GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Lack of association of variants of the renal salt reabsorption-related genes *SLC12A3* and *ClC-Kb* and hypertension in Mongolian and Han populations in Inner Mongolia

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Genet. Mol. Res. 10 (2): 948-954 (2011)
Received November 29, 2010
Accepted January 24, 2011
Published May 24, 2011
DOI 10.4238/vol10-2gmr1165

ABSTRACT. Abnormalities in renal sodium chloride and water reabsorption play important roles in the development of hypertension. Mutations in the genes involved in renal sodium chloride reabsorption can affect blood pressure. Recently, the *R904Q* variant of the sodium/chloride transporters, member 3 (*SLC12A3*) gene and the T481S variant of the chloride channel *Kb* (*ClC-Kb*) gene were found to be implicated in essential hypertension. We investigated a possible role of the *SLC12A3* and *ClC-Kb* genes in the prevalence of essential hypertension in the Mongolian and Han ethnic groups. The study population comprised 308 unrelated Mongolians with essential hypertension, 271 Mongolian normotensives, 285 unrelated Han with essential hypertension, and 194 Han normotensives living in Inner Mongolia. The presence of the *SLC12A3 R904Q* and *ClC-Kb-T481S* polymorphisms was determined using TaqMan PCR. The risk factors for hypertension were age, body

mass index, alcohol consumption, total plasma cholesterol, and low-density lipoprotein cholesterol. The genotype and allele frequencies of *SLC12A3 R904Q* and *ClC-Kb-T481S* were not significantly different between hypertensive patients and controls in the Mongolian (*SLC12A3 R904Q*, P = 0.471 and P = 0.494, *ClC-Kb-T481S*, P = 0.960 and P = 0.960, respectively) and Han (*SLC12A3 R904Q*, P = 0.765 and P = 0.777, *ClC-Kb-T481S*, P = 0.100 and P = 0.103, respectively) populations. There was no significant association between the *SLC12A3 R904Q* variant and the *ClC-Kb-T481S* variant and essential hypertension in either ethnic group.

Key words: Essential hypertension; Mongolian population; *SLC12A3*; *ClC-Kb*; Gene polymorphism

INTRODUCTION

Essential hypertension (EH) is one of the most common cardiovascular diseases, and it causes a major public health problem by affecting approximately 25-30% of adults worldwide. Hypertension is involved in strokes, heart attacks and renal failure, all of which have high mortality rates. The pathogenesis of EH stems from a complex interaction between environmental and genetic factors. The genes involved in renal sodium chloride reabsorption are critical for the long-term control of blood pressure (Guyton, 1991), and variations in these genes can cause severe hypertension (Lifton et al., 2001). *SLC12A3 R904Q* and *ClC-Kb-T481S* are two polymorphisms that play important roles in the development of hypertension.

Solute carrier family 12 (sodium/chloride transporters) member 3 (*SLC12A3*) is a 1021-amino acid glycoprotein with a general topology similar to that of Na-K-Cl cotransporter 1 (*NKCC1*) and 2 (*NKCC2*). These proteins share a central core containing a 12-transmembrane domain. *SLC12A3* has been reported to play a major role in sodium chloride reabsorption in the distal convoluted tubule, which is responsible for the reabsorption of 5-10% of the filtered sodium chloride (Obermüller et al., 1995) and accounts for a significant portion of the total renal sodium reabsorption. Mutations in the human *SLC12A3* gene affect blood pressure regulation, as seen in Gitelman syndrome, a disease characterized by sodium wasting and low blood pressure. This cotransporter is the target of thiazide diuretics used to treat high blood pressure (Simon et al., 1996). Recently, Melander et al. (2000) found a novel *SLC12A3* gene molecular variant, *Q904*, that was associated with hypertension. *Q904* homozygotes were over-represented in hypertensive patients compared with control subjects, suggesting that the *Q904* variant increased the risk for developing EH in southern Sweden.

The chloride channel *Kb* (*ClC-Kb*) gene is also important for sodium chloride reabsorption in the thick ascending limb. Mutations in the *CLCNKB* gene lead to classic Bartter syndrome, which is characterized by mild sodium chloride wasting. Jeck and co-workers (2004a) showed that the common *ClC-Kb-T481S* allele results in a current of Cl^- ions 7-fold larger than that produced by the *481T* allele *in vitro*, and that carriers of the mutant *418S* allele were associated with higher systolic blood pressure (SBP) and diastolic blood pressure (DBP) in a German population. Furthermore, individuals carrying the *481S* allele had significantly higher plasma Na^+ concentrations and lower glomerular filtration rates (Jeck et al., 2004b).

The genetic variants involved in sodium chloride reabsorption may behave differently

in different populations. The aim of this study was to investigate the influence of *SLC12A3* and *ClC-Kb* genetic variants on hypertension in two Chinese ethnic populations, Mongolian and Han, and to determine if there were any correlations among genotype, age, gender, body mass index (BMI), blood fat, smoking, drinking, and blood pressure.

MATERIAL AND METHODS

Subjects

The present study consisted of subjects aged 20-75 years, who were recruited from two villages in Duolun County and three villages in Erenhot of Xilin Gol League in Inner Mongolia. A total of 579 unrelated Mongolian herdsmen and 479 Han farmers were enrolled, including 308 Mongolian EH patients, 271 Mongolian normotensives (controls), 285 Han EH patients, and 194 Han normotensives (controls). The following inclusion criteria were applied. Each subject was from a family that had been living in Inner Mongolia for at least three generations without a history of mixed marriage. EH was diagnosed according to WHO criteria: SBP ≥140 mmHg, and/or DBP ≥90 mmHg, and/or current treatment for hypertension with antihypertensive medication. The normotensive group was selected based on the following criteria: SBP <135 mmHg, DBP <85 mmHg, and no previous diagnosis of EH. None of the subjects had a history of secondary hypertension, coronary heart disease, diabetes, kidney failure, or thyroid gland disease.

The subjects were gathered in a quiet room and prevented from smoking, exercising and drinking alcohol, tea or coffee for at least 1 h before the physical examination. The following data were recorded for each subject: name, age, gender, ethnicity, height, weight, BMI, history of drinking and tobacco use (smoking was defined as smoking at least 1 cigarette per day for at least 1 year, and drinking was defined as consuming 50 g or more alcohol per day for at least 1 year), and blood pressure (SBP and DBP). Blood pressure was measured three times, with a 2-min interval between each measurement. SBP was recorded to the nearest 2 mmHg at the appearance of the first Korotkoff sound (phase I), and DBP was recorded to the nearest 2 mmHg at the disappearance of the fifth Korotkoff sound (phase V). The SBP and DBP values were calculated as the means of three consecutive physician-obtained measurements. Body weight and height were measured with subjects wearing only light indoor clothing and no shoes. BMI was calculated by dividing weight (kg) by height squared (m^2). Blood samples were collected after an overnight fast, and total plasma cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured within 8 h in a local hospital. Informed consent was obtained from all subjects. Our experiment was approved by the Inner Mongolia Medical College Affiliated Hospital Ethics Committee.

DNA isolation and TaqMan assay

Genomic DNA was extracted from peripheral blood using a commercial blood DNA extraction kit (Genomic DNA purification kit; TaKaRa Biotechnology, Dalian, China) and stored at -20°C. Genotyping was performed blinded to clinical status using a TaqMan assay (Applied Biosystems, Foster City, CA, USA). The allele-specific probes used in the Taq-

Man assay were as follows: *SLC12A3 R904Q* forward primer 5'-CCA CAT CCT CCC TGA CAT CAA-3' and reverse primer 5'-GAA GCC CCA AAA CAG AAC TTA CTG-3', TaqMan forward probe 5'-ACC CCT CAG GCT GAG-3'-VIC and reverse probe 5'-ACC CTC GGG CTG AG-3'-FAM, *ClC-Kb-T481S* forward primer 5'-GGG CTG CAG CCT TCT CA-3' and reverse primer 5'-GGC CGG TCA CCT CGA A-3' and TaqMan forward probe 5'-CCA CAC CAT CTC CA3'-VIC and reverse probe 5'-CCA CTC CAT CTC CA -3'-FAM. A total of 10 ng genomic DNA was used per assay. The reaction conditions were as follows: an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 92°C for 15 s and annealing and extension at 60°C for 60 s. The results were analyzed using the ABI Prism 7300HT detection system and the SDS 1.2 software (Applied Biosystems).

Statistical analysis

The comparison of clinical characteristics between EH patients and normotensives was performed using the Wilcoxon rank test, and the results are reported as median ± interquartile range. Deviation from Hardy-Weinberg equilibrium for the variants was determined using the chi-square test. Allele and genotype frequencies between groups were compared using the chi-square test. Associations between genotypes and hypertensive category were determined using the multivariate logistic regression. A P value of <0.05 was considered to be significant. Statistical analyses were performed using the SPSS software (version 13.0; SPSS, Chicago, IL, USA).

RESULTS

Clinical characteristics and EH

The clinical characteristics of the Mongolian and Han subjects are shown in Table 1. Significant differences in SBP, DBP, age, BMI, TG, HDL-C, and smoking (%) were observed between EH patients and controls in both the Mongolian and Han populations ($P < 0.05$); TC, LDL-C and drinking (%) were markedly higher in EH patients than the Mongolian control population ($P < 0.01$).

Table 1. Characteristics of normotensives (NT) and hypertensives (HT) in Mongolian and Han participants.

	Mongolian		P	Han		P
	NT (N = 271)	HT (N = 308)		NT (N = 194)	HT (N = 285)	
Male/female	108/163	146/162	0.068	112/82	181/104	0.203
Age (years)	42.00 ± 16.00	51.00 ± 18.00	0.000**	44.00 ± 13.00	49.00 ± 15.50	0.000**
BMI (kg/m^2)	22.54 ± 5.47	24.76 ± 4.43	0.000**	23.23 ± 3.89	25.06 ± 3.35	0.000**
SBP	109.06 ± 12.32	160.33 ± 26.57	0.000**	119.44 ± 11.42	146.77 ± 18.03	0.000**
DBP	71.44 ± 9.01	104.51 ± 14.67	0.000**	75.04 ± 8.44	90.20 ± 12.37	0.003**
HDL-C (mM)	1.54 ± 0.66	1.46 ± 0.94	0.046*	1.37 ± 0.97	1.24 ± 0.36	0.003**
TG (mM)	1.21 ± 1.01	1.87 ± 1.27	0.000**	3.55 ± 3.07	1.58 ± 1.63	0.000**
TC (mM)	3.97 ± 1.47	5.33 ± 2.32	0.000**	4.98 ± 1.16	4.91 ± 1.19	0.110
LDL-C (mM)	2.45 ± 1.02	3.46 ± 2.02	0.000**	3.07 ± 0.87	3.03 ± 1.03	0.088
Smoking (No/Yes)	254/17	206/102	0.000**	134/60	222/63	0.030*
Drinking (No/Yes)	252/19	120/188	0.000**	130/64	195/90	0.746

Data are reported as median ± interquartile range or as number of individuals. BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure; HDL-C = high-density lipoprotein-cholesterol; TG = triglycerides; TC = total cholesterol; LDL-C = low-density lipoprotein-cholesterol. *P < 0.05, **P < 0.01.

SLC12A3 R904Q and *ClC-Kb-T481S* variants and EH

The genotype distribution of the *SLC12A3 R904Q* and *ClC-Kb-T481S* variants did not significantly deviate from Hardy-Weinberg equilibrium. The distribution of genotypes and alleles of both ethnic groups is presented in Table 2. The minor allele frequencies of *ClC-Kb-T481S* and *SLC12A3 R904Q* were 1.6 and 8.4% in Mongolian subjects and 2.1 and 7.7% in Han subjects, respectively. The distribution of genotype and allele frequencies of *SLC12A3 R904Q* and *ClC-Kb-T481S* was not significantly different between EH patients and controls in both the Mongolian and Han populations ($P > 0.05$). Further analyses performed by adjusting age, gender, BMI, LDL-C, and TG in logistic regression did not show any association between *SLC12A3 R904Q* and *ClC-Kb-T481S* and EH.

Table 2. Association between gene polymorphisms and hypertension.

Gene	Genotype/allele	Mongolian		P	Han		P
		NT	HT		NT	HT	
SLCA12A3 R904Q	GG	219 (80.8%)	256 (83.1%)	0.471	160 (82.55%)	232 (81.4%)	0.765
	AG	52 (19.2%)	52 (16.9%)		34 (17.5%)	53 (18.6%)	
	G	490 (90.4%)	564 (91.6%)	0.494	354 (91.2%)	517 (92.3%)	0.777
	A	52 (9.6%)	52 (8.4%)		34 (8.8%)	53 (7.7%)	
ClC-Kb-T481S	AA	262 (96.7%)	298 (96.8%)	0.960	191 (98.5%)	273 (95.8%)	0.100
	AT	9 (3.3%)	10 (3.2%)		3 (1.5%)	12 (4.2%)	
	A	533 (98.3%)	606 (98.4%)	0.960	385 (99.2%)	558 (97.9%)	0.103
	T	9 (1.7%)	10 (1.6%)		3 (0.8%)	12 (2.1%)	

Data are reported as number with percent in parentheses. NT = normotensives; HT = hypertensives. *P < 0.05, **P < 0.01.

The risk factors of EH

Age, BMI, drinking (%), TC, and LDL-C were shown to be the risk factors for hypertension using logistic regression analysis by the forward stepwise (Wald) method (Table 3). Age (each additional decade), drinking (%), and high BMI, TC and LDL-C levels increased the risk for developing high blood pressure 1.73-, 2.41-, 2.56-, 1.75-, and 3.26-fold, respectively, in Inner Mongolia.

Table 3. Logistic regression analysis, the risk factors of hypertension in Inner Mongolia region.

	β	SEM	Wald	P	OR	95%CI
Drinking	0.879	0.165	28.278	0.000**	2.407	1.741-3.328
Age	0.545	0.078	48.283	0.000**	1.725	1.479-2.012
BMI	0.940	0.142	44.156	0.000**	2.561	1.941-3.380
TC	0.560	0.188	8.892	0.003**	1.750	1.211-2.528
LDL-C	1.181	0.165	51.470	0.000**	3.259	2.360-4.500

SEM = standard error of the mean; OR = odds ratio; CI = confidence interval; BMI = body mass index; TC = total cholesterol; LDL-C = low-density lipoprotein cholesterol. *P < 0.05, **P < 0.01.

DISCUSSION

In the present study, we attempted to test the hypothesis that subtle genetic variants at the renal salt reabsorption-related genes (*SLC12A3 R904Q* and *ClC-Kb-T481S*) may be at the origin of essential hypertension in Mongolian and Han populations. Unfortunately, we did not find an association between *SLC12A3 R904Q* and *ClC-Kb-T481S* variants with essential hypertension in Mongolian and Han populations.

In a previous study, Jeck et al. (2004a) demonstrated that coexpressing the *ClC-Kb-T481S* variant with the Barttin subunit-induced currents that were significantly larger than those produced by the wild-type *ClC-Kb* variant and the Barttin subunit when expressed in *Xenopus* oocytes. In a follow-up study, Jeck et al. (2004) noted that *ClC-Kb-T481S* was more prevalent in a West African population (22%) than in European whites (12%) and that there was an association of the minor allele frequency with hypertension. Subsequent studies performed on Australian, Swedish, Italian, and Japanese populations have failed to demonstrate a correlation between the *ClC-Kb-T481S* polymorphism and hypertension (Speirs et al., 2005; Kokubo et al., 2005; Fava et al., 2007; Barlassina et al., 2007). In contrast, Sile et al. (2009) observed that *ClC-Kb-T481S* was associated with EH in males within the Ghanaian population; however, cultured mammalian cells that heterologously expressed *ClC-Kb-T481S* and the Barttin subunit did not perform significantly differently from the wild-type cells. In our study, we did not find an association between the *ClC-Kb-T481S* variant and essential hypertension in the Mongolian and Han populations.

Melander et al. (2000) reported that *SLC12A3 Q904* homozygotes were over-represented in southern Swedish hypertensive patients compared with control subjects and concluded that *SLC12A3 Q904* homozygotes had an increased risk for the development of essential hypertension. They further hypothesized that this mutation may represent an activating variant that contributes to elevated blood pressure. In a study designed to replicate the findings made by Melander et al. (2000), Matsuo et al. (2004) found that the *SLC12A3 R904Q* variant was significantly associated with the prevalence of hypertension in a Japanese population, but they failed to confirm this mutant in a thiazide-loading test. Another study performed on a Canadian population failed to demonstrate a correlation between the *SLC12A3 R904Q* polymorphism and hypertension (Keszei et al., 2007).

The results obtained for the *ClC-Kb-T481S* variant in the Mongolian and Han populations contradicted the observations made in German and Ghanaian subjects and replicated those in the Australian, Swedish, Italian, and Japanese subjects. The results obtained for *SLC12A3 R904Q* in the Mongolian and Han populations contradicted the observations made in the Swedish and Japanese subjects and replicated those in the Canadian subjects. The conflicting results from these studies increase the difficulty of drawing any conclusions regarding the association of the *SLC12A3 R904Q* and *ClC-Kb-T481S* variants with essential hypertension.

Different allele frequencies of the *SLC12A3 R904Q* and *ClC-Kb-T481S* variants in diverse ethnic groups may help explain the varying results found in different studies. Genetic heterogeneity between populations could be the cause of these conflicting results. In addition to different genetic backgrounds among ethnic groups, variation in environmental factors and gene-environment interactions could also play an important role. Polymorphisms in the genes involved in renal sodium chloride reabsorption could behave differently in diverse populations exposed to different environments, thus leading to variable disease prevalences.

The Inner Mongolia Autonomous Region is located in northwestern China and includes the Xilin Gol League, which is a desert with an arid and cold climate. The local Mongolian herdsmen subsist primarily upon animal meat with little fruit and vegetables, and their sodium intake is exceptionally high due to their tradition of adding salt to every bowl of milk tea they drink (Zhou et al.,1989). Although this practice has been related to renal salt retention, extracellular volume expansion and volume hypertension, no relationship between the two renal sodium chloride reabsorption gene variants and essential hypertension was identified in the Mongolian population. In

our study, age, BMI, drinking (%), TC, and LDL-C were the risk factors associated with hypertension in the Mongolian population, demonstrating that genetic background, living habits and other environmental factors in the area affect the occurrence of hypertension.

In conclusion, the *SLC12A3 R904Q* and *ClC-Kb-T481S* gene variants were examined in a case-control study of Mongolian and Han populations. There was no association between the *SLC12A3 R904Q* variant and the *ClC-Kb-T481S* variant and essential hypertension in the Mongolian and Han populations in Inner Mongolia. Further population genetic studies need to be conducted to investigate the role of these two variants in essential hypertension in other populations living in different environments.

ACKNOWLEDGMENTS

We appreciate the support and assistance provided by the Duolun County Center for Disease Prevention and Control and the Department of Epidemiology of Inner Mongolia Medical School.

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