the Mongolian population. Further research is needed to validate the functional role of *SLC12A3* polymorphisms in hypertension.

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Introduction

Hypertension or persistent high blood pressure (BP) is a condition that significantly increases the risk of heart and cerebrovascular diseases, thus posing a severe threat to human health. Worldwide, more than one billion people have hypertension [1], which is the leading cause of mortality, accounting for approximately 7.5 million deaths each year [2–4]. Essential or primary hypertension, defined as high BP for which there are no secondary causes such as renovascular disease and renal failure, is the most common form, accounting for 90%–95% of all cases; secondary hypertension due to kidney, artery, heart, or endocrine system disorders covers the remaining 5%–10% [5]. Previous genome-wide association studies [6, 7] have identified genetic loci associated with hypertension, suggesting that it results from a complex interaction of genetic mechanisms and environmental factors. However, although a number of genetic polymorphisms and rare genetic variants associated with slight or significant effects on BP and hypertension have been identified [8, 9], the genetic basis of hypertension is still poorly understood.

It is established that salt homeostasis maintained by the kidneys is directly associated with BP and that renal sodium reabsorption plays an important role in the development of hypertension. The up-regulation of renal sodium reabsorption triggers BP elevation via the concomitant increase in water reabsorption to maintain plasma sodium concentration at or near 140 mM; the resultant increase in intravascular volume augments venous blood return to the heart, thereby raising cardiac output and directly leading to hypertension [10]. The thiazide-sensitive Na⁺-Cl⁻ cotransporter (NCC) in the kidney transports sodium and chloride ions from the surrounding fluid into the cells of nephron distal convoluted tubule, and accumulating evidence [11] indicates that genetic variation of the NCC-encoding *SLC12A3* gene is associated with kidney sodium reabsorption and hypertension via effects on NCC functional activity. For example, *SLC12A3* mutation, Arg919Cys in exon 24, increase NCC activity, resulting in the up-regulation of kidney sodium reabsorption [11]. In addition to this, a recent study on pendrin and NCC dKO mice concluded that pendrin and NCC are predominantly active during salt depletion (and or in response to aldosterone) [12]. Association studies have shown that *SLC12A3* mutation (Arg904Gln and G2736A) demonstrated a significant relationship with the risk of hypertension [13, 14]. Chang et al. [15] have reported positive association of tag single nuclear polymorphism (SNP) rs7204044 with hypertension in both the Mongolian and Han populations in China. Genetic variants of the *SLC12A3* gene have also been shown to affect the response to BP-reducing drugs such as thiazide diuretics [16]. However, other studies have not found correlation between *SLC12A3* polymorphisms and hypertension. Zhang et al. [17] have reported the lack of association between Arg904Gln substitution and essential hypertension based on a meta-analysis, while other studies did not observe the relationship of reported *SLC12A3* polymorphisms with hypertension in rats [18] and humans [19]. Similarly, the Arg904Gln polymorphism in NCC did not show correlation with hypertension, as evidenced by the investigation of two ethnic groups in Northwest China [20] and our earlier study in the Uyghurs, Kazaks, and Mongolians [21], ethnic minorities in China. However, these and other related studies based on random or specific populations have not performed stratification for genetic admixture which may be a confounding factor affecting the conclusion about the link between *SLC12A3* genetic variants and hypertension. In addition, the results have not been validated in replication studies.

In our previous study, we have investigated the relationship between the *SLC12A3* gene and hypertension and found that two tag SNPs are predisposing genetic factors for hypertension in the Han population dominant in China [21]. In the current study, we aimed to analyze the association of the common *SLC12A3* variants with the occurrence of essential hypertension among the inhabitants of the Inner Mongolia region, which has not been yet established. To address this, we used a family-based association test (FBAT) to study a cohort of hypertensive ethnic Mongolians with extended pedigree.

Materials and Methods

Subjects

The investigation of a family-based cohort of hypertensive and normotensive individuals was performed at two separate locations of Xilingol League in the Inner Mongolia Autonomous Region of China, the Xianghuang county, and the city of Xilinhot. Subjects with essential hypertension were identified based on systolic BP (SBP) ≥ 140 mmHg and/or diastolic BP (DBP) ≥ 90 mmHg, or by the use of at least one antihypertensive drug, and were included in the study as probands. The relatives of the probands were tracked and diagnosed based on the same criteria using the same procedure; normotensive relatives were also recruited. Probands with secondary hypertension, severe cardiovascular disease, kidney disease and diabetes mellitus, as well as their relatives were excluded. In total, 424 individuals (236 hypertensive patients and their relatives; among them, 211 women and 213 men) comprising 88 extended pedigrees were tracked, recruited, and investigated. All participants were ethnic Mongolians. The study was conducted in accordance with the ethical standards established by the United States National Institute of Health and was approved by the Ethical Committee of Affiliated Hospital of Inner Mongolia Medical University. Written informed consent was obtained from all participants or their parents/guardians.

Subjects' evaluation

Participants' characteristics, including age, gender, ethnicity, and family structure, were recorded. Patients were in sitting position when blood pressure was measured as described previously [21]: 1) three times with 2-min intervals between the measurements; 2) SBP and DPB were recorded to the nearest 2 mmHg at the appearance of the first (phase I) and disappearance of the fifth (phase V) Korotkoff sounds, respectively; 3) three consecutive measurements were averaged. Body mass index (BMI) was calculated as the body weight in kilograms divided by the square of height in meters. Such clinical parameters as total plasma cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and fasting blood glucose were measured after 8-h overnight fasting at a local hospital using routine methods. Indirect clinical diagnosis was also made by physicians for incapacitated, deceased, or absent family members based on the information offered by their guardians or close relatives.

Genotyping

In this study, *SLC12A3* was considered as a candidate gene for the evaluation of genetic polymorphism effects on hypertension. SNPs were selected from the dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) and the International HapMap Project (<http://hapmap.ncbi.nlm.nih.gov/>) datasets using the HaploView 3.2 software package [22] by the method of Gabriel et al. [23]. Briefly, the strategy and criteria for tag SNP selection were as follows: a) location in the target gene or 1,000-bp flanking regions, b) preference for potentially functional SNPs, c) minor allele frequency (MAF) $> 10\%$ in the Asian population (Han Chinese in Beijing + Japanese in Tokyo); and d) r^2 threshold > 0.80 . Finally, 15 SNPs in the *SLC12A3* gene were selected (Table 1); they were located within 3.4 kb, one SNP was mapped 2.0 kb upstream of the *SLC12A3* gene, one synonymous codon was mapped to the exon region, and the rest were located in the intron region.

Genomic DNA was obtained from 1 mL venous blood using the AxyPrep-96 DNA Extraction Kit (Axygen, Union City, CA, USA) and subjected to multiplex PCR-ligase detection. Then, 1 μ L of the amplified product was mixed with 1 μ L of GS-500 ROX fluorescent molecular weight standards (Applied Biosystems, Foster City, CA, USA) and 1 μ L deionized formamide, denatured at 95°C for 2 min, and resolved by electrophoresis in 5% polyacrylamide gels containing 5 mol/L ureophil. Images were captured using the GENESCANTM 672 software and analyzed with GeneMapper 3.0. Strict quality control measures were implemented during genotyping, with more than 99% concordance in duplicate randomly selected samples.

Statistical analysis

The demographic information and baseline data of hypertensive and normotensive family members were assessed by compare means test (Independent – Sample T Test) and exact Chi-square test using the SPSS13.0 software (SPSS Inc., Chicago, IL, USA). In order to reduce influence of relationship, only parents (166 female and 148 male) were included and analyzed for demographic information and baseline comparison. Prior to the analysis, family samples were tested with the PEDCHECK program [24] to identify Mende-

Table 1. The location of selected tag SNPs and Hardy-Weinberg equilibrium test results

SNPs	Position ^a	Distance (kb) ^b	Location	ObsHET ^c	HWp ^d	MAF ^e	Alleles
rs4784733	16:56865094	-	Upstream	0.359	1.0000	0.231	C:T
rs2304478	16:56865628	0.53	Intron	0.226	0.9086	0.133	G:A
rs13306673	16:56867019	1.39	Intron	0.189	0.8189	0.099	T:C
rs2289119	16:56878991	11.97	Intron	0.347	0.1190	0.272	G:A
rs8043560	16:56883033	4.04	Intron	0.389	0.8321	0.251	C:T
rs2304483	16:56884041	1.01	Intron	0.409	0.2380	0.345	G:A
rs5803	16:56887057	3.02	Coding exon	0.369	1.0000	0.246	T:C
rs7187932	16:56897792	10.74	Intron	0.418	0.3198	0.260	G:A
rs6499858	16:56901203	3.41	Intron	0.523	0.2846	0.400	T:C
rs11644728	16:56905634	4.43	Intron	0.226	0.5920	0.138	G:C
rs8049280	16:56906216	0.58	Intron	0.267	0.5946	0.169	C:T
rs7204044	16:56908797	2.58	Intron	0.221	0.4835	0.115	G:A
rs2010501	16:56911627	2.83	Intron	0.405	0.6189	0.305	C:T
rs2399594	16:56912285	0.66	Intron	0.421	0.6763	0.323	A:G
rs711746	16:56912892	0.61	Intron	0.451	0.2574	0.451	A:G

^aPhysical position of SNPs in the GRCh38 data set. ^bDistance from the adjacent marker. ^cObsHET, observed heterozygosity. ^dHWp, p values of the Hardy-Weinberg equilibrium test. ^eMAF, minimum allele frequency.

lian inconsistencies in the pedigree data. Family-based association analyses were performed using the FBAT Program 1.5.5 (<http://www.biostat.harvard.edu/~fbat/default.html>) which represents a unified approach to testing valid associations between marker alleles and traits applicable to any phenotype, sampling structure, and pattern of missing marker alleles [25, 26]; similar to the transmission/disequilibrium test (TDT), FBAT is immune to confounding by admixture. With missing parents, FBAT is similar to, but more powerful than, the reconstruction-combined TDT and considerably more powerful than the sib TDT [27]. If Mendelian inconsistencies were detected, pedigree sub-families were not considered useful for association analyses, although the FBAT approach is completely robust against model misspecification. Individual marker genotypes were tested for the Hardy-Weinberg equilibrium and analyzed for linkage disequilibrium (LD) by Haploview [22]. We first tested each marker separately for excessive allele transmission under difference genetic models (e.g. additive, dominant and recessive models). As Horvath et al. [28] suggested, the results under additive model were prior to consider, since the true genetic model was unknown. Then we analyzed excessive haplotype transmission for the consistency with the markers identified by single-site analysis. Haplotypes were reconstructed using the HBAT command in FBAT, which utilizes the expectation-maximization algorithm for SNPs with obvious overtransmission ($p < 0.10$). The statistical power of this investigation with the present sample size was estimated by the Quanto 1.2.4. program for power and sample size calculations in genetic studies [29]. Statistical significance was considered at $p < 0.05$. To control the risk of false discoveries, a so-called q -value which estimates the proportion of false discoveries among all significant markers [30] was calculated for each p -value used as the threshold for declaring significance.

In silico analysis was performed to evaluate the potential biological significance of SNPs related to the following: 1) transcription factor binding site or promoter region; 2) splice site alteration; and 3) expression of a novel protein with different structure-functional properties. The TFSEARCH program (http://www.gene-regulation.com/links_tools.html) was used to search transcription factor binding sites identified in vertebrates [31] using the 85.0 threshold score within 50-bp sequence around each SNP. The Neural Network Promoter Prediction, Promoter 2.0 Prediction, and McPromoter MM:II tools were used to detect promoter sites generated or lost because of alternative SNP alleles. Splice site estimation was performed with the MasEnNCCan software based on the maximum entropy principle [32] and the splice site predictor at the Berkeley Drosophila Genome Project based on neural networks [33]. Furthermore, 300-bp SNP flanking regions were analyzed by the ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and Conserved Domain Database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) to identify possible ORFs,

conserved domains, and related protein families. The signature-recognition tools Phobius (<http://www.ebi.ac.uk/Tools/pfa/phobius/>) and InterProScan (<http://www.ebi.ac.uk/interpro/>) were used to evaluate the effects of SNPs on the structural features of polypeptides.

Results

Demographic and baseline characteristics of the participants

The present study included 424 participants with familial hypertension covering 88 extended pedigrees of more than 100 nuclear families; for each family, one to nine patients were identified. Participants' demographic and baseline data are presented in Table 2. For 314 individuals, the data on BP, gender, age, and five basic clinical parameters were available. Information on the heritage and pedigree genetic structure of the remaining 110 participants could be obtained and these individuals were also included in the FBAT analysis, although the related basic variables were missing. Normotensive and hypertensive subjects showed significant differences in gender, age, SBP, and DBP (Table 2). More men had hypertensive status than women ($p = 0.002$), and normal BP was more characteristic of younger people ($p < 0.001$). Hypertensive subjects had significantly higher SBP (mean = 154.29 mmHg, 95%CI [151.03–157.55]) and DBP (mean = 100.84 mmHg, 95%CI [99.10–102.58]) than normotensive subjects (SBP, mean = 117.16 mmHg, 95%CI [115.32–119.00] and DBP, mean = 75.80 mmHg, 95%CI [74.60–77.00]) ($p < 0.001$). There was a statistically significant difference in two clinical variables, TG and LDL-C, which were higher in hypertensive participants ($p = 0.031$ and $p = 0.049$, respectively).

Genotyping profile and LD structure of SLC12A3 SNPs

The identified 15 SNPs did not show significant deviation from Hardy-Weinberg distribution (Table 1). Heterozygosity ranged from 18.9% to 52.3% and most MAFs were more than 10%, except for the rs13306673 site (MAF = 9.9%). Figure 1 shows the LD structure created by HaploView, which identified three blocks of tag SNPs with higher LD, consistent with the rough distribution in the HapMap data set.

Table 2. Demographic and clinical characteristics of study participants

Characteristics	Normotensive group [95% CI] (n = 135)	Hypertensive group [95% CI] (n = 179)	p value
Median age, years (range)	40 (15–82)	53 (15–87)	< 0.001 ^b
Gender, female/male	85/50	81/98	0.002 ^b
SBP, mmHg ^a	117.16 [115.32, 119.00]	154.29 [151.03, 157.55]	< 0.001 ^b
DBP, mmHg ^a	75.80 [74.60, 77.00]	100.84 [99.10, 102.58]	< 0.001 ^b
BMI, kg/m ²	25.17 [24.37, 25.98]	25.00 [24.21, 25.80]	0.430
TC, mM	4.42 [4.21, 4.63]	4.79 [4.54, 5.05]	0.106
TG, mM	1.26 [1.07, 1.45]	1.71 [1.36, 2.06]	0.031 ^b
HDL-C, mmol/L	1.39 [1.33, 1.44]	1.33 [1.27, 1.40]	0.134
LDL-C, mmol/L	2.89 [2.70, 3.04]	3.14 [2.96, 3.31]	0.049 ^b
FBG, mmol/L	6.39 [6.22, 6.57]	6.32 [6.01, 6.63]	0.699

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride; FBG, fasting blood glucose. ^aMean values were used for multiple testing. ^bSignificant p values (< 0.05) are in bold.

Fig. 1. Markers used in the association analyses. Symbols and positions of the markers relative to the *SLC12A3* gene and adjacent genes are shown. Haplotype frequencies for three unfavorable SNPs with MAFs of $\geq 5\%$ are indicated. Fifteen SNPs are grouped into three blocks (B1, B2, and B3) using the method of Gabriel et al. with the $r^2 > 0.8$ threshold [23]. *SLC12A3*, solute carrier family 12 (sodium/chloride transporter), member 3; *UNP93*, nuclear pore complex protein Nup93; *HERPUD1*, ubiquitin-like domain member 1; *GRCh38*, new human genome assembly (GenBank, December 2013).

Genotype and allele distributions of *SLC12A3* SNPs

Single marker association analysis identified two SNPs significantly associated with hypertension in our family cohort in both additive biallelic and multi-allelic models. As shown in Table 3, the G allele of rs2304478 showed significant positive correlation with hypertension ($z = 2.36$, $p = 0.02$ in additive model and dominant model), while the T allele of rs5803 transmitted in families with one or more hypertensive members showed significant negative correlation with hypertension ($z = -1.95$, $p = 0.05$ in additive model, $p = 0.01$ in dominant model). The same results were obtained for rs7204044, where the G allele also showed an obvious trend for transmission in hypertensive patients ($z = 1.81$, $p = 0.07$) under additive model. However, the data for three SNPs did not withstand correction for multiple testing.

Haplotype analysis

Structure analysis of haplotypes containing three SNPs and showing obvious overtransmission in hypertensive patients is presented in Table 4. The HBAT test indicated that three haplotypes with two-window size showed significant association with hypertension ($p = 0.012$ for rs2304478(G)/rs5803(C), $p = 0.016$ for rs5803(C)/rs7204044(G), and $p = 0.006$ for rs2304478(G)/rs7204044(G)). Furthermore, the haplotype G/C/G containing three SNPs was also transmitted among hypertensive patients showing positive association ($z = 2.65$, $p = 0.008$). All p values withstood correction for multiple testing and demonstrated statistically significant association between the haplotypes and hypertension (all $p < 0.05$) (Table 4 and Fig. 1).

In silico functional analysis of SNPs

We next performed detailed *in silico* analysis of SNPs that showed significant association with hypertension as single markers, including rs2304478, rs5803, and rs7204044. No transcription factor binding sites or promoters were affected by these SNPs. rs5803 was likely to be located within a splice site determined by MasEnNCCan based on the maximum entropy principle (the maximum entropy model score -1.38 for the T allele and -9.13 for the C allele). The analysis with ORF Finder showed that rs2304478 (a 300-bp sequence) might encode a

59-amino acid peptide having 94% identity with solute carrier family 12 member 3 isoform X1 of *Chrysemys picta bellii*, whereas a putative peptide comprising the rs5803 site had 91% identity with *Homo sapiens* Na⁺-Cl⁻ electro neutral NCC (46 amino acids) containing the 2a30 domain of a K⁺-Cl⁻ cotransporter. No match was found for a putative rs7204044-encoded peptide. However, the rs2304478 and rs5803 alternative alleles did not show obvious effects on the putative encoded peptides.

Statistical power analysis of genetic testing

Statistical power analysis by QUANTO 1.2.4 indicated that the present study based on more

Table 3. Family-based association test for tag SNPs under additive and dominant genetic models

Marker	Alleles ^a	Allele frequency	Test statistics		<i>p</i> -FBAT	
			<i>z</i> ^b	CHISQ ^c	Additive	Dominant
rs4784733	C:T	0.22/0.78	1.36	1.84	0.17	0.41
rs2304478	G:A	0.86/0.14	2.36	5.56	0.02 ^d	0.02 ^d
rs13306673	T:C	0.10/0.90	0.69	0.48	0.49	0.75
rs2289119	G:A	0.73/0.27	1.42	2.02	0.16	0.07
rs8043560	C:T	0.25/0.75	-0.41	0.17	0.68	0.46
rs2304483	G:A	0.65/0.35	1.24	1.54	0.22	0.22
rs5803	T:C	0.26/0.74	-1.95	3.80	0.05 ^d	0.01 ^d
rs7187932	G:A	0.74/0.26	-0.43	0.18	0.67	0.07
rs6499858	T:C	0.38/0.62	1.07	1.34	0.28	0.37
rs11644728	G:C	0.88/0.12	0.06	0.01	0.95	0.86
rs8049280	C:T	0.16/0.84	-0.56	0.32	0.57	0.49
rs7204044	G:A	0.89/0.11	1.81	3.26	0.07	0.16
rs2010501	C:T	0.28/0.72	-0.77	0.59	0.44	0.13
rs2399594	A:G	0.69/0.31	-0.67	0.45	0.50	0.27
rs711746	A:G	0.56/0.44	-0.83	0.70	0.41	0.45

^aAlleles detected in more than 10 informative families. ^b*z*-score calculated based on a biallelic marker model; positive *z* values and *p* < 0.05 indicate a high-risk haplotype. ^cCHISQ, a statistic parameter calculated based on a multiple marker model. ^dSignificant *p* values (< 0.05) are in bold.

Table 4. Haplotype-specific analysis of identified SNPs associated with hypertension

Haplotypes	Frequency ^a	Var (S) ^b	Test statistics (<i>z</i>)	<i>p</i> -HBAT ^c	Global <i>p</i> ^d
rs2304478/ rs5803					0.042 ^e
G/C	0.70	8.98	2.50	0.012 ^e	
G/T	0.16	5.87	-1.03	0.302	
rs5803/ rs7204044					0.046 ^e
C/G	0.69	8.45	2.41	0.016 ^e	
T/G	0.22	6.22	-1.34	0.181	
rs2304478/ rs7204044					0.022 ^e
G/G	0.76	9.63	2.72	0.006 ^e	
A/G	0.15	6.50	-1.63	0.102	
rs2304478/ rs5803/ rs7204044					0.027 ^e
G/C/G	0.61	9.89	2.65	0.008 ^e	
G/T/G	0.15	5.08	-0.44	0.657	

^aHaplotypes with higher frequencies (> 0.10) in more than 10 informative families. ^bEstimated variance for general statistics compared with a null model. ^c*p* values of the HBAT test. ^d*p* values of the asymptotic global HBAT test for all haplotypes with more than 0.10 frequencies within one-window size. ^eSignificant *p* values (< 0.05) are in bold.

than 100 trio families (in which both parents and at least one child were affected by hypertension) had >80% power in the FBAT test (MAF > 0.25, OR > 2.0, *k_p* = 0.20), which guarantees the reliability and accuracy of our analysis results.

Discussion

Hypertension results from complex interactions of genetic traits and environmental factors, where genetic background could account for 30%–50% of BP variations in the general population [34]. The Mongolians, one of the minor ethnic groups in China, have a high prevalence of hypertension: the crude hypertension rate amounts to 50% among elderly inhabitants (age > 55 years) of a pastoral area of the Inner Mongolia Autonomous Region. Moreover, there is a tendency for familial aggregation [35], suggesting a critical role of genetic factors in the high prevalence of hypertension among the Mongolians. In the present study, we focused on the effects of genetic variation in the *SLC12A3* gene on hypertension in the Mongolian population in China. To the best of our knowledge, this is the first study to investigate the effect of *SLC12A3* genetic variants on hypertension in a family-based cohort. The association we observed was particularly evident in an extended family cohort which allowed elimination or effective reduction of the influence of environmental factors and is immune to confounding by admixture [36].

Genetic variation, including SNPs, plays an important role in the regulation of gene expression via mRNA splicing, degradation, and translation, and may affect protein structure and biological function [37]. Recent efforts in exploring molecular mechanisms of hypertension have revealed polymorphisms in the genes encoding renal sodium and potassium transporters, which contribute to hypertension susceptibility in the general population [38]. The variations in the *SLC12A3* gene, including common and rare SNPs and other mutations, affect the regulation of solute transport and sodium reabsorption in the kidneys and show a close relationship with hypertension and Gitelman's syndrome characterized by low BP [11, 13, 39]. In the present study, two SNPs (rs2304478 and rs5803) in the *SLC12A3* gene showed significant association with hypertension in the Mongolian population; one of the alleles was transmitted in the family and positively (rs2304478) or negatively (rs5803) correlated with hypertension. rs7204044 was also included in our analysis based on previous findings in which it is positively associated with hypertension [15], although in this study, it showed statistically insignificant positive association with hypertension. The results of haplotype analysis were consistent with those of single-site analysis in that haplotypes containing pedigree-transmitted SNP alleles showed significant association with hypertension, both for window sizes of two and three SNP haplotypes by the HBAT method. No significant association with hypertension was detected for other selected SNPs in the current study on the Mongolians, as well as in previous studies on other populations [15, 20, 21].

The biological functions of the three identified SNPs and the influence of the underlying regulatory genetic mechanisms on clinical outcome for hypertensive patients remain to be elucidated. The *in silico* analysis which included SNP 150-bp flanking regions indicated that rs5803, a synonymous SNP, may be involved in the alternative splicing of the *SLC12A3* mRNA of electroneutral NCC harboring the 2a30 domain of the K⁺-Cl[−] cotransporter, which regulates a number of physiological processes, including BP [40]. A part of the putative rs2304478-containing peptide (17 amino acids) showed high identity with solute carrier family 12 member 3 isoform X1 from *C. picta bellii*. Thus, we cannot rule out a possibility that rs5803 and rs2304478 can be biologically meaningful SNPs, although their functional significance has not been determined. *SLC12A3*-encoded NCC involved in Na⁺-Cl[−] reabsorption in the kidney is important for electrolyte homeostasis and is a potential target for treating high BP. Therefore, *SLC12A3* genetic polymorphisms can affect renal electrolyte reabsorption via regulation of NCC functional activity, and are likely to be associated with BP control and hypertension. Our present investigation showing the correlation between SNPs in the *SLC12A3* gene and hypertension confirms this notion.

Our study has several limitations. First, in this association analysis of complex traits, the possibility of spurious correlations must be considered. Two approaches utilized in the current study might have reduced the likelihood of false-positive findings: a family-based cohort, which excludes confounding due to population admixture, and validation of stati-

stically significant association using multimarker haplotype and global tests, which ensures that true association is revealed.

Second, the analyzed sample size was relatively modest. Several SNPs, including rs7204044, which did not achieve nominal statistical significance, had low MAFs (<15%), and it is possible that some of these SNPs may demonstrate correlation with hypertension in a larger population. Thus, despite a possibility of a type II error, the association we revealed can still be considered robust.

Third, as in most association studies, it is unclear which, if any, of the tested SNPs may be causally related to the development of hypertension phenotype, although certain functional evaluation was performed in silico. Among the examined SNPs, only one (rs5803) is located in the *SLC12A3* coding sequence. This SNP which represents a synonymous change in the Ala714 site was found to be associated with hypertension in this study, possibly because of its potential effect on alternative splicing [41]. However, no functional significance had been reported for rs2304478 and rs7204044 at the time of our investigation (September 2014), suggesting the need for further research to identify causal associations.

Fourth, it was difficult to determine the contribution of individual markers and their inter-relationship because the three SNPs were not located in a single region with strong LD; therefore, the influence of each SNP on hypertension was independent of others or still unknown. Modest sample size allowed only the analysis of direct associations and precluded that of indirect associations occurring through LD and conditioning markers; thus, cumulative effects of unfavorable alleles could not be estimated.

Fifth, the effect of clinical variables may still have an impact on our conclusions. Despite sample control, significant differences were still found between hypertensive and normotensive participants (gender, age, TG, and LDL-C); therefore, further stratification and adjustment are required.

Sixth, functional gain-of-function SNPs in *SLC12A3* gene would be expected to be associated with hyperkalemia, metabolic acidosis and hypercalciuria (the opposite of Gitelman and in the same direction as seen in Gordon syndromes). Nevertheless, these parameters were not measured at that time of our collecting samples. It is a limitation for the present study and these parameters will be tested in further association study. Finally, the *p* values determined by single-marker analysis did not withstand correction for multiple comparisons, and the possibility of a type I error cannot be excluded.

Conclusion

In summary, we observed an association between the *SLC12A3* gene and hypertension in a family-based cohort from the population of Inner Mongolia. Further studies are needed to clarify the effect of specific *SLC12A3* polymorphisms on BP and risk for hypertension, which would provide scientific basis for the development of novel therapeutic approaches to treat hypertension using NCC-mediated signaling pathways as drug targets.

Disclosure Statement

The authors declare no conflicts of interest.

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