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POLYMORPHISMS OF *NRF2*, AN ANTIOXIDATIVE GENE, ARE ASSOCIATED WITH BLOOD PRESSURE IN JAPANESE

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ABSTRACT

Nuclear factor-erythroid 2 (NF-E2)-related factor 2 (*Nrf2*) is a transcription factor that regulates the expression of antioxidant genes by activating *Nrf2*-antioxidant response element (ARE) pathway. This study aimed to investigate association of *Nrf2* gene single nucleotide polymorphisms (SNPs), rs35652124 (A→G) and rs6721961 (C→A), with various laboratory data in 464 health evaluation examinees. The genotyping of these SNPs was performed using polymerase chain reaction with confronting two-pair primers (PCR-CTPP) assay. The genotype frequencies of rs35652124 SNP were 21.1% for AA, 44.0% for AG, and 34.9% for GG. The frequency of A allele was 0.431. In male subjects, cholinesterase was significantly high, and HDL cholesterol was significantly low in (AG+GG) carriers. In female subjects, diastolic blood pressure (BP) was significantly low in (AG+GG) carriers. The genotype frequencies of rs6721961 SNP were 55.2% for CC, 34.7% for CA, and 10.1% for AA. The frequency of A allele was 0.275. In male subjects, systolic BP, diastolic BP and cholinesterase were significantly low, and iron was significantly high in (CA+AA) carriers. In female subjects, cholinesterase was significantly high in (CA+AA) carriers, and diastolic BP was significantly high in AA carriers. In conclusion, *Nrf2* polymorphisms are associated with BP in Japanese.

Key Words: *Nrf2*, blood pressure, SNP; cholinesterase; HDL cholesterol

INTRODUCTION

Nuclear factor-erythroid 2 (NF-E2)-related factor 2 (*Nrf2*) is a member of the cap'n'collar family of basic leucine zipper transcription factors that regulate the expression of many antioxidant pathway genes.[1)] *Nrf2* is maintained at basal levels in cells by binding to its inhibitor protein, Kelch-like erythroid-cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1).[2,3)]

A large number of studies revealed that *Nrf2* protects many cell types and organ systems from a broad spectrum of toxic insults and disease pathogenesis. For example, *Nrf2* protects lung from butylated hydroxytoluene-induced acute respiratory distress syndrome,[4)] hyperoxic injury,[5)]

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and bleomycin-mediated pulmonary fibrosis.[6] *Nrf2* increased sensitivity to acetaminophen-induced centrilobular hepatocellular necrosis and hepatotoxicity.[7,8] *Nrf2* also contributes to neuroprotection. Activation of the *Nrf2*-antioxidant response element pathway protects neuroblastoma cells from oxidative glutamate toxicity[9] and H_2O_2-induced apoptosis.[10] Thus, *Nrf2* is called the "multi-organ protector".[11]

Many single nucleotide polymorphisms (SNP) have been identified in the *Nrf2* gene.[12-14] Of special relevance are the rs35652124 (A→G) polymorphism and the rs6721961 (C→A) polymorphism, which are located in the promotor region of the gene. Both SNPs were found to reduce the transcription activity of *Nrf2*,[12] presumably resulting in decreased *Nrf2*-dependent gene transcription. Furthermore, a correlation between individuals carrying the rs6721961CA genotype and increased incidence of acute lung injury, has been reported.[12] This study aimed to investigate the relationship between these *Nrf2* SNPs and laboratory data in Japanese subjects.

MATERIALS AND METHODS

Study subjects

This study included 464 Japanese subjects who underwent health evaluation at Nagoya University Hospital. The general characteristics of the subjects were as follows: The subjects included 285 men and 179 women. The mean age was 49.7±12.7 (SD) years. Almost all the laboratory parameters were within the normal range for Japanese (Table 1). Written informed consent was obtained from all the subjects, and the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983. The subject enrollment was approved by Ethics Committee of Nagoya University School of Medicine in 2004 for Nagoya University Hospital.

Anthropometric, laboratory measurement

Height, weight, systolic blood pressure (BP) and diastolic BP were measured for all participants. Body mass index (BMI) was calculated by dividing the weight (kg) with the square of height (m). The body fat percentage was measured by an electrical body-fat-percentage measuring instrument (Tanita Inc., Tokyo, Japan). Blood samples were obtained after fasting for 12 h. The following biochemical parameters were determined by standard laboratory methods based on Japan Society of Clinical Chemistry: red blood cell, hemoglobin, white blood cell, platelet, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (γ-GTP), total protein, albumin, cholinesterase, total bilirubin, amylase, fasting glucose, total cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, uric acid, iron and C-reactive protein.

Genotyping of Nrf2 SNPs

Nrf2 SNPs, rs35652124 and rs6721961, located in the promoter were selected from the HapMap database. The genotyping was performed using polymerase chain reaction with confronting two-pair primers (PCR-CTPP) assay.[15] Confronting pairs of primers (four primers in all) are as follows:

rs35652124
Forward primer 1: CTTTTATCTCACTTTACCGCCCGAG
Forward primer 2: GCAGTCACCCTGAACGCCCT
Reverse primer 1: GACACGTGGGAGTTCAGAGGG

Table 1 General characteristics of subjects

		Total	Male	Female
n		464	285	179
Age	(year)	49.7±12.7	50.6±12.9	48.2±12.3
Height	(cm)	164.6±8.6	169.5±6.1	156.7±5.8
Body weight	(kg)	61.9±11.4	67.6±9.2	52.8±8.1
BMI	(kg/m^2)	22.7±3.1	23.5±2.7	21.5±3.3
Body fat percentage	(%)	20.7±6.4	18.8±5.7	23.8±6.2
Waist circumference	(cm)	77.3±10.2	82.2±8.1	69.6±8.1
Bone mineral density	(g/cm^2)	0.729±0.092	0.773±0.062	0.658±0.087
Systolic BP	(mmHg)	122.9±17.4	125.0±15.8	119.7±19.3
Diastolic BP	(mmHg)	78.0±11.8	80.8±11.1	73.5±11.6
%vital capacity	(%)	112.3±16.0	113.4±16.1	110.6±15.7
Forced expiratory volume 1.0sec %	(%)	90.2±7.0	89.9±6.7	90.7±7.4
Red blood cell	($\times10^6/\mu l$)	4.56±0.45	4.71±0.46	4.33±0.32
Hemoglobin	(g/dl)	14.1±1.4	14.8±1.0	12.9±1.2
White blood cell	($\times10^3/\mu l$)	5.13±1.45	5.25±1.50	4.95±1.36
Platelet	($\times10^3/\mu l$)	232±53	222±51	247±54
Creatinine	(mg/dl)	0.78±0.16	0.87±0.13	0.65±0.11
AST	(IU/l)	22.2±8.8	23.7±9.4	19.9±7.1
ALT	(IU/l)	23.4±17.8	27.4±19.9	17.0±11.1
ALP	(IU/l)	210.5±62.9	213.4±59.9	205.9±67.3
γ-GTP	(IU/l)	42.0±44.9	53.1±52.6	24.5±18.0
Total protein	(g/dl)	7.29±0.42	7.28±0.42	7.31±0.42
Albumin	(g/dl)	4.41±0.22	4.44±0.22	4.36±0.21
Cholinesterase	(ΔpH)	1.04±0.23	1.06±0.20	0.99±0.27
Total bilirubin	(mg/dl)	0.95±0.51	1.00±0.37	0.88±0.67
Amylase	(IU/l)	87.4±31.2	83.5±26.5	93.4±36.7
Fasting glucose	(mg/dl)	91.7±13.9	94.3±14.7	87.6±11.5
Total cholesterol	(mg/dl)	202.8±36.4	203.9±34.8	201.0±38.7
Triglyceride	(mg/dl)	108.8±77.2	121.0±89.0	89.3±47.1
HDL cholesterol	(mg/dl)	55.9±14.0	52.9±13.7	60.7±13.1
LDL cholesterol	(mg/dl)	125.2±33.9	126.9±33.4	122.6±34.5
Uric acid	(mg/dl)	5.62±1.37	6.24±1.21	4.64±0.99
Iron	(μg/dl)	114.9±41.6	123.1±39.4	102.0±41.7
C-reactive protein	(mg/dl)	0.100±0.239	0.110±0.265	0.084±0.190

Data are expressed as mean±SD

Reverse primer 2: GGGGTTCCCGTTTTTCTCCC
The region containing this polymorphism was amplified by PCR with these primers with the initial denature at 95°C for 10 min followed by PCR with these primers with the initial denature at 95°C for 10 min followed by 30 cycles at 95°C for 1 min, at 66°C for 1 min, at 72°C for 1 min and additionally at 72°C for 5 min. PCR products were visualized on a 2% agarose gel with ethidium bromide staining. Genotyping was performed as follows; 317, 145 bp for AA genotype, 317, 212, 145 bp for AG genotype, and 317, 212 bp for GG genotype.

rs6721961
Forward primer 1: CCCTGATTTGGAGGTGCAGAACC
Forward primer 2: GGGGAGATGTGGACAGCG
Reverse primer 1: GCGAACACGAGCTGCCGGA
Reverse primer 2: CTCCGTTTGCCTTTGACGAC
The region containing this polymorphism was amplified by PCR with these primers with the initial denature at 95°C for 10 min followed by PCR with these primers with the initial denature at 95°C for 10 min followed by 30 cycles at 95°C for 1 min, at 58°C for 1 min, at 72°C for 1 min and additionally at 72°C for 5 min. PCR products were visualized on a 2% agarose gel with ethidium bromide staining. Genotyping was performed as follows; 282, 113 bp for CC genotype, 282, 205, 113 bp for CA genotype, and 282, 205 bp for AA genotype.

Statistical analysis

All results are expressed as mean±SD, and significance was defined as a p value of <0.05. The analysis was done by using PASW statistics 18 (SPSS Japan Inc., Tokyo, Japan). Hardy-Weinberg equilibrium testing was performed by using the X^2 test. Student's t test and multivariate analysis adjusted for age were performed in comparison of the mean values between the different genotype groups.

RESULTS

Incidence of Nrf2 SNPs

Table 1 shows general characteristic of subjects. In this study, the genotype frequencies of the rs35652124 polymorphism were 21.1% for AA (n=98), 44.0% for AG (n=204), and 34.9% for GG (n=162). The frequency of A allele was 0.431, which was not in compliance with Hardy-Weinberg equilibrium (p=0.026). The genotype frequencies of the rs6721961 polymorphism were 55.2% for CC (n=256), 34.7% for CA (n=161), and 10.1% for AA (n=47). The frequency of A allele was 0.275, which was not in compliance with Hardy-Weinberg equilibrium (p=0.005). We consider that the difference between the observed and expected values could happen by accident.

Table 2 shows genotype frequencies of *Nrf2* gene. A strong linkage was observed between these two SNPs ($D'=0.944$, $r^2=0.445$). AA genotype of rs35652124 and AA genotype of rs6721961 had more cases (43 cases) than expected (32.8 cases).

Association of Nrf2 gene SNPs with various variables

Table 3 shows association of SNP rs35652124 with several variables. In male subjects, cholinesterase was significantly high, and HDL cholesterol was significantly low in (AG+GG) carriers. In female subjects, diastolic BP was significantly low in (AG+GG) and GG carriers, and total cholesterol was significantly low in GG carriers. The other variables did not show any significant association.

Table 2 Genotype frequencies of *Nrf2* gene

		rs6721961			
		CC	CA	AA	Total
rs35652124	AA	18	37	43	98
	AG	79	121	4	204
	GG	159	3	0	162
	Total	256	161	47	464

Table 3 Variables according to polymorphism rs35652124 in *Nrf2* gene

		rs35652124					
		AA	AG+GG	p value	AA+AG	GG	p value
Male							
n		59	226				
Cholinesterase	(ΔpH)	1.01±0.20	1.08±0.20	0.028			
HDL cholesterol	(mg/dl)	56.3±16.2	52.1±12.9	0.033			
Female							
n		39	140		116	63	
Diastolic BP	(mmHg)	77.3±12.5	72.4±11.2	0.017	74.9±11.7	70.9±11.2	0.026
Total cholesterol	(mg/dl)				206.0±39.5	191.8±35.7	0.019

Table 4 Multivariate analysis of polymorphism rs35652124 in *Nrf2* gene (adjusted for age)

	rs35652124	
	AA vs AG+GG	AA+AG vs GG
	p value	p value
Male		
Cholinesterase	0.037	ns
HDL cholesterol	0.036	ns
Female		
Diastolic BP	0.050	ns
Total cholesterol	ns	ns

Table 4 shows multivariate analysis of rs35652124 adjusted for age. In male subjects, cholinesterase was significantly high, and HDL cholesterol was significantly low in (AG+GG) carriers. In female subjects, diastolic BP was significantly low in (AG+GG) carriers.

Table 5 shows association of SNP rs6721961 with several variables. In male subjects, systolic BP, diastolic BP and cholinesterase were significantly low in (CA+AA) carriers, and iron was

Table 5 Variables according to polymorphism rs6721961 in *Nrf2* gene

		rs6721961					
		CC	CA+AA	p value	CC+CA	AA	p value
Male							
n		154	131				
Systolic BP	(mmHg)	126.9±17.8	122.6±12.8	0.019			
Diastolic BP	(mmHg)	82.2±12.4	79.3±9.1	0.024			
Cholinesterase	(ΔpH)	1.09±0.21	1.04±0.19	0.024			
Iron	(μg/dl)	118.4±39.3	128.5±39.1	0.033			
Female							
n		102	77		160	19	
Cholinesterase	(ΔpH)	0.96±0.29	1.05±0.22	0.024			
Diastolic BP	(mmHg)				72.9±11.0	78.5±15.5	0.045

Table 6 Multivariate analysis of polymorphism rs6721961 in *Nrf2* gene (adjusted for age)

	rs6721961	
	CC vs CA+AA	CC+CA vs AA
	p value	p value
Male		
Systolic BP	0.008	ns
Diastolic BP	0.013	ns
Cholinesterase	0.044	ns
Iron	0.025	ns
Female		
Cholinesterase	0.030	ns
Diastolic BP	ns	0.040

significantly high in (CA+AA) carriers. In female subjects, cholinesterase was significantly high in (CA+AA) carriers, and diastolic BP was significantly high in AA carriers. The other variables did not show any significant association.

Table 6 shows multivariate analysis of rs6721961 adjusted for age. In male subjects, systolic BP, diastolic BP and cholinesterase were significantly low, and iron was significantly high in (CA+AA) carriers. In female subjects, cholinesterase was significantly high in (CA+AA) carriers, and diastolic BP was significantly high in AA carriers.

DISCUSSION

Nrf2 is a member of the cap'n'collar family of basic leucine zipper transcription factors that regulate the expression of many antioxidant pathway genes in the so-called phase 2 response.[1] Under oxidative stress, phase 2 enzymes such as NAD(P)H: quinone oxidoreductase-1 (NQO1), glutathione peroxidase 2 (GPX2), and heme oxygenase-1 (HO-1), are induced to provide anti-oxidant and anti-inflammatory effects.[16] This process is mediated by activating the *Nrf2*-ARE pathway.[1] Therefore, *Nrf2* protects cells from oxidative stress.

This study first demonstrated that *Nrf2* polymorphisms are associated with BP in Japanese subjects. In male subjects, systolic BP and diastolic BP were significantly low in rs6721961 (CA+AA) carriers. In female subjects, diastolic BP was significantly low in rs35652124 (AG+GG) carriers. Nitric oxide can be degraded by superoxide free radicals, and oxidative stress can thus increase BP.[17] *Nrf2* may be involved in the regulation of anti-oxidative gene expression or anti-oxidant enzyme activities in the vessels. Marzec *et al.*[12] reported that *Nrf2* gene transcription activity was significantly high in rs6721961 C wild-type compared to promoter constructs bearing rs35652124 G and rs6721961 A variants. These promoter polymorphisms were predicted to have functional significance, and rs6721961 affects basal *Nrf2* expression and function.[12] Thus, different transcription activity due to *Nrf2* gene polymorphisms might affect BP by modulating protection against vascular oxidative stress.

In male subjects, cholinesterase was significantly high in rs35652124 (AG+GG) carriers. HDL cholesterol was significantly low in rs35652124 (AG+GG) carriers. Cholinesterase was significantly low in rs6721961 (CA+AA) carriers. Because cholinesterase has been related with fatty liver, the *Nrf2* polymorphism might be associated with lipid metabolism. Iron was significantly high in rs6721961 (CA+AA) carriers. *Nrf2* participates in the expression of HO-1.[18] HO-1 degrades heme into biliverdin, carbon monoxide and iron. Thus, *Nrf2* polymorphism may also be related with this metabolic pathway with HO-1.

In the present study, *Nrf2* rs35652124 G allele frequency was 0.571, and rs6721961 A allele frequency was 0.276. These alleles may decrease *Nrf2* transcriptional activity.[12] Hapmap database reported that rs35652124 G allele frequency was about 0.3, and rs6721961 A allele frequency was 0.1 in European descent. Japanese seem to have higher frequencies of low-*Nrf2*-activity alleles. In fact, rs35652124 AA genotype and rs6721961 CC genotype were observed only in 18 per 464 cases (Table 2).

In conclusion, the novel finding of the present study is that *Nrf2* polymorphisms are associated with BP in Japanese subjects. Antioxidative activity of *Nrf2* might be involved in the regulatory mechanism of BP.

CONFLICTS OF INTEREST

None has anything to declare conflicts of interest.

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