J Physiol Sci (2009) 59:81–86
DOI 10.1007/s12576-008-0011-9

ORIGINAL PAPER

Association of Ala589Ser polymorphism of *WNK4* gene with essential hypertension in a high-risk Chinese population

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Received: 25 February 2008 / Accepted: 13 November 2008 / Published online: 17 December 2008

Abstract Recent studies on the association between particular single nucleotide polymorphisms of serine–threonine kinase with no lysine (K) 4 gene (*WNK4*) and essential hypertension have yielded controversial results. Here, frequencies of Ala589Ser polymorphism within exon 8 of the *WNK4* gene were assessed among 259 unrelated ethnic Chinese patients with essential hypertension and 235 strictly matched normotensive controls. All subjects were derived from a relatively isolated population identified in the Kerqin desert region in Zhangwu county of Liaoning, northeastern China, which features a dry climate and the people having a high dietary salt intake, in addition to a significantly higher prevalence (~35%) of essential hypertension. Genotypes were verified with polymerase chain reaction-restriction fragment length polymorphism and confirmed by direct sequencing. Expression pattern and regulatory mechanisms of the WNK4 gene were also explored using Northern blotting and in vitro hormone stimulation assays. Strong associations between the Ala589Ser polymorphism and both raised systolic and diastolic blood pressures were identified. In addition to the kidneys, *WNK4* gene expression was also found in many other organs. Several *cis*-acting elements had been discovered in the promoter region of the gene. As revealed by preliminary experiment, various hormones can down-regulate the expression of *WNK4*, among which glucocorticoid hormone seems to act in a dose-dependent manner. The *WNK4* gene probably plays an important role in the pathogenesis of essential hypertension. As a missense mutation, the Ala589Ser polymorphism may bring changes to the enzyme's function(s), resulting in increased susceptibility to the disease.

Keywords Association · Essential hypertension · *WNK4* · SNP

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Introduction

The newly cloned serine–threonine kinase with no lysine (K) 4 gene (*WNK4*) has been mapped at 17q12-21, a hot locus for blood pressure regulation [1, 2]. Mutations in *WNK4* can cause a Mendelian trait featuring pseudohypoaldosteronism type II (PHAII; Online Mendelian Inheritance in Man No. 145260) [3], an autosomal dominant disorder characterized by severe hypertension, hyperkalemia and renal tubular acidosis caused by impaired K^+ and H^+ secretion. *WNK4* is predominantly expressed in the distal convoluted tubule, connecting tubule and cortical collecting duct of the kidneys, which are crucial areas for regulation of salt and water reabsorption [3, 4].

In 1999, our group started to investigate an isolated population identified in the Kerqin desert region in Zhangwu county of Liaoning, northeastern China, where a high prevalence of essential hypertension was discovered. The region also features a dry climate and the people having a high dietary salt intake. The standardized

morbidity from essential hypertension in this region reaches 35%, much higher than other areas in China [5].

This group has provided an ideal population for genetic epidemiological research on essential hypertension. In recent years, we have systematically searched among the population for particular single nucleotide polymorphisms (SNPs) within candidate genes, including endothelial channel beta-subunit (*ENAC*), G-protein β_3 subunit (*GNB3*) and β-adrenergic receptor (*β-AR*) family, whose roles in hypertension have already been confirmed [6, 7].

Following the discovery of *WNK4* gene, a number of studies have been conducted to investigate the association between particular SNPs within the gene and the onset of hypertension. So far, however, results of such studies have been controversial [8–13]. By sequencing the entire coding regions of *WNK4*, Kokubo et al. [14] identified 21 polymorphisms among 771 hypertensive subjects and 1,047 controls randomly sampled in Suita city in Japan. Their results indicated that systolic blood pressure in men with the CT + TT genotype for *WNK4* C14717T was 3.1 mmHg higher than those with the CC genotype ($P = 0.042$). In addition, three missense mutations of the *WNK4* gene, clustered into a small region within exon 7 but distant from the catalytic kinase domain, were identified in PHA II families [3]. Any of these can lead to a functional reduction of *WNK4*, whose normal function is to suppress the expression of Na–Cl co-transporter (NCCT) on the cell surface, therefore reducing the reabsorption of salt and water [15, 16]. In our previous work, we screened *WNK4* exon 7 and found only one G/A polymorphism (nucleotide position: 1155547 in sequence NT_010840.8) for which the frequency of A allele was significantly higher in the hypertensive group [17]. However, this turned out to be a synonymous polymorphism. Here, we have tested a G/T polymorphism (Ala589Ser) in exon 8 of *WNK4* (nucleotide position: 1155942 in sequence NT_010840.8) by case-control study and assessed the relevance of this SNP to the clinical phenotypes. In addition, the expression pattern and regulatory mechanisms of the *WNK4* gene were also explored using Northern blotting and in vitro hormone stimulation assays.

Materials and methods

Subjects

A total of 259 unrelated patients with essential hypertension and 235 unrelated normal controls were recruited. All subjects were from Zhangwu County and diagnosed with the criteria that systolic blood pressure (SBP) was above 140 mmHg and/or diastolic blood pressure (DBP) was above 90 mmHg, or usage of antihypertensive agents. Blood pressure was measured three times on the right arm of seated participants and averaged after at least 5 min resting. All subjects had routine laboratory tests for plasma electrolytes, glucose, total cholesterol (T-chol), low-density lipoprotein cholesterol (LDL-chol), high-density lipoprotein cholesterol (HDL-chol) and triglyceride (TG). Ethical approval and informed consent were obtained from the local ethics committee as well as from all subjects.

Genotyping

Genomic DNA was extracted from peripheral blood by samples phenol and chloroform. The targeted polymorphism of *WNK4*, Ala589Ser, was detected by polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP). PCRs were performed with forward primer 5′-TGGAAACCCATTTTCCCCTGG-3′ and reverse primer 5′-AGGTGGTGAGGCCTAGAAAGT-3′ at the annealing temperature of 62°C. Ten microliters of PCR product was digested overnight at 37°C with 5 units of restriction endonuclease AlwNI (New England Biolab, Ipswich, MA). Digested products were run on 1.2% agarose gel and stained with ethidium bromide to check their patterns. Wild-type (A589) was cut into 180 and 112 bp, whereas variant type (S589) was uncut (Fig. 1). Genotypes were confirmed by direct sequencing (Shenggong Inc., Shanghai, China).

Statistical analysis

All statistical analyses were performed with SPSS version 11.5 for Windows. χ^2 test was used to examine whether the genotype distributions differed from expected with Hardy–Weinberg equilibrium. Subjects were compared with respect to plasma glucose, T-chol, TG, LDL-chol and

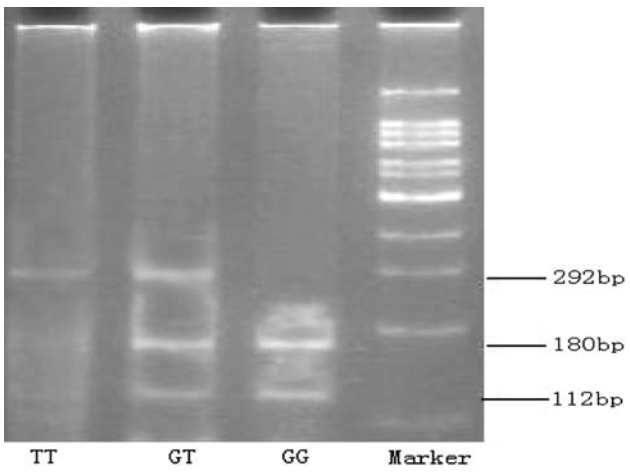

Fig. 1 Detection of Ala589Ser polymorphism of *WNK4* gene using PCR-RFLP. PCR products were digested with AlwNI. Wild-type (A589) was cut into 180 and 112 bp, whereas variant type (S589) was uncut

HDL-chol using the independent sample *t*-test. Statistical significance for differences in genotypes and allele frequencies between patients and controls and between different populations was assessed using the χ^2 test. Association between genotype and hypertension was evaluated by multiple linear regression analysis, where SBP and DBP were regarded as dependent variables, and other parameters were sequentially entered as independent variables. Ala589Ser carriers were grouped together because of low TT prevalence. *P* values less than 0.05 were considered to be statistically significant.

Northern blotting

Total RNA was extracted from various human tissues (with informed consent obtained) using TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA (20 mg) was fractionated in a 1.2% agarose-formaldehyde gel and then transferred onto a nylon membrane Hybond-N (Amersham Biosciences, Piscataway, NJ). A 321-bp antisense cRNA from *hWNK4* cDNA was labeled, hybridized and detected by CDP-Star using DIG Northern starter kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Signals of hybridization bands were detected on X-ray film and quantified by densitometric analysis.

Influence of various hormones on the expression of *hWNK4*

COS-7 cells derived from African green monkey SV40-transferred kidney fibroblast were maintained in Dulbecco's Modified Eagle's Medium (Gibco/BRL, Bethesda, MD) with 10% fetal bovine serum, 100 unit/ml penicillin and 100 mg/ml streptomycin at 37.8°C in a humidified atmosphere containing 5% CO_2. To assess the influence of various hormones on the expression of *WNK4* gene, cells cultures were switched to serum-free media and then exposed to particular hormones for 24 h (detailed dosages please refer to Fig. 3). To assess the influence of various dosages of dexamethasone on the expression of *WNK4*, COS-7 cells were transfected with a pCAT-*WNK4* promotor and stimulated with 1 and 10 nM of dexamethasone. Twenty-four hours later, absorbance at 405 nm was measured using an ELISA plate reader; the ratios between the sample protein and total protein concentrations were then calculated.

Results

Clinical characteristics of subjects

Characteristics of hypertensive patients and normotensive controls are summarized in Table 1. Compared with the control group, hypertensive groups had significantly higher levels of blood glucose, T-chol, TG, LDL-chol and BMI in addition to raised systolic and diastolic blood pressures.

Table 1 Characteristics of subjects in the hypertensive and control groups

Variable	Hypertensives	Controls	*P*
Number of subjects	259	235	
Male:female	118:141	99:136	0.443
Age (years)	51.45 ± 12.09	49.50 ± 13.97	0.097
SBP (mmHg)	156.12 ± 25.56	113.23 ± 12.73	<0.0001
DBP (mmHg)	96.88 ± 11.92	73.78 ± 8.99	<0.0001
T-chol (mmol/l)	5.09 ± 1.02	4.39 ± 1.08	<0.0001
TG (mmol/l)	1.68 ± 1.36	1.06 ± 0.86	<0.0001
HDL-chol (mmol/l)	1.59 ± 0.40	1.58 ± 0.37	0.929
LDL-chol (mmol/l)	3.02 ± 0.80	2.57 ± 0.80	<0.0001
Blood glucose (mmol/l)	4.72 ± 1.58	4.59 ± 0.89	0.048
Serum ferrum (μmol/l)	16.35 ± 6.81	16.56 ± 6.97	0.735
Serum calcium (mmol/l)	2.40 ± 0.09	2.38 ± 0.19	0.095
Serum natrium (mmol/l)	142.67 ± 13.22	141.72 ± 10.14	0.375
Serum potassium (mmol/l)	4.07 ± 0.53	4.04 ± 0.44	0.542
BMI (kg/m^2)	25.37 ± 6.54	22.02 ± 3,57	<0.0001

Variables are presented as mean ± SD

SBP Systolic blood pressure, *DBP* diastolic blood pressure (DBS), *T-chol* total cholesterol, *LDL-chol* low-density lipoprotein cholesterol, *HDL-chol* high-density lipoprotein cholesterol, *TG* triglyceride

Frequencies of Ala589Ser polymorphisms of *WNK4*

For the 494 subjects in the two populations, the genotype and allele frequencies of *WNK4* gene did not deviate from Hardy–Weinberg equilibrium ($\chi^2 = 2.73$, $P = 0.10$), and the overall frequencies were similar to those of African Americans [9] ($P > 0.05$) (Table 2). However, the frequency of T allele in the hypertensive group was significantly higher than that of controls (25.9 vs. 20.2%, $P = 0.035$). OR for hypertension to carry T allele was 1.38 (95% CI 1.02–1.86). GT and TT genotypes were closely associated with both raised systolic and diastolic blood pressures (Table 3).

Association of Ala589Ser polymorphism with particular clinical characteristics

In addition to the significant differences in both systolic and diastolic blood pressures between subjects with different genotypes (Table 4), stepwise regression analysis, in which plasma electrolytes, glucose, T-chol, TG, LDL-chol,

Table 2 Frequency of Ala589Ser polymorphism in Chinese and African American populations

	Cases	Genotype frequency, *n* (%)			Allele frequency, *n* (%)	
		GG	GT	TT	G	T
Chinese	494	285 (57.69)	189 (38.26)	20 (4.05)	759 (76.82)	229 (23.18)
African American	172	103 (59.88)	61 (35.47)	8 (4.65)	267 (77.62)	77 (22.38)

Table 3 Comparison of *WNK4* gene Ala589Ser polymorphism between hypertension and control groups

	Cases	Genotype frequency, *n* (%)			Allele frequency, *n* (%)	
		GG	GT	TT	G	T
Hypertensives	259	136 (52.51)	112 (43.24)	11 (4.25)	384 (74.13)	134 (25.87)
Controls	235	149 (63.40)	77 (32.77)	9 (3.83)	375 (79.79)	95 (20.21)
					$\chi^2 = 4.43$	$P = 0.035$

Table 4 Characteristics of subjects with different genotypes of *WNK4* Ala589Ser polymorphism

Clinical phenotype	GG	GT + TT	*P*
SBP (mmHg)	133.39 ± 28.44	138.89 ± 30.99	0.041
DBP (mmHg)	84.59 ± 14.81	87.66 ± 16.68	0.032
T-chol (mmol/l)	4.77 ± 1.11	4.74 ± 1.10	0.725
TG (mmol/l)	1.36 ± 1.07	1.41 ± 1.33	0.620
HDL-chol (mmol/l)	1.57 ± 0.38	1.60 ± 0.39	0.372
LDL-chol (mmol/l)	2.85 ± 0.83	2.76 ± 0.83	0.233
Blood glucose (mmol/l)	4.77 ± 1.48	4.62 ± 1.01	0.196
Serum ferrum (μmol/l)	16.47 ± 7.30	16.43 ± 6.28	0.956
Serum calcium (mmol/l)	2.39 ± 0.18	2.39 ± 0.10	0.585
Serum natrium (mmol/l)	141.87 ± 12.69	142.70 ± 10.62	0.441
Serum potassium (mmol/l)	4.06 ± 0.50	4.05 ± 0.50	0.772

Variables are presented as mean ± SD

SBP Systolic blood pressure, *DBP* diastolic blood pressure (DBS), *T-chol* total cholesterol, *LDL-chol* low-density lipoprotein cholesterol, *HDL-chol* high-density lipoprotein cholesterol, *TG* triglyceride

Table 5 Multiple regression analysis of blood pressure

	β-Coefficient	*P*
SBP		
Constant	67.034	<0.0001
TG	4.526	<0.0001
LDL	10.215	<0.0001
Natrium	0.427	0.002
Genotype	5.308	0.039
	$R^2 = 0.135$	
DBP		
Constant	64.889	<0.0001
TG	2.424	<0.0001
LDL	4.676	<0.0001
Genotype	3.328	0.015
	$R^2 = 0.124$	

Dependent variables: SBP, DBP

SBP Systolic blood pressure, *DBP* diastolic blood pressure (DBS), *LDL* low-density lipoprotein, *TG* triglyceride

HDL-chol, age, body mass index (BMI) and genotype (0 = GG, 1 = GT + TT) were considered as independent variables, also suggested TG, LDL-chol and genotype to be significantly associated with SBP and DBP in all subjects (Table 5).

Expression pattern of the *WNK4* gene and its potential regulatory mechanisms

As revealed by Northern blotting, the *WNK4* gene was also found to express in many other organs besides the kidneys (Fig. 2). Using software including TRANSFAC 4.0 (available at http://transfac.gbf.de/TRANSFAC/), TSSG/TSSH (available at http://www.cbs.dtu.dk/services/Promoter/) and NSITE (available at http://www.softberry.com/berry.phtml), a number of cis-acting elements, e.g., AP1, SP1, GRE and GATA, were identified upstream (0 to −600 bp) of the gene. As indicated with a RT-PCR assay, expression of *WNK4* may be down-regulated by various hormones (Fig. 3). Among

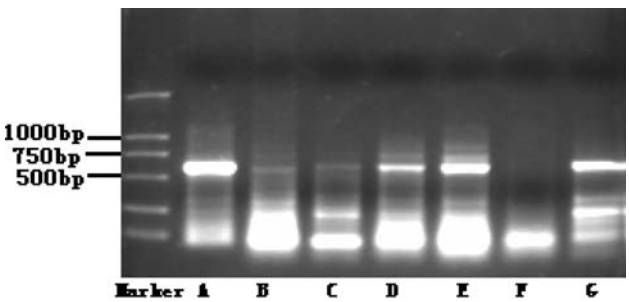

Fig. 2 Expression of *WNK4* gene in selected human tissues except the liver. *Lane A* kidney, *B* heart, *C* brain, *D* small intestine, *E* spleen, *F* liver, *G* lungs

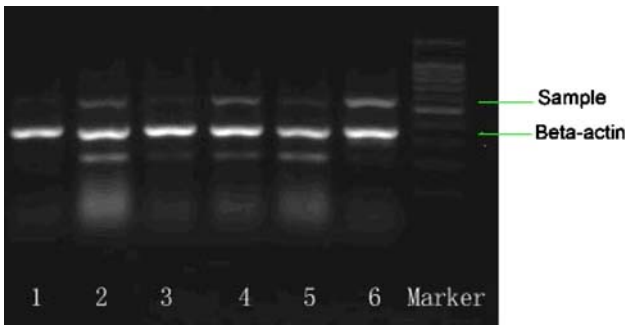

Fig. 3 Down-regulating effect of various hormones on the expression of *WNK4* gene. COS-7 cells were treated with the above hormones [*lane 1* estrogen (final concentration 1 μmol/l), *2* insulin (100 nmol/l), *3* dexamethasone (1 nmol/l), *4* angiotensin II (5 μmol/l), *5* growth hormone (6 nmol/l), *6* blank control] for 24 h. RNA of the cells were then extracted with the Trizol method and quantified with RT-PCR

Table 6 Influence of dexamethasone on the transcription activity of *WNK4* gene

	Blank control	Dexmethasone (1 nM)	Dexmethasone (10 nM)
Total protein in cell lyse OD595	0.567	0.691	0.689
Total protein (mg/ml)	14.28	18.55	18.48
CAT value OD405	0.143	0.116	0.177
Target protein concentration (mg/ml)	2.043	2.020	2.072
Target protein/total protein	0.143	0.109	0.112

these, glucocorticoid hormones, e.g., dexamethasone, seem to act in a dose-dependent manner (Table 6).

Discussion

A great discrepancy seems to exist between our findings and the results by Erlich et al. [9], which rejected the association between the Ala589Ser polymorphism and essential hypertension in American populations. A similar discrepancy was reported by Speirs et al. [11], who failed to detect the association between an intron 10 polymorphism and hypertension. Notably, the relatively smaller size and greater genetic heterogeneity in their subjects may have contributed to the bias. The population in our study derives from a relatively isolated region featuring a high prevalence of hypertension. The dry climate, high dietary salt intake, low migration and high morbidity of hypertension have made it a typical and ideal subject for genetic epidemiological research on the disease. Our previous studies on the same population have identified an association between polymorphisms of β_1-adrenergic receptor gene and essential hypertension, but excluded those with β_2-adrenergic receptor, β_3-adrenergic receptor or G-protein β_3 subunit genes [6, 7].

The frequency of S598 polymorphism of *WNK4* was found to be elevated in hypertensives in this study, which also found support from subsequent multiple linear regression analysis. We also analyzed other blood pressure-related characteristics; however, besides TG, LDL-chol and the Ala589Ser polymorphism in *WNK4* exon 8, no other parameters could be associated with increased risk for the disease. How much of the effect is attributable to the polymorphism? Which parameters fell out of the analysis? Smoking? Obesity? Gender?

As a missense mutation, the Ala589Ser polymorphism can result in substitution of a nonpolar residue alanine by a polar residue serine. The location of this polymorphism is close to three mutations clustered to the first putative coil domain of the *WNK4* and thereby may cause PHA II by diminishing the function(s) of its product. It seems that this change in polarity can trigger a spatial conformation change for the functional domain of *WNK4* kinase, which may affect its activity or interaction(s) with downstream protein(s).

Taken together, our results have suggested that various hormones may influence the expression of *WNK4* gene in the kidneys. As a missense mutation, the Ala589Ser polymorphism may play an important role in the increased susceptibility for essential hypertension.

Acknowledgements This study was sponsored by a grant from the Natural Scientific Foundation of China (no. 30300204) and a Doctoral Degree Starting Grant from Liaoning Province (no. 20051041). We are grateful to all patients and their families for their support.

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