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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,\(^5\) and...
and bleomycin-mediated pulmonary fibrosis. Nrf2 increased sensitivity to acetaminophen-induced centrilobular hepatocellular necrosis and hepatotoxicity. Nrf2 also contributes to neuroprotection. Activation of the Nrf2-antioxidant response element pathway protects neuroblastoma cells from oxidative glutamate toxicity and H$_2$O$_2$-induced apoptosis. Thus, Nrf2 is called the "multi-organ protector".

Many single nucleotide polymorphisms (SNP) have been identified in the Nrf2 gene. Of special relevance are the rs35652124 (A→G) polymorphism and the rs6721961 (C→A) polymorphism, which are located in the promoter region of the gene. Both SNPs were found to reduce the transcription activity of Nrf2, 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. 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. Confronting pairs of primers (four primers in all) are as follows:

rs35652124
Forward primer 1: CTTTTATCTCACTTTACCGCCCGAG
Forward primer 2: GCAGTCACCCTGAACGCCCT
Reverse primer 1: GACACGTGGGAGTTCAGAGGG
## 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/m²)**      | 22.7±3.1  | 23.5±2.7  | 21.5±3.3  |
| **Body fat percentage (%)** | 20.7±6.4  | 18.5±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/cm²)** | 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 (×10⁶/μl)** | 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 (×10³/μl)** | 5.13±1.45 | 5.25±1.50 | 4.95±1.36 |
| **Platelet (×10³/μl)** | 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: GGGTTCCCGTTTTTCTCCC
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: CTCCGTTTGCTTTTGACGAC
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 X² 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, r²=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

| rs35652124 | 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 |
| 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 |
|------------|--------------|--------------|
| 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
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 antioxidant 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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