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Association Study of G Protein-Coupled Receptor Kinase 4 Gene Variants with Essential Hypertension in Northern Han Chinese

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Summary

To investigate the association between polymorphisms in the G protein-coupled receptor kinase 4 gene (*GRK4*) (*R65L*, *A142V* and *A486V*) and essential hypertension in northern Han Chinese, we conducted a case-control study consisting of 503 individuals with essential hypertension (HT) and 490 age-, gender-, and area-matched normotensive (NT) controls. The three *GRK4* variants were genotyped by PCR-RFLP analysis. Both haplotype and single locus analysis were used to process the genotyping data. The *A486* allele showed a significant association with HT ($P < 0.001$). A total of 6 haplotypes were observed in the entire population, with the haplotypes *L-V-A* and *R-A-A* being found to be significantly related to hypertension ($P = 0.001$).

Keywords: G protein-coupled receptor kinase 4, essential hypertension, association study, polymorphism

Introduction

It has been suggested that the G protein-coupled receptor kinase 4 protein was associated with essential hypertension (HT), as it is involved in the desensitization of G protein-coupled receptors including the D_1 receptor. Dopamine exerts its natriuretic actions via the D_1-like and D_2-like receptors located in the renal proximal tubule. In conditions of sodium excess, locally produced dopamine acts on renal tubule cells to inhibit sodium reabsorption (Jose *et al.* 2003).

Three variants of the γ isoform of *GRK4*, *R65L*, *A142V* and *A486V* have been reported to affect GRK activity, resulting in increased serine phosphorylation of D_1 receptors and uncoupling of the receptor from its G-protein complex. Bengra *et al.* (2002) deserved a significant association ($P = 0.034$) between *V486* and an Italian population of mildly hypertensive patients. In another study the *V486* allele was also found to be associated with HT ($P = 0.02$), and additionally the *L65* and the *V142* alleles tracked with elevation in diastolic blood pressure (DBP), even though this was seen only in male HTs ($P = 0.009$; $P = 0.002$, respectively) (Speirs *et al.* 2004). Haplotype frequency differences between the HT and NT groups were also observed in this study, particularly for the *R-V-V* haplotype containing *R65L*, *A142V* and *A486V* (Speirs *et al.* 2004).

Considering the functional importance of *GRK4*, and the results from studies in Caucasian populations, we performed a case-control design using three variants (*R65L*, *A142V* and *A486V*) in the *GRK4* gene, aiming at examining the role of these polymorphisms in the development of the HT in Northern

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Han Chinese by single locus analysis and haplotype analysis.

Materials and Methods

Subjects

In this study all of the DNA samples and clinical data from participants were collected from the International Collaborative Study of Cardiovascular Disease in Asia (InterASIA in China) (Gu *et al*. 2002). The local bioethical committee approved the protocol, and informed consent was obtained from each participant. We enrolled 503 unrelated HT subjects and 490 unrelated NT subjects from Beijing City, Jilin, Shaanxi and Shandong Province, where high prevalences of cardiovascular morbidity and mortality have been observed. Three BP measurements were obtained from each participant by trained and certified observers, according to a standard protocol recommended by the American Heart Association (Perloff *et al.* 1993). Hypertension was defined as an average systolic blood pressure (SBP) $\geq$160 mmHg or an average DBP $\geq$ 100 mmHg. The NT subjects had SBP <140 mmHg and DBP <90 mmHg. Blood pressure values from subjects remaining on antihypertensive medications were adjusted for treatment effects according to the algorithm used in the analyses of Framingham data (Levy *et al*. 2000). Using serum and urinary tests, and checking their detailed clinical history, we excluded subjects with secondary hypertension, coronary heart disease and diabetes from the study.

Genotyping

The genomic sequence for *GRK4* was obtained from NCBI Entrez (http://www.ncbi.nlm.nih.gov/). The *R65L*, *A142V* and *A486V* polymorphisms in the *GRK4* gene were genotyped by means of polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). The sequences of PCR primers and conditions for RFLP are described in Table 1. Samples were subjected to denaturation at 94°C for 5 min, followed by 38 cycles of 94°C for 15 s, annealing at the 60°C for 15 s, then extension at 72°C for 30s and a final step at 72°C for 2 or 7 min. Each 10 μl reaction consisted of approximately 25 ng of genomic DNA, 2pmol of each primer (both the *A142V* and *A486V* polymorphisms were detected using primers containing a single base pair mismatch, to introduce a restriction site into the mutant gene sequence), 0.2 mM of each deoxynucleoside triphosphate, 1 unit of *Hot start Taq* Enzyme (TaKaRa) and 1 μl reaction buffer. Restriction enzymes were obtained from TaKaRa or MBI Fermentas and digests (10 μl) containing 1 unit of restriction enzyme were incubated at 37°C for 10 hours (Table 1). The digests were then separated on a 3% agarose gel and visualized by ethidium bromide staining.

Statistical Analysis for Single Markers

Statistical analyses were conducted using the SAS program (SAS Institute Inc., Cary, NC, USA) and the HWE program (Guo *et al.* 1992). Quantitative data were expressed as mean $\pm$ SD. Hardy-Weinberg equilibrium was assessed by Fisher's exact test using HWE.

The differences in clinical characteristics between HT and NT groups were assessed by the *t*-test for quantitative variables and chi-square test for categorical ones. The frequencies of the alleles and genotypes between HT and NT subjects were compared by the chi-square test. To further examine the association between the *GRK4* genotypes and blood pressure levels we analyzed

Table 1 Primer sequences and restriction enzymes

Polymorphisms	Primer sequences	Restriction enzymes (T)
448G → T, R65L	5′-TTGCTTCTTATCCCTTTGC-3′(F) 5′-TTTGAGACGGAGTCTTGCT-3′(R)	*Aat*II, (37°C)
679C → T, A142V	5′-GCAGAAGGTTGGGTGGTGT-3′(F) 5′-AAGGAGGAGAACCCTTCCAAAAAGG-3′(R)	*Hae*III, (37°C)
1711C → T, A486V	5′-AGAGTGGCGGTGTTTATGCG-3′(F) 5′-GGTGTCCAGGTAGATCCCTTTCAGC-3′(R)	*Hin*6I, (37°C)

F indicates forward primer; R, reverse primer; T, incubation temperature.
Underlined loci were mismatched.

their mean values according to the *GRK4* genotypes by ANOVA. For the single locus analyses the Bonferroni corrections were performed to control the family-wise type I error probability. That is, a *P*-value less than 0.004 (0.05/12) was considered as statistical significance. In the logistic regression analysis for single markers odds ratios (ORs) and 95% confidence intervals (CIs) were computed by the SAS program.

Haplotype Analysis

Pair-wise linkage disequilibrium coefficients were calculated with estimated haplotype frequencies in the NT cohort using the 2LD program (http://www.iop.kcl.ac.uk/IoP/Departments/PsychMed/GepiBst/software.shtml), with D′ expressing the extent of linkage disequilibrium. The EM algorithm-based function haplo.em in the Haplo.stats package (version 1.2.1, Mayo Clinic/Foundation, Rochester, Minn., USA, http://www.mayo.edu/hsr/people/schaid.html) of the statistical language R (http://www.r-project.org) was used to estimate each individual's haplotypes for the entire sample. The estimated data was then used by the function haplo.score for a haplotype global test and haplotype-specific test, and by haplo.glm for logistic regression analysis based on the genotyping data (Schaid *et al.* 2002).

Result

The demographic and clinical data from all individuals are shown in Table 2. Aside from BP and BMI, HDL-C, LDL-C, TG, Glu and Cr levels were significantly higher in cases than in controls. No significant differences were found between the cases and controls for smoking and drinking status.

R65L, A142V and A486V Polymorphisms

The genotype and allele frequency distributions of *R65L*, *A142V* and *A486V*, which were examined by the chi-square test, are shown in Table 3. Significant association of *A486V* with HT was observed after Bonferroni correction ($P < 0.001$). After analyzing the association of blood levels with both the genotypic and allelic frequency distributions of the three polymorphisms using the ANOVA test, we found SBP levels were proportional to the number of *A* alleles at the *A486V* locus, i.e., the more *A* alleles an individual carried the higher their SBP level would be ($P < 0.001$). A

Table 3 Genotypic and allelic frequency distributions of three polymorphisms in GRK4

Genotype	Cases (%)	Controls (%)	*P*
R65L			
RR/RL/LL	79.5/19.3/1.2	75.9/22.3/1.8	0.334
R/L	89.2/10.8	87.0/13.0	0.162
A142V			
AA/AV/VV	68.4/28.4/3.2	63.1/31.8/5.1	0.023
A/V	82.6/17.4	79.0/21.0	0.043
A486V			
AA/AV/VV	33.6/43.3/23.1	19.6/46.1/34.3	<0.001*
A/V	55.5/44.5	42.7/57.3	<0.001

*Probability was computed by Armitage's Test.

	Cases (n = 503)	Controls (n = 490)	*P*
Gender, M/F	262/241	257/233	0.91
Age, y	53.57 ± 9.34	53.51 ± 9.23	0.92
SBP, mm Hg	177.07 ± 28.05	117.47 ± 11.64	<0.0001
DBP, mm Hg	104.34 ± 12.28	75.05 ± 8.01	<0.0001
BMI, kg/m^2	26.32 ± 3.85	24.30 ± 3.56	<0.0001
HDL-C, mmol/L	1.25 ± 0.30	1.32 ± 0.34	0.0012
LDL-C, mmol/L	3.19 ± 0.86	3.09 ± 0.87	0.0544
TG, mmol/L	1.70 ± 1.06	1.43 ± 0.86	<0.0001
Glu, mmol/L	5.93 ± 1.79	5.60 ± 1.68	0.0026
Cr, μmol/L	71.22 ± 14.59	46.35 ± 11.56	0.0162
Smokers	204	211	0.42
Drinkers	173	164	0.76

BMI indicates body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; Cr, creatinine; Tg, triglycerides; HDL-C, high-density lipoprotein cholesterol; Glu, glucose; LDL-C, low-density lipoprotein cholesterol

Table 2 Characteristics of study participants

Table 4 Logistic regression parameters of single loci

Variables	OR (95% CI)	*P*
BMI	1.16 (1.11-1.20)	<0.0001
Glu	1.12 (1.03-1.22)	0.0108
A486V	AA vs. VV: 3.157 (2.145-4.646)	<0.0001
	AV vs. VV: 1.527 (1.092-2.134)	0.2771

OR indicates odds ratio; CI, confidence interval. ORs were adjusted for gender, age, BMI, smoking, drinking and blood lipid levels.

Table 6 Logistic regression parameters of haplotypes and environment covariates

Variables	OR (95% CI)	*P*
BMI	1.16 (1.11 to 1.20)	<0.0001
Glu	1.12 (1.03 to 1.22)	0.0081
R-A-A	1.39 (1.14 to 1.69)	<0.0001
R-V-V	0.29 (0.10 to 0.87)	0.0276
L-V-A	5.71 (1.66 to 19.68)	0.0005

R-A-A, *L-V-A*, in the order of *R65L*, *A142V* and *A486V*; *R-A-V* was specified as the baseline in the model.

similar trend also existed between DBP levels and genotypes of *A486V* ($P = 0.0013$). When other risk factors were adjusted for, it could be shown that *AA* carriers had more than a three-fold higher risk of becoming hypertensive than *VV* carriers (Table 4).

Haplotypes Analysis

A total of 6 haplotypes involving the *R65L*, *A142V* and *A486V* loci were observed in all subjects (Table 5). The D′ value of *R65L* and *A142V* was 0.98, which indicated strong LD between them, while the LD between *A142V* and *A486V* was relative weak ($D' = 0.44$). Both haplotypes *L-V-A* and *R-A-A* (in the order *R65L*, *A142V* and *A486V*) occurred more frequently in the HT group than in the NT group ($P = 0.001$, $P < 0.001$, respectively). However haplotypes *R-A-V, R-V-V* and *L-V-V* occurred less frequently in the HT group than in the NT group ($P < 0.001$, $P = 0.003$, $P = 0.008$, respectively). After analyzing all the estimated haplotypes by logistic regression, we observed that haplotypes *L-V-A*, *R-A-A* and *R-V-V* were still significantly associated with hypertension status after adjusting for effects of other risk factors. In particular, the haplotype *L-V-A* rendered about a six-fold higher risk of hypertension to carriers than the *R-A-V* haplotype (Table 6).

Table 5 Comparison of frequencies of estimated haplotypes between cases and controls

	Haplotypes					
	R65L	A142V	A486V	Cases	Controls	*P*
1	R	A	V	0.360	0.421	<0.001
2	R	V	V	0.008	0.028	0.003
3	L	V	V	0.080	0.124	0.008
4	R	V	A	0.063	0.054	0.684
5	L	V	A	0.026	0.005	0.001
6	R	A	A	0.460	0.370	<0.001

Discussion

Although we have found that the *A486V* polymorphism is associated with HT in the northern Chinese Han population, this result contradicts those found in studies of Caucasian Italians (Bengra *et al.* 2002) and Caucasian Australians (Speirs *et al.* 2004). In their studies the *V486* allele was found to be the risk factor for HT, while in ours *A486* was shown to be associated with HT. After analyzing the *R65L* and *A142V* variants, we found no association of these two variants with HT. This is somewhat in accordance with the results from Bengra *et al.* (2002) and Speirs *et al.* (2004). Additionally, we saw tracking of the *R* allele of the *R65L* variant with an elevation in DBP; this is also the opposite to the result found by Speirs *et al.* These controversial results might be due to population-specific differences in the extent of LD between *A486V* and a putative functional SNP, either in the *GRK4* gene or in nearby regions. Thus, further studies covering more genomic regions than the present study may help to test this hypothesis. Different sample sizes may also contribute to discrepant results. In the Bengra study 60 HTs and 60 NTs were included, and in Speirs's study 168 HTs and 60 NTs (Bengra *et al.* 2002; Speir *et al.* 2004); our sample sizes are considerably larger.

In the present study, the chi-square test was used to examine the association of HT with both genotypic and allelic frequency distributions of three polymorphisms, and the ANOVA test was used to examine the association of blood levels with these genetic factors. To

limit the type I error to a reasonable level (in our case, 0.05), corrections for multiple testing by the Bonferroni method were applied, though this approach was judged to be conservative, in the sense of enabling fewer variants to be identified as associated with hypertension status.

Haplotypes composed of adjacent SNPs are considered to have a greater information content than single SNPs, and thus have more power to explore the association between candidate genes and complex diseases (Akey *et al.* 2001; Daly *et al.* 2001; Johnson *et al.* 2001; Rioux *et al.* 2001). The program Haplo.stats provides several different global tests of association of haplotypes with a wide variety of traits, as well as haplotype-specific tests, which gives a meaningful advantage in attempts to understand the roles of many different haplotypes (Schaid *et al.* 2002). Using this programme, we found that both haplotypes *R-A-A* and *L-V-A* were positively associated with hypertension, whereas haplotype *R-V-V* was negatively associated. Since the susceptibility allele *A486* was present in both *R-A-A* and *L-V-A* it was apparent that most of the effect of *R-A-A* may be due to the *A486* allele. Thus, in our study the haplotype analyses had a similar degree of power to a single marker analysis. Because the frequency of haplotype *L-V-A* was low in both cases and controls, haplotypes could not be assigned with complete certainty. Accordingly, caution should be taken in interpreting our results about the risk of becoming hypertensive, given the wide CIs for the predicted OR values.

The SNP *A486V* was not in Hardy-Weinberg equilibrium in the HT group in this study, even though it was re-genotyped by direct sequencing of the genomic segment it is found in. Generally, deviation from Hardy-Weinberg equilibrium may be due to various factors including biological and non-biological influences. To accommodate the influence of departure from Hardy-Weinberg equilibrium we analyzed the genotype frequencies of *A486V* by Armitage's Test (Sasieni, 1997).

In summary, the results of our study in a Northern Chinese Han population showed great differences from those found in Italian and Australian Caucasian populations, even though all studies found the same result: that the *A486V* variant is significantly associated with HT. Considering the complex nature of essential hypertension, and population-specific differences, a further study employing more extensive SNPs and a great number of subjects in different populations may elucidate the functional SNPs in the *GRK4* gene.

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