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**Association of *TNFRSF4* gene polymorphisms with essential hypertension**

**Short title:** *TNFRSF4* gene and essential hypertension

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**Conflicts of interest**

None

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**Abbreviations**

95% CI, 95% confidence interval; *Agt*, mouse angiotensinogen gene; ANOVA, analysis of variance; APC, antigen-presenting cell; CRP, C-reactive protein; DBP, diastolic blood pressure; EH, essential hypertension; HT, hypertensive; LD, linkage disequilibrium; MI, myocardial infarction; NT, normotensive; OR, odds ratio; PCR, polymerase chain reaction; SBP, systolic blood pressure; SD, standard deviation; SNP, single nucleotide polymorphism; TaqMan-ASA, TaqMan allele-specific amplification; TNFRSF4 (OX40), tumor necrosis factor receptor superfamily, member 4; TNFSF4 (OX40L), tumor necrosis factor (ligand) superfamily, member 4; UTR, untranslated region.

**Abstract**

Essential hypertension is a complex disorder that results from the interaction of a number of susceptibility genes and environmental factors. The *TNFRSF4* (*tumor necrosis factor receptor superfamily, member 4*) gene was one of the genes that showed altered renal expression in long-term salt loading in mice. Moreover, association of the *TNFRSF4* and *TNFSF4* (*tumor necrosis factor (ligand) superfamily, member 4*) genes with myocardial infarction was recently reported. Since essential hypertension is a well-known risk factor for myocardial infarction, we hypothesized that *TNFRSF4* could be a susceptibility gene for essential hypertension. We performed a case-control study of *TNFRSF4* in two independent populations. Extensive investigation of SNPs of the entire gene suggested that it resided in one linkage disequilibrium block, and 4 SNPs in the 5' flanking region sufficiently represented major haplotypes. In the combined population, the frequency of the most frequent haplotype, C-C-A-A, was significantly lower ($P = 8.07 \times 10^{-5}$) and that of the second most frequent haplotype, C-T-G-A, was significantly higher ($P = 6.07 \times 10^{-4}$) in hypertensive subjects than in control subjects. This difference was observed only in female patients. The C-T-G-A haplotype showed a lower promoter activity than other haplotypes, suggesting a relationship with disease susceptibility. Our results suggest that *TNFRSF4* is a female-specific susceptible gene for essential hypertension.

**Condensed Abstract**

We selected the *TNFRSF4* (*tumor necrosis factor receptor superfamily, member 4*) gene as a candidate gene for essential hypertension susceptibility and performed a case-control study of the gene in two independent populations. Haplotype analysis using 4 SNPs in the 5' flanking region of the gene revealed that the frequency of the most frequent haplotype was significantly lower and that of the second most frequent haplotype was significantly higher than controls, in female patients only. Furthermore, the second most frequent haplotype showed a lower promoter activity than other haplotypes. Our results suggest that *TNFRSF4* is a female-specific susceptible gene for essential hypertension.

**Key Words**

hypertension, essential; association studies; SNPs; haplotype; *TNFRSF4* gene

## Introduction

Hypertension affects more than 25% of the adult population worldwide [1]. Essential hypertension (EH) accounts for more than 90% of hypertension cases and is a multifactorial disorder resulting from the interaction of a number of susceptibility genes and environmental factors. It is estimated that the genetic contribution to blood pressure variation ranges from 30 to 50% [2]. Identification of susceptibility genes for hypertension would provide a clue to the pathophysiology of the disease.

There are several approaches for genetically dissecting EH; candidate-gene linkage studies, genome-scanning linkage studies, candidate-gene association studies, genetic studies in animal models, and gene expression profiling in animal models [3]. Each approach has its own strengths and weaknesses, and some argue integration of the approaches is a more efficient way forward [4]. The Millennium Genome Project for Hypertension in Japan has adopted the candidate-gene association strategy because of its relatively higher statistical power and convenience of collecting samples [5]. Candidate genes are selected based on the accumulation of experimental evidence (expression profiling in animal models) and information in the literature. As a first step in this project, we performed DNA microarray experiments in mice to screen genes whose renal expression was changed by long-term salt loading, because genes that showed salt sensitivity were considered to be candidate genes for EH. The results showed that more than 300 genes were either downregulated or upregulated. For the genetic association study, from these 300 genes we nominated 121 that had been reported in the literature as candidate genes. To date, 70 genes have been screened, 10 of which showed significant association with EH on haplotype-based analysis. Three of these 10 genes were positive in both the expression profiling and genetic association studies. The *tumor necrosis factor receptor superfamily, member 4* (*TNFRSF4*) gene was one of the three.

TNFRSF4 (OX40) is a member of the tumor-necrosis factor receptor (TNFR) superfamily, and is primarily expressed as a transmembrane protein on activated $CD4^+$ T cells after antigen recognition [6-9]. Tumor necrosis factor (ligand) superfamily, member 4 (TNFSF4, also called OX40L) [10], the ligand for TNFRSF4 on activated $CD4^+$ T cells, is expressed on antigen-presenting cells (APCs) including activated B cells, macrophages and dendritic cells, as well as on endothelial cells and some activated T cells [11-14]. The TNFRSF4–TNFSF4 interaction between T cell and APC contributes to proinflammatory T cell function. In particular, TNFRSF4–TNFSF4 interactions are crucial for the generation of memory $CD4^+$ T cells by promoting the survival of effector T cells [15-18]. Thus, it is suggested that the TNFRSF4-TNFSF4 pathway is involved in inflammation and immune response.

T lymphocyte activation involving several receptor–ligand pairs such as TNFRSF4–TNFSF4 is suggested to promote atherosclerosis [12, 19, 20], which is now considered to be an inflammatory disease [21]. Recently, *TNFSF4* was identified as a susceptibility gene for atherosclerosis and a genetic variation in *TNFSF4* was reported to be associated with myocardial infarction (MI) and severity of coronary artery disease [22]. Genetic variation in *TNFRSF4* was also shown to be associated with MI [23]. These reports suggested that the TNFRSF4–TNFSF4 pathway plays an important role in the pathogenesis of atherosclerosis and MI in humans. It is generally believed that hypertension is one of the major risk factors for atherosclerosis and MI [24]; however, MI and hypertension often coexist, as seen in the SHEEP study cohort in which MI patients were significantly associated with hypertension [25]. Thus, the association between MI and TNFRSF4/TNFSF4 in human subjects may be due to not only atherogenesis but also hypertension itself. We hypothesized that *TNFRSF4* and/or *TNFSF4* were potential candidate genes for EH.

The aim of the present study is to investigate the association between genetic variations of the *TNFRSF4* gene and EH in the Japanese population. We performed a case-control study

using two independent populations of Japanese patients with EH.

## Methods

*Study Subjects*

Initial screening of candidate genes involved 1035 subjects with EH (762 males and 273 females) and 1058 age-matched controls (792 males and 266 females) who were recruited through the study group of the Millennium Genome Project for Hypertension [5]. Six medical institutes took part in the collaborative study and recruited subjects in Japan. Recruitment procedures, case-control criteria, and clinical characteristics are described in detail elsewhere [5].

The clinical characteristics of the subjects included in this study for *TNFRSF4* gene analyses are shown in (Table 1) and (Table 2). Subjects in population 1 were a part of the population recruited through the study group of the Millennium Genome Project for Hypertension [5]. Subjects in population 2 were recruited from Ohasama, a cohort in a rural community of northern Japan [26].

Each subject was assigned to one of the blood pressure diagnostic categories defined by the criteria of the 1999 WHO/ISH guidelines for the management of hypertension [27]. Hypertensive (HT) subjects had systolic blood pressure (SBP) ≥140 mmHg or diastolic blood pressure (DBP) ≥90 mmHg or were patients currently taking chronic antihypertensive medication. Normotensive (NT) subjects had SBP/DBP < 140/90 mmHg, and had never been treated with antihypertensive medication. Informed consent was obtained from each individual as per the protocol approved by each institution's ethics committee.

*DNA microarray experiments in mice*

In DNA microarray experiments, we used two lines of mice having different numbers of the functional mouse angiotensinogen gene (*Agt*) [28, 29], kindly donated by Professor Oliver

Smithies (Department of Pathology, University of North Carolina). To observe distinct effects by long-term salt loading, *Agt* 2/2 mice (with four wild-type copies of the *Agt* gene) were fed a high-salt diet containing 8% NaCl for six months, while *Agt* 0/1 mice (with one wild-type copy of the *Agt* gene) were fed a low-salt diet containing 0.3% NaCl. Total RNA was isolated from the kidneys of mice and differences in gene expression were examined using mouse cDNA microarray (Incyte Genomics, Inc.), which contains 9222 mouse cDNA clones.

*Screening of candidate genes*

We selected a total of 121 candidate genes (Supplemental Table 1) based on the following criteria: (i) were genes reported as candidates in the literature or with functions relevant to the blood pressure regulation, and (ii) were human homolog of genes in which renal expression was changed by long-term salt loading in mice. For an initial screening of these candidate genes, some of the available single nucleotide polymorphisms (SNPs) per gene were selected from the Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/) or dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) and were genotyped in 1035 patients and 1058 controls using the PCR-SSP-FCS method [30]. Haplotype-based association analyses were performed using SNPAlyze v4.1 Pro software (DYNACOM, Mobara, Japan) based on an expectation/maximization (EM) algorithm. *P* values for overall distribution of haplotypes were calculated by permutation test at 1000 iterations. *P* values less than 0.05 were considered statistically significant.

*Screening for polymorphisms in TNFRSF4*

To identify genetic variants of the human *TNFRSF4* gene, we sequenced all 7 exons, the adjacent intronic sequence, 4 kb of the 5' flanking region and 1.5 kb of the 3' flanking region in 32 control subjects. Nineteen primer sets were designed on the basis of the *TNFRSF4*

genomic and mRNA sequences from the GenBank database (accession numbers NT_004350.18 and NM_003327, respectively). All polymerase chain reaction (PCR) products were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit and an ABI PRIZM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequences were analyzed and polymorphisms identified using the Genetyx program (Genetyx Corp., Tokyo, Japan).

*Genotyping of polymorphisms in TNFRSF4*

Genotyping of four SNPs in the *TNFRSF4* gene (P1: -3948C>T, P2: -3606C>T, P8: -1725A>G and P12: -530A>G) was performed using either the TaqMan™ allele-specific amplification (TaqMan-ASA) method [31] or the Custom TaqMan™ Genomic Assays kit (Applied Biosystems). In the TaqMan-ASA method, specific primers were designed on the basis of the *TNFRSF4* genomic sequence from the GenBank database (accession number NT_004350.18). The primer sequences are shown in (Table 3). The PCR mixture for the TaqMan-ASA method contained 5 μl of 2× TaqMan™ Universal Mix (Applied Biosystems), 0.4 μmol/L of each PCR primer, 0.12 μmol/L of TaqMan probe, and 5 ng of template DNA in a final volume of 10 μl. The samples were analyzed with an ABI PRIZM 7000 Sequence Detection system (Applied Biosystems). The thermoprofiles were 50ºC for 2 min, 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min.

*Luciferase assay*

*TNFRSF4* reporter constructs of 3970 bp (nt -3968 to +2) were created by means of PCR amplification of genomic DNA from homozygous subjects who had alternative haplotypes with the use of following primers: forward, 5'-GGGGTACCGTGCCACATGGCTGGAATTTAC-3' (including *KpnI* site) and reverse,

5’-TCTAGCTAGCGTCTCTGCTGTCGCCAGAGTC-3’ (including *NheI* site). Amplicons of three haplotypes (Pr-H1, Pr-H2 and Pr-H5) were cloned into the pGL4.10[luc2] vector (Promega, Madison, WI). Promoter constructs that contained one polymorphic change (Pr-P2-T, Pr-P3-T, Pr-P4-del, Pr-P6-G, Pr-P8-G, Pr-P9-G, Pr-P10-T, and Pr-P11-G) were created by site-directed mutagenesis carried out in the Pr-H1 plasmid using the GeneEditor$^{TM}$ *in vitro* site-directed mutagenesis system (Promega). All constructs were verified by sequencing. COS-7 cells (monkey kidney, SV40 T antigen-transformed) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. HEK293 cells (human embryonic kidney) were cultured in minimum essential medium supplemented with 2 mmol/L L-glutamine, 1% non-essential amino acids, 10% fetal bovine serum and antibiotics. Cells in 12-well plates at 50-70% confluence were transfected with 500 ng of each construct and 10 ng of pGL4.74[hRluc/TK] *Renilla* luciferase vector (Promega) as an internal control for transfection efficiency, using 1.5 μl of FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland). After 24 hours of transfection, the cells were harvested, and firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System and a TD-20/20 luminometer (Promega). Each experiment was repeated five or six times, and each sample was studied in triplicate.

*Statistical analysis*

Haploview version 3.32 (http://www.broad.mit.edu/mpg/haploview/index.php) was used to analyze and visualize the linkage disequilibrium (LD) and haplotypic patterns. Hardy-Weinberg equilibrium was assessed by $\chi^2$ analysis. Overall distributions of the genotypes or alleles were analyzed by $\chi^2$ analysis using $2\times3$ or $2\times2$ contingency tables between NT controls and HT patients. Haplotype frequencies were estimated using SNPAlyze v4.1 Pro software. The distributions of each haplotype between NT controls and HT patients

were calculated both by $\chi^2$ tests of one haplotype against the others (haplotype-wise test) and by permutation tests with 1000 iterations using SNPAlyze software. We calculated odds ratios (ORs) with 95% confidence intervals (CIs) using logistic regression analyses with or without clinical covariates (age, body mass index, total cholesterol, high-density lipoprotein cholesterol, and triglyceride). To estimate the contribution of the gene to the total variance of blood pressure, the variance component procedure with the analysis of variance (ANOVA) type III variance estimates was used. Comparisons in reporter assays were performed using Student's *t*-test or ANOVA. All statistical analyses were performed with SPSS software (SPSS Japan Inc, Tokyo, Japan) unless otherwise stated. *P* values less than 0.05 were considered statistically significant.

## Results

*DNA microarray experiments in mice*

We used cDNA microarray analyses to compare the expression profiles of 9222 genes in the kidneys of *Agt* 2/2 mice (with four wild-type copies of the *Agt* gene) with a high-salt diet vs. those of *Agt* 0/1 mice (with one wild-type copy of the *Agt* gene) with a low-salt diet. Differential expression values greater than 1.3 based on internal quality control data are summarized in (Supplemental Table 2) and (Supplemental Table 3). We found that 119 genes were downregulated in the kidneys of *Agt* 2/2 mice by 1.3- to 3.1-fold compared with *Agt* 0/1 mice, and 192 genes were upregulated by 1.3- to 1.9-fold. Murine *TNFRSF4* gene (*Tnfrsf4*) was the gene downregulated 1.3 fold.

*Screening of candidate genes by haplotype association study*

We selected a total of 121 candidate genes (Supplemental Table 1) based on the following criteria: (i) were genes reported as candidates in the literature or with possible involvement of blood pressure regulation, and (ii) were human homolog of genes in which renal expression was changed by long-term salt loading in mice. We excluded genes whose genotype data were not available due to the following reasons: no SNP data was available in the databases; minor allele frequencies of SNPs in Japanese were too low (< 5%); or the genotyping of some SNPs was difficult. So far, 191 SNPs in 70 genes have been successfully genotyped for genetic association tests, and the genotyping of only a single SNP was completed in 8 genes. A haplotype-based association test was performed in 62 genes and a single SNP association study in 8 genes. *P* values for difference in overall distribution of the haplotype or genotype frequencies between NT and HT in total (male + female), male, and female subjects are shown in (Supplemental Table 4). Significant *P* values were observed for 10 genes;

*Aquaporin-2* (*AQP2*), *Estrogen receptor 2* (*ESR2*), *Glycogen synthase 1* (*GYS1*), *Kallikrein 1* (*KLK1*), *Nephrin* (*NPHN*), *Solute carrier family 1 (glial high affinity glutamate transporter), member 2* (*SLC1A2*), *Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3* (*SLC9A3*), *Steroidogenic acute regulatory protein* (*STAR*), *Syntaxin binding protein 1* (*STXBP1*) and *TNFRSF4*. Three genes (*STAR*, *STXBP1*, and *TNFRSF4*) are the human homologs to the mouse genes that showed changes in renal expression in the salt-loading experiment. The *P* value for overall distribution of the haplotype of *TNFRSF4* was significant only in female subjects.

*Identification of polymorphisms in TNFRSF4*

We searched for polymorphisms in the *TNFRSF4* gene, including 4 kb of the 5' flanking region and 1.5kb of the 3' flanking region. By direct sequencing in 32 Japanese individuals, a total of 44 polymorphisms were identified; 20 in the 5' flanking region, 4 in exons, 7 in introns, and 13 in the 3' flanking region. Of those, 27 polymorphisms (P1-P27) with minor allele frequencies (MAF) $\geq$ 5% (in 32 DNA samples) are presented in (Table 4). A graphical overview of the structure of the human *TNFRSF4* gene showing the location of the 27 polymorphisms identified in this study is shown in (Fig. 1). Pair-wise LD measuring $r^2$ between polymorphisms and defined haplotype block structures in this region was evaluated using the solid spine of LD method in Haploview (Fig. 1). Three haplotype blocks (blocks 1, 2 and 3) were defined in the *TNFRSF4* gene region with this method. Blocks 1 and 2 appear to be separated because P8 showed low LD to other polymorphisms and blocks 2 and 3 were separated by P22 for the same reason. However, strong LDs were observed among certain blocks, such as between P4 and P27 ($r^2$ = 0.91). In addition, multiallelic D' values between these blocks were high (0.86, between blocks 1 and 2; 1.0, between blocks 2 and 3). Thus, we decided to handle an entire gene region as one block, which could be analyzed by tag SNPs

from the entire region. Four SNPs in the 5' flanking region (P1: -3948C>T, P2: -3606C>T, P8: -1725A>G and P12: -530A>G) were employed for further analysis. The 4-SNP haplotypes constructed from these SNPs covered more than 85% of haplotype diversity of the entire *TNFRSF4* gene when P22 was not included for analysis.

*Case-control study for TNFRSF4 polymorphisms*

The clinical characteristics of the normotensive (NT) and hypertensive (HT) subjects in population 1 are summarized in (Table 1). Difference in age between the NT and HT subjects was significant when males and females were jointly compared ($P = 0.011$), whereas it was not significant when males and females were separately compared.

In population 1, 4 SNPs (P1, P2, P8 and P12) were genotyped in 562 NT controls and 587 HT patients. All of these SNPs were in Hardy-Weinberg equilibrium in the NT group. (Table 5) shows the distribution of genotypic and allelic frequencies of the 4 SNPs in each group. The overall distribution of genotype and allele did not significantly differ between the HT and NT groups for total, male or female subjects. The $P$ value of $\chi^2$ test for the difference in the genotypic frequency of P8 between the male HT and NT groups was 0.044, which was not significant after Bonferroni's correction (multiplied by 4).

We next analyzed the 4-SNP haplotype in population 1 (Table 6). Six common haplotypes (H1 to H6) covered approximately 99% of subjects in the HT and NT groups. The frequencies of each haplotype in males did not differ between the HT and NT groups. However, in females, the frequency of the major C-C-A-A haplotype (H1) of the HT subjects was significantly lower than that of the NT subjects ($P = 0.031$). Multiple logistic regression in females revealed that the association of the H1/H1 diplotype with hypertension remained significant ($P = 0.006$) after adjustment for age, body mass index, total cholesterol, high-density lipoprotein cholesterol, and triglyceride. The OR of the H1/H1 diplotype against

the others was 0.56 with a 95% CI of 0.37-0.85. The frequency of the minor C-C-A-G haplotype (H5) of the HT subjects was significantly higher than that of the NT subjects ($P$ = $6.78 \times 10^{-5}$). H5 haplotype was significantly associated with hypertension in a dominant model ($P$ = 0.004) after adjustment for the above factors. The OR of the H5/H5+H5/other diplotype against the others was 6.93 with a 95% CI of 1.88-25.5.

To confirm an association of the 4-SNP haplotype with female EH, we genotyped 4 SNPs using the second case-control population (population 2) comprising 925 NT controls and 732 HT patients. (Table 2) presents the clinical features of the NT controls and HT patients in population 2. All genotype results of 4 SNPs in each group were consistent with Hardy-Weinberg equilibrium. (Table 7) shows the distribution of genotypic and allelic frequencies of 4 SNPs in each group of population 2. The overall distribution of genotype and allele of all 4 SNPs did not significantly differ between the HT and NT groups for total or male subjects. However, among women, significant differences were observed in the allelic frequencies of P2 ($P$ = 0.005) and the genotypic and allelic frequencies of P8 ($P$ = 0.005 and 0.003, respectively) between the HT and NT subjects even after Bonferroni's correction (multiplied by 4). P2 was still significantly associated with hypertension in females in both a dominant ($P$ = 0.007) and recessive model ($P$ = 0.038) after adjustment for age, body mass index, total cholesterol, high-density lipoprotein cholesterol, and triglyceride. The OR of T/T+C/T against C/C (dominant model) was 1.22 with a 95% CI of 1.05-1.40, and the OR of T/T against C/T+C/C (recessive model) was 1.94 with a 95% CI of 1.04-3.62. P22 was also significantly associated with hypertension in females in both a dominant model ($P$ = 0.011) and recessive model ($P$ = 0.002) after adjustment for the above factors. The OR of G/G+A/G against A/A (dominant model) was 1.20 with a 95% CI of 1.04-1.38, and the OR of G/G against A/G+G/G (recessive model) was 1.49 with a 95% CI of 1.16-1.92.

(Table 8) shows the frequency of 4-SNP haplotypes in population 2. Among females, the

HT subjects showed a significantly lower frequency of H1 (C-C-A-A) ($P = 8.48 \times 10^{-4}$) and a significantly higher frequency of H2 (C-T-G-A) ($P = 6.46 \times 10^{-4}$) than the NT subjects, whereas in males no significant difference in frequencies of haplotypes between the HT and NT groups was observed. Multiple logistic regression in females revealed that the association of H1 haplotype with hypertension remained significant in both a dominant ($P = 0.006$) and recessive model ($P = 0.005$) after adjustment for age, body mass index, total cholesterol, high-density lipoprotein cholesterol, and triglyceride. The OR of the H1/H1+H1/other diplotype against the others (dominant model) was 0.52 with a 95% CI of 0.32-0.83, and the OR of the H1/H1 diplotype against the others (recessive model) was 0.67 with a 95% CI of 0.50-0.89. The H2 haplotype was also significantly associated with hypertension in females in a dominant model ($P = 0.001$) after adjustment for the above factors. The OR of the H2/H2+H2/other diplotype against the others was 1.40 with a 95% CI of 1.18-1.65. In population 2, the frequency of H5 did not significantly differ between the HT and NT groups for females.

Although trends of frequency changes in the H1 and H2 haplotypes among women in the two independent populations were the same, the frequency of H2 showed a significant difference not in population 1 but in population 2. This discrepancy could have been caused by difference in the sample size. When we analyzed the differences in frequencies of each haplotype between the HT and NT groups in combined samples of the two populations (Table 9), female HT subjects showed a significantly lower frequency of H1 ($P = 8.07 \times 10^{-5}$) and a significantly higher frequency of H2 ($P = 6.07 \times 10^{-4}$) than the NT subjects. The frequency of H5 of female HT subjects was still significantly higher than that of NT subjects ($P = 0.003$). No significant difference in haplotype frequencies between male HT and NT groups was observed.

*Variance component estimation of TNFRSF4*

The variance estimates of the *TNFRSF4* diplotype and the residual in SBP of the control females of population 1 were 5.5 and 79.6, respectively. Therefore, the *TNFRSF4* gene explains 6.5% of the variation of SBP in this group. The values in DBP were 2.8 and 52.1, respectively, with the gene contributing 5.2% of the variation.

*Transcriptional effects of polymorphisms in the promoter region*

To study transcriptional effects of the polymorphisms, we transfected COS-7 cells and HEK293 cells with promoter constructs containing the haplotypes in the *TNFRSF4* gene (Pr-H1, Pr-H2 and Pr-H5). In COS-7 cells, promoter activity of the Pr-H2 construct was significantly lower than that of the Pr-H1 or Pr-H5 construct (0.89 for Pr-H2/Pr-H1, $P$ = 0.008 and 0.91 for Pr-H2/Pr-H5, $P$ = 0.026; Fig 2a). The same results were observed in HEK293 cells (0.92 for Pr-H2/Pr-H1, $P$ = 0.001 and 0.88 for Pr-H2/Pr-H5, $P$ = 0.001; Fig 2c). There was no significant difference in promoter activity between the Pr-H1 and Pr-H5 constructs in both cells. These results suggest that expression of TNFRSF4 mRNA in cells is lower in individuals who have the H2 haplotype than in cells from individuals who have other types of haplotypes. To clarify the responsible SNP(s) for the lower promoter activity of Pr-H2, we performed an additional assay using a series of promoter constructs that contained only one polymorphic change (Pr-P2-T, Pr-P3-T, Pr-P4-del, Pr-P6-G, Pr-P8-G, Pr-P9-G, Pr-P10-T, and Pr-P11-G). In COS-7 cells, promoter activities of Pr-P2-T, Pr-P6-G and Pr-P11-G were significantly lower than that of Pr-H1 (0.69 for Pr-P2-T/Pr-H1, $P < 0.0001$, 0.90 for Pr-P6-G/Pr-H1, $P$ = 0.016 and 0.88 for Pr-P11-G/Pr-H1, $P$ = 0.015; Fig 2b). In HEK293 cells, as in COS-7 cells, Pr-P2-T showed significantly lower promoter activity when compared with Pr-H1 (0.71 for Pr-P2-T/Pr-H1, $P$ = 0.0001; Fig. 2d). The results of other constructs, however, were different: promoter activities of Pr-P8-G and Pr-P11-G were

significantly higher than that of Pr-H1 (1.04 for Pr-P8-G/Pr-H1, $P$ = 0.002 and 1.10 for Pr-P11-G/Pr-H1, $P$ = 0.003; Fig 2d). Only Pr-P2-T showed consistent change in promoter activity in the two different cell lines. These results suggest that P2 had the largest impact on the decreased promoter activity of the H2 haplotype.

## Discussion

The significance of *TNFRSF4* in the pathogenesis of female EH was indicated in two independent sets of populations. Haplotype analysis using 4 SNPs (P1: -3948C>T, P2: -3606C>T, P8: -1725A>G and P12: -530A>G) in the 5' upstream region showed that the frequency of H1 (C-C-A-A) was significantly low among female HT patients when compared with female NT controls in both population 1 ($P = 0.031$) and population 2 ($P = 8.48 \times 10^{-4}$). The frequency of H2 (C-G-T-A) of female HT patients was significantly higher than that of female NT controls in population 2 ($P = 6.46 \times 10^{-4}$), but not in population 1. In the combined population, both significantly lower frequency of H1 ($P = 8.07 \times 10^{-5}$) and significantly higher frequency of H2 ($P = 6.07 \times 10^{-4}$) were observed in female HT patients compared to female NT controls. No difference in haplotype frequencies between the HT and NT groups was observed in the male subjects of either the combined or separate populations. These results of association of the *TNFRSF4* haplotype with hypertension suggested that the H1 haplotype is a protective allele and that the H2 haplotype is a high-risk allele for EH in women. The promoter activity of the H2 haplotype was significantly lower than that of the H1 and H5 (C-C-A-G) haplotypes. Furthermore, the Pr-P2-T construct showed lower promoter activity than other constructs. Allelic association of P2 (-3606C>T, rs12036216) with female HT patients was significant in population 2 and the combined population (data not shown), but not in population 1. These data suggested that P2 is the responsive SNP that modifies the risk for hypertension in females, although it is possible that unidentified variant(s) in linkage disequilibrium with this haplotype have function(s) that influence disease susceptibility. We also observed a significant difference of frequency in the H5 haplotype in the combined population ($P = 0.003$) and in population 1 ($P = 6.78 \times 10^{-5}$), but not in population 2. However, we could not find any transcriptional effect of H5 haplotype.

The TNFRSF4–TNFSF4 interactions on T lymphocytes enhance proliferation and differentiation of the cells as well as generation and survival of memory $CD4^+$ T cells in the process of inflammation and immune response [15-18]. Several inflammatory markers, such as soluble leukocyte adhesion molecules, cytokines, specific growth factors, heat shock proteins, CD40L, and C-reactive protein (CRP), were reported to increase in patients with EH [32-41]. Although the relationship between inflammation and hypertension has not been well established, a growing body of evidence indicates that vascular inflammation may be involved in both the initiation and development of hypertension [42-46]. Sesso *et al.* showed that elevated plasma CRP, a well-known marker of inflammation, was associated with the future development of hypertension in a dose-dependent manner [46]. Furthermore, hypertension has been suggested to trigger inflammation through the increased expression of several mediators, including leukocyte adhesion molecules, chemokines, specific growth factors, heat shock proteins, endothelin-1, and angiotensin [47-54]. Given our findings that variants of the *TNFRSF4* gene, which might affect the inflammatory cascade, were associated with EH among women, it is likely that inflammation may play a role in initiation and/or development of hypertension.

Inflammatory process [21] and T lymphocyte activation [12, 19, 20] are implicated to be involved in the pathogenesis of atherosclerosis. Thus alteration(s) in the TNFRSF4–TNFSF4 pathway could influence atherosclerosis formation. Indeed, Wang *et al.* found that polymorphisms of *TNFSF4* are associated with myocardial infarction (MI) in women [22]. Furthermore, a polymorphism in *TNFRSF4* was also reported to be associated with MI [23]. These studies strongly suggested that genes involved in the TNFRSF4–TNFSF4 pathway play a role in the pathogenesis of atherosclerosis and MI, particularly in women.

Our findings combined with those of the reports mentioned above suggested that genetic variations in the TNFRSF4–TNFSF4 pathway may be involved in the pathogenesis of both

atherosclerosis and hypertension. So, which comes first, atherosclerosis or hypertension? Hypertension is one of the principal risk factors for atherosclerosis and MI [24], but the exact mechanism underlying the association is not fully understood. Although arterial stiffness, which is a predictor of atherosclerosis [55, 56], has been thought to be the result of hypertension rather than its cause, recent studies suggested that arterial stiffness is related to the development of hypertension [57, 58]. These data indicated that the relationship between hypertension and arterial stiffness may be bidirectional [59]. Therefore, three different scenarios are possible to explain the results that genetic variations in the TNFRSF4–TNFSF4 pathway are associated with both hypertension and MI. First, inflammation may directly increase arterial stiffness and induce the development of an atherosclerotic lesion, which may lead to the development of hypertension. Second, inflammation may induce hypertension, which may result in increasing arterial stiffness and atherosclerosis. Third, inflammation may promote the development of hypertension and atherosclerosis by different pathways. Although it is not clear whether atherosclerosis is a cause of hypertension, our findings and previous studies indicate that the inflammation may be an important part of the link between hypertension and atherosclerosis and cardiovascular events, such as MI.

*TNFSF4* is also a potential candidate for a susceptibility gene involved in the pathogenesis of female EH. We therefore examined the putative association between polymorphisms in the *TNFSF4* gene and hypertension in population 1. The allele frequencies of 4 SNPs (rs1234315, rs3850641, rs1234313, and rs3861950) and its haplotype did not significantly differ between the HT group and the NT group for females (data not shown). In contrast to the case of MI where susceptibility was affected by variations of both *TNFRSF4* and *TNFSF4*, susceptibility for hypertension may be affected only by *TNFRSF4*, though more extensive studies are required before we conclude an association of *TNFSF4* with hypertension.

In the present study, we found that variations of *TNFRSF4* affected hypertension susceptibility only in females. This is an interesting similarity to female-specific MI susceptibility exerted by *TNFSF4* and *TNFRSF4*. Some case-control studies have identified gene variants associated with gender-specific susceptibility to EH [5, 60, 61]. Recently, Nakayama *et al.*[5] reported that an SNP in the 5'-untranslated region of the *follicle-stimulating hormone receptor* (*FSHR*) gene, in which mutations were reported to cause hereditary hypergonadotropic ovarian failure [62], was associated with EH in women and affected the levels of transcriptional activity. In this study the functional mutation of the gene was clearly identified in patients with EH in a gender-specific manner. Currently, the reason for female-specific association of *TNFRSF4* with EH is an open question. One possibility is the involvement of the female sex hormone, estrogen. After menopause, females' risk for inflammatory cardiovascular diseases such as atherosclerosis and coronary heart disease increases, suggesting that estrogens modulate the initiation and progress of inflammation [63-65]. Recently, Xing *et al.* [66] suggested that estrogen may exert anti-inflammatory effects by inhibiting tumor necrosis factor-α-mediated chemokine production in vascular smooth muscle cells. However, estrogen is also known to increase CRP, which is an inflammatory marker [63]. These findings indicate that estrogen may modulate production of several proinflammatory molecules in distinct pathways. It is possible that TNFRSF4 and estrogen cross-talk in inflammation networks.

In conclusion, the present study revealed that haplotypes of the *TNFRSF4* gene were associated with EH among women in two Japanese populations, suggesting an involvement of the *TNFRSF4* gene in the pathogenesis of female essential hypertension.

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**Tables**

(Table 1) Characteristics of subjects in population 1

| | Total subjects | | | Male subjects | | | Female subjects | | |
|---|---|---|---|---|---|---|---|---|---|
| Parameters | NT | HT | *P* | NT | HT | *P* | NT | HT | *P* |
| No. of subjects | 562 | 587 | | 301 | 316 | | 261 | 271 | |
| Age (years) | 61.6±9.2 | 60.1±11.2 | 0.011* | 59.9±9.0 | 58.5±11.1 | 0.083 | 63.6±9.1 | 62.0±11.1 | 0.056 |
| BMI ($kg/m^2$) | 22.2±2.8 | 23.9±3.3 | < 0.001* | 22.1±2.9 | 23.8±3.1 | < 0.001* | 22.3±2.7 | 24.0±3.6 | < 0.001* |
| SBP (mmHg) | 111.7±8.9 | 163.7±21.1 | < 0.001* | 111.8±8.8 | 162.1±18.4 | < 0.001* | 111.5±9.1 | 166.1±24.4 | < 0.001* |
| DBP (mmHg) | 68.9±7.3 | 98.3±14.8 | < 0.001* | 69.4±7.3 | 98.7±14.0 | < 0.001* | 68.0±7.3 | 97.6±16.0 | < 0.001* |
| TC (mg/dl) | 205.5±38.0 | 207.1±34.9 | 0.596 | 195.8±35.7 | 198.7±33.0 | 0.294 | 216.2±37.6 | 216.0±34.7 | 0.970 |
| HDL-C (mg/dl) | 57.3±15.1 | 58.3±17.2 | 0.314 | 55.3±15.0 | 56.8±17.5 | 0.263 | 59.5±15.0 | 60.0±16.7 | 0.697 |
| TG (mg/dl) | 123.8±87.4 | 141.7±84.7 | 0.003* | 132.1±106.2 | 147.0±94.5 | 0.133 | 116.7±67.4 | 135.4±71.3 | 0.007* |

Values are mean ± SD. NT, normotensive; HT, hypertensive; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-C, HDL cholesterol; TG, triglyceride.
* Significant difference.

(Table 2) Characteristics of subjects in population 2

| | Total subjects | | | Male subjects | | | Female subjects | | |
|---|---|---|---|---|---|---|---|---|---|
| Parameters | NT | HT | *P* | NT | HT | *P* | NT | HT | *P* |
| No. of subjects | 925 | 732 | | 317 | 323 | | 608 | 409 | |
| Age (years) | 54.6±11.5 | 61.6±9.7 | < 0.001* | 55.8±11.1 | 61.5±10.2 | < 0.001* | 54.0±11.6 | 61.7±9.3 | < 0.001* |
| BMI ($kg/m^2$) | 23.4±3.1 | 24.2±3.3 | < 0.001* | 23.5±3.0 | 23.6±3.1 | 0.506 | 23.4±3.1 | 24.6±3.4 | < 0.001* |
| SBP (mmHg) | 123.9±9.8 | 142.2±12.1 | < 0.001* | 125.4±8.6 | 143.9±11.4 | < 0.001* | 123.1±10.3 | 140.8±12.5 | < 0.001* |
| DBP (mmHg) | 70.3±7.1 | 80.2±9.1 | < 0.001* | 71.6±6.9 | 81.9±9.4 | < 0.001* | 69.6±7.2 | 78.8±8.6 | < 0.001* |
| TC (mg/dl) | 193.4±34.2 | 195.0±33.8 | 0.358 | 186.4±33.8 | 183.9±34.0 | 0.352 | 197.1±33.9 | 203.8±31.0 | 0.001* |
| HDL-C (mg/dl) | 55.3±14.1 | 53.8±14.6 | 0.028* | 51.2±14.1 | 52.8±14.4 | 0.180 | 57.4±13.7 | 54.4±14.7 | 0.001* |
| TG (mg/dl) | 128.9±73.4 | 142.5±89.8 | 0.001* | 139.1±85.1 | 146.3±103.0 | 0.340 | 123.7±66.1 | 139.5±77.9 | 0.001* |

Values are mean ± SD. NT, normotensive; HT, hypertensive; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-C, HDL cholesterol; TG, triglyceride.
* Significant difference.

(Table 3) Primer sequence (5' → 3') for TaqMan-ASA genotyping

| SNP | Allele specific primer | Common primer | TaqMan probe [a] |
|---|---|---|---|
| P1 | CACATGGCTGGAATTTACCATC<br>CACATGGCTGGAATTTACCTCT | CTCAGCAGTGGGAGAAAAACAA | CCTCTGAAGCGTTTTCACTGGTATCATGTGT |
| P2 | GTCGCCTTTCCCCCTCCG<br>GTCGCCTTTCCCCCTCCA | GCTGCAGCCAATAGGCACCTT | AATAGCCACTTCGTGCGGCTGG |
| P8 | GTCACAGGTCCAAGAAAGCCGT<br>GTCACAGGTCCAAGAAAGCCGC | GCAGGCTGCCTTACAGACCTT | TGAGCTCTGGGTCAGTGTCCA |
| P12 | GGTCAGGAGTTCAAGACCAGTGT<br>GGTCAGGAGTTCAAGACCAGTTC | CCACGCCCGAATAATTTTTG | AGTAGAGACGGGATTTCGCCATGTTAGC |

[a] TaqMan probes contained a 5' FAM (6-carboxyfluorescein) reporter fluorophore and a 3' TAMRA (6-carboxytetramethylrhodamine) quencher.

(Table 4) Polymorphisms with minor allele frequencies ≥ 5 % detected in the *TNFRSF4* genomic region in 32 Japanese controls

| Name | Polymorphism [a] | Location | Amino acid change | MAF [b] | dbSNP ID | JSNP ID |
|---|---|---|---|---|---|---|
| P1 | -3943C>T | 5' Flanking | | 0.06 | | |
| P2 | -3601C>T | 5' Flanking | | 0.27 | rs12036216 | |
| P3 | -3119G>T | 5' Flanking | | 0.27 | rs11721 | |
| P4 | -2577delA | 5' Flanking | | 0.22 | | |
| P5 | -2568C>G | 5' Flanking | | 0.06 | | |
| P6 | -2461C>G | 5' Flanking | | 0.27 | | |
| P7 | -2167C>T | 5' Flanking | | 0.06 | | |
| P8 | -1720A>G | 5' Flanking | | 0.30 | rs3813201 | JST-IMS173304 |
| P9 | -936A>G | 5' Flanking | | 0.19 | rs34115518 | |
| P10 | -699C>T | 5' Flanking | | 0.16 | rs35339498 | |
| P11 | -669C>G | 5' Flanking | | 0.19 | rs35659545 | |
| P12 | -525A>G | 5' Flanking | | 0.11 | rs35107976 | |
| P13 | 150+47G>C | Intron1 | | 0.11 | rs35737009 | |
| P14 | 376-16C>G | Intron3 | | 0.11 | rs34108055 | |
| P15 | 442+32ins35bp | Intron4 | | 0.25 | | |
| P16 | 442+248C>T | Intron4 | | 0.19 | rs9661697 | |
| P17 | 539G>A | Exon5 | Glu178Glu | 0.25 | rs17568 | |
| P18 | 639+25C>T | Intron5 | | 0.19 | rs2298212 | JST-IMS053053 |
| P19 | 640-31T>G | Intron5 | | 0.20 | rs2298211 | JST-IMS053052 |
| P20 | 921C>T | Exon7 (3' UTR) | | 0.11 | rs2298210 | JST-IMS053051 |
| P21 | 989C>G | Exon7 (3' UTR) | | 0.11 | rs2298209 | JST-IMS053050 |
| P22 | 1067+308G>A | 3' Flanking | | 0.08 | rs2298208 | JST-IMS053049 |
| P23 | 1067+941G>C | 3' Flanking | | 0.20 | rs34067070 | |
| P24 | 1067+1224delTT | 3' Flanking | | 0.20 | | |
| P25 | 1067+1240G>C | 3' Flanking | | 0.20 | rs34279802 | |
| P26 | 1067+1266T>C | 3' Flanking | | 0.20 | rs35916760 | |
| P27 | 1067+1296C>T | 3' Flanking | | 0.20 | rs36057244 | |

[a] Numbering according to the cDNA sequence of *TNFRSF4* (accession number NM_003327).
[b] Minor allele frequency (MAF) on the basis of the sequencing of 32 DNA samples.

(Table 5) Genotype and allele frequencies among NT and HT subjects in population 1

| | | Total subjects | | | Male subjects | | | Female subjects | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | NT n = 562 | HT n = 587 | *P* [a] | NT n = 301 | HT n = 316 | *P* [a] | NT n = 261 | HT n = 271 | *P* [a] |
| P1 | Genotype | | | | | | | | | |
| | CC | 448 (0.799) | 455 (0.776) | | 238 (0.793) | 245 (0.775) | | 210 (0.805) | 210 (0.778) | |
| | CT | 109 (0.194) | 124 (0.212) | | 60 (0.200) | 67 (0.212) | | 49 (0.188) | 57 (0.211) | |
| | TT | 4 (0.007) | 7 (0.012) | 0.524 | 2 (0.007) | 4 (0.013) | 0.691 | 2 (0.008) | 3 (0.011) | 0.722 |
| | Allele | | | | | | | | | |
| | C | 1005 (0.896) | 1034 (0.882) | | 536 (0.893) | 557 (0.881) | | 469 (0.898) | 477 (0.883) | |
| | T | 117 (0.104) | 138 (0.118) | 0.305 | 64 (0.107) | 75 (0.119) | 0.506 | 53 (0.102) | 63 (0.117) | 0.429 |
| P2 | Genotype | | | | | | | | | |
| | CC | 324 (0.578) | 319 (0.544) | | 166 (0.553) | 170 (0.538) | | 158 (0.605) | 149 (0.552) | |
| | CT | 208 (0.371) | 220 (0.375) | | 121 (0.403) | 117 (0.370) | | 87 (0.333) | 103 (0.381) | |
| | TT | 29 (0.052) | 47 (0.080) | 0.129 | 13 (0.043) | 29 (0.092) | 0.055 | 16 (0.061) | 18 (0.067) | 0.455 |
| | Allele | | | | | | | | | |
| | C | 856 (0.763) | 858 (0.732) | | 453 (0.755) | 457 (0.723) | | 403 (0.772) | 401 (0.743) | |
| | T | 266 (0.237) | 314 (0.268) | 0.089 | 147 (0.245) | 175 (0.277) | 0.203 | 119 (0.228) | 139 (0.257) | 0.263 |
| P8 | Genotype | | | | | | | | | |
| | AA | 284 (0.506) | 280 (0.478) | | 144 (0.480) | 154 (0.487) | | 140 (0.536) | 126 (0.467) | |
| | AG | 238 (0.424) | 248 (0.423) | | 137 (0.457) | 125 (0.396) | | 101 (0.387) | 123 (0.456) | |
| | GG | 39 (0.070) | 58 (0.099) | 0.182 | 19 (0.063) | 37 (0.117) | 0.044 | 20 (0.077) | 21 (0.078) | 0.250 |
| | Allele | | | | | | | | | |
| | A | 806 (0.718) | 808 (0.689) | | 425 (0.708) | 433 (0.685) | | 381 (0.730) | 375 (0.694) | |
| | G | 316 (0.282) | 364 (0.311) | 0.129 | 175 (0.292) | 199 (0.315) | 0.376 | 141 (0.270) | 165 (0.306) | 0.202 |
| P12 | Genotype | | | | | | | | | |
| | AA | 401 (0.716) | 393 (0.671) | | 209 (0.699) | 215 (0.680) | | 192 (0.736) | 178 (0.659) | |
| | AG | 148 (0.264) | 175 (0.299) | | 84 (0.281) | 88 (0.278) | | 64 (0.245) | 87 (0.322) | |
| | GG | 11 (0.020) | 18 (0.031) | 0.179 | 6 (0.020) | 13 (0.041) | 0.318 | 5 (0.019) | 5 (0.019) | 0.144 |
| | Allele | | | | | | | | | |
| | A | 950 (0.848) | 961 (0.820) | | 502 (0.839) | 518 (0.820) | | 448 (0.858) | 443 (0.820) | |
| | G | 170 (0.152) | 211 (0.180) | 0.069 | 96 (0.161) | 114 (0.180) | 0.355 | 74 (0.142) | 97 (0.180) | 0.093 |

[a] Significant *P*-value after Bonferroni's correction for four loci is 0.0125 (0.05/4).

(Table 6) 4-SNP haplotype (P1-P2-P8-P12) frequency among NT and HT subjects in population 1

| | Haplotype [a] | Male subjects | | | | Female subjects | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | NT n = 298 | HT n = 316 | *P* [b] | Permutation *P* | NT n = 261 | HT n = 270 | *P* [b] | Permutation *P* |
| H1 | C-C-A-A | 404 (0.677) | 413 (0.653) | 0.371 | 0.363 | 376 (0.720) | 356 (0.659) | 0.031 | 0.021 * |
| H2 | C-T-G-A | 81 (0.136) | 96 (0.152) | 0.419 | 0.420 | 60 (0.116) | 75 (0.138) | 0.267 | 0.259 |
| H3 | T-T-G-G | 63 (0.106) | 73 (0.115) | 0.584 | 0.559 | 52 (0.099) | 62 (0.115) | 0.405 | 0.402 |
| H4 | C-C-G-G | 16 (0.026) | 21 (0.033) | 0.484 | 0.470 | 17 (0.033) | 14 (0.027) | 0.578 | 0.617 |
| H5 | C-C-A-G | 17 (0.029) | 18 (0.029) | 0.967 | 0.958 | 1 (0.002) | 19 (0.036) | $6.78 \times 10^{-5}$ * | < 0.001 * |
| H6 | C-C-G-A | 12 (0.021) | 5 (0.008) | 0.063 | 0.066 | 9 (0.018) | 12 (0.021) | 0.646 | 0.682 |
| | others | 3 (0.005) | 6 (0.010) | | | 7 (0.013) | 2 (0.004) | | |
| Entire distribution | | | | | 0.722 [c] | | | | 0.003 [c,] * |

[a] Four loci are P1, P2, P8, P12, and six predominant haplotypes are listed; "others" category includes minor haplotypes with < 1%frequency.
[b] Significant *P*-value after Bonferroni correction for major six haplotypes is 0.0083 (0.05/6).
[c] *P*-value for the entire distribution with permutation test.
* Significant difference.

(Table 7) Genotype and allele frequencies among NT and HT subjects in population 2

| | | Total subjects | | | Male subjects | | | Female subjects | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | NT n = 925 | HT n = 732 | *P* [a] | NT n = 317 | HT n = 323 | *P* [a] | NT n = 608 | HT n = 409 | *P* [a] |
| P1 | Genotype | | | | | | | | | |
| | CC | 729 (0.792) | 573 (0.786) | | 253 (0.801) | 249 (0.778) | | 476 (0.788) | 324 (0.792) | |
| | CT | 181 (0.197) | 147 (0.202) | | 58 (0.184) | 66 (0.206) | | 123 (0.204) | 81 (0.198) | |
| | TT | 10 (0.011) | 9 (0.012) | 0.929 | 5 (0.016) | 5 (0.016) | 0.770 | 5 (0.008) | 4 (0.010) | 0.949 |
| | Allele | | | | | | | | | |
| | C | 1639 (0.891) | 1293 (0.887) | | 564 (0.892) | 564 (0.881) | | 1075 (0.890) | 729 (0.891) | |
| | T | 201 (0.109) | 165 (0.113) | 0.721 | 68 (0.108) | 76 (0.119) | 0.530 | 133 (0.110) | 89 (0.109) | 0.927 |
| P2 | Genotype | | | | | | | | | |
| | CC | 550 (0.598) | 403 (0.553) | | 176 (0.555) | 182 (0.567) | | 374 (0.620) | 221 (0.542) | |
| | CT | 323 (0.351) | 282 (0.387) | | 118 (0.372) | 123 (0.383) | | 205 (0.340) | 159 (0.390) | |
| | TT | 47 (0.051) | 44 (0.060) | 0.176 | 23 (0.073) | 16 (0.050) | 0.488 | 24 (0.040) | 28 (0.069) | 0.017 |
| | Allele | | | | | | | | | |
| | C | 1423 (0.773) | 1088 (0.746) | | 470 (0.741) | 487 (0.759) | | 953 (0.790) | 601 (0.737) | |
| | T | 417 (0.227) | 370 (0.254) | 0.069 | 164 (0.259) | 155 (0.241) | 0.477 | 253 (0.210) | 215 (0.263) | 0.005 * |
| P8 | Genotype | | | | | | | | | |
| | AA | 464 (0.508) | 342 (0.472) | | 146 (0.465) | 157 (0.489) | | 318 (0.530) | 185 (0.458) | |
| | AG | 384 (0.420) | 316 (0.436) | | 139 (0.443) | 143 (0.445) | | 245 (0.408) | 173 (0.428) | |
| | GG | 66 (0.072) | 67 (0.092) | 0.189 | 29 (0.092) | 21 (0.065) | 0.436 | 37 (0.062) | 46 (0.114) | 0.005 * |
| | Allele | | | | | | | | | |
| | A | 1312 (0.718) | 1000 (0.690) | | 431 (0.686) | 457 (0.712) | | 881 (0.734) | 543 (0.672) | |
| | G | 516 (0.282) | 450 (0.310) | 0.080 | 197 (0.314) | 185 (0.288) | 0.321 | 319 (0.266) | 265 (0.328) | 0.003 * |
| P12 | Genotype | | | | | | | | | |
| | AA | 630 (0.686) | 479 (0.659) | | 214 (0.677) | 208 (0.650) | | 416 (0.691) | 271 (0.666) | |
| | AG | 265 (0.289) | 220 (0.303) | | 93 (0.294) | 100 (0.313) | | 172 (0.286) | 120 (0.295) | |
| | GG | 23 (0.025) | 28 (0.039) | 0.213 | 9 (0.028) | 12 (0.038) | 0.690 | 14 (0.023) | 16 (0.039) | 0.301 |
| | Allele | | | | | | | | | |
| | A | 1525 (0.831) | 1178 (0.810) | | 521 (0.824) | 516 (0.806) | | 1004 (0.834) | 662 (0.813) | |
| | G | 311 (0.169) | 276 (0.190) | 0.128 | 111 (0.176) | 124 (0.194) | 0.405 | 200 (0.166) | 152 (0.187) | 0.231 |

[a] Significant *P*-value after Bonferroni's correction for four loci is 0.0125 (0.05/4).

* Significant difference.

(Table 8) 4-SNP haplotype (P1-P2-P8-P12) frequency among NT and HT subjects in population 2

| | Haplotype [a] | Male subjects | | | | Female subjects | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | NT n = 303 | HT n = 299 | *P* [b] | Permutation *P* | NT n = 584 | HT n = 388 | *P* [b] | Permutation *P* |
| H1 | C-C-A-A | 403 (0.665) | 403 (0.674) | 0.743 | 0.714 | 839 (0.718) | 502 (0.647) | $8.48\times10^{-4}$ * | < 0.001 * |
| H2 | C-T-G-A | 86 (0.142) | 75 (0.125) | 0.400 | 0.388 | 115 (0.098) | 116 (0.149) | $6.46\times10^{-4}$ * | 0.001 * |
| H3 | T-T-G-G | 66 (0.109) | 71 (0.119) | 0.592 | 0.593 | 125 (0.107) | 84 (0.108) | 0.939 | 0.926 |
| H4 | C-C-G-G | 30 (0.049) | 24 (0.040) | 0.429 | 0.443 | 47 (0.040) | 45 (0.058) | 0.067 | 0.074 |
| H5 | C-C-A-G | 11 (0.018) | 20 (0.034) | 0.095 | 0.113 | 21 (0.018) | 18 (0.023) | 0.434 | 0.451 |
| H6 | C-C-G-A | 8 (0.014) | 4 (0.007) | 0.265 | 0.374 | 15 (0.013) | 8 (0.010) | 0.606 | 0.583 |
| | others | 2 (0.003) | 1 (0.002) | | | 6 (0.005) | 3 (0.004) | | |
| Entire distribution | | | | | 0.533 [c] | | | | 0.026 [c,] * |

[a] Four loci are P1, P2, P8, P12, and six predominant haplotypes are listed; “others” category includes minor haplotypes with <1%frequency.
[b] Significant *P*-value after Bonferroni correction for major six haplotypes is 0.0083 (0.05/6).
[c] *P*-value for the entire distribution with permutation test.
* Significant difference.

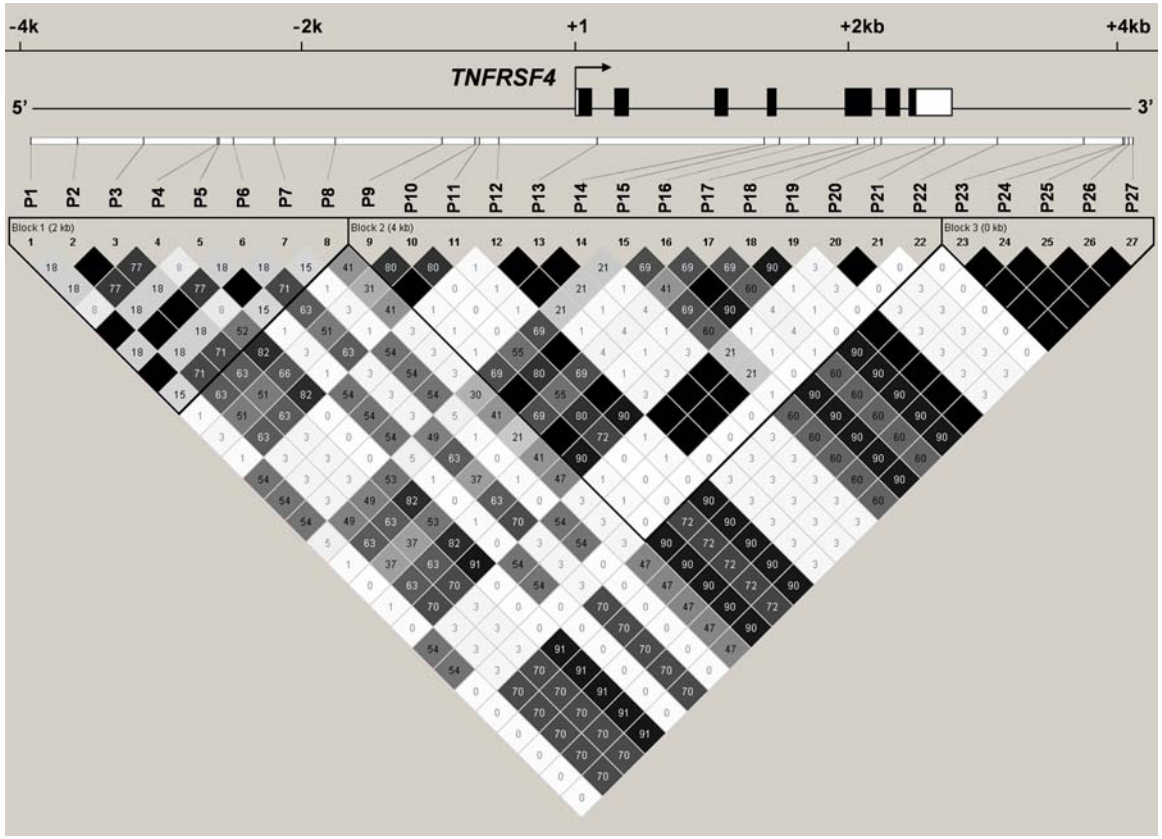
(Table 9) 4-SNP haplotype (P1-P2-P8-P12) frequency among NT and HT subjects in the combined population (populations 1 and 2)

| | | Male subjects | | | Female subjects | | |
|---|---|---|---|---|---|---|---|
| | Haplotype [a] | NT n = 602 | HT n = 615 | *P* [b] | NT n = 845 | HT n = 658 | *P* [b] |
| H1 | C-C-A-A | 806 (0.671) | 816 (0.663) | 0.682 | 1215 (0.719) | 858 (0.652) | $8.07\times10^{-5}$ * |
| H2 | C-T-G-A | 167 (0.139) | 171 (0.139) | 0.989 | 175 (0.104) | 191 (0.145) | $6.07\times10^{-4}$ * |
| H3 | T-T-G-G | 129 (0.107) | 144 (0.117) | 0.446 | 177 (0.104) | 146 (0.111) | 0.578 |
| H4 | C-C-G-G | 45 (0.038) | 45 (0.036) | 0.846 | 64 (0.038) | 60 (0.045) | 0.306 |
| H5 | C-C-A-G | 29 (0.024) | 38 (0.031) | 0.263 | 22 (0.013) | 37 (0.028) | 0.003 * |
| H6 | C-C-G-A | 21 (0.017) | 9 (0.008) | 0.033 | 24 (0.014) | 20 (0.015) | 0.906 |
| | others | 5 (0.004) | 7 (0.006) | | 13 (0.008) | 6 (0.004) | |

[a] Four loci are P1, P2, P8, P12, and six predominant haplotypes are listed; “others” category includes minor haplotypes with <1%frequency.
[b] Significant *P*-value after Bonferroni correction for major six haplotypes is 0.0083 (0.05/6).
* Significant difference.

## Figures

(Fig. 1)



(Fig. 2)

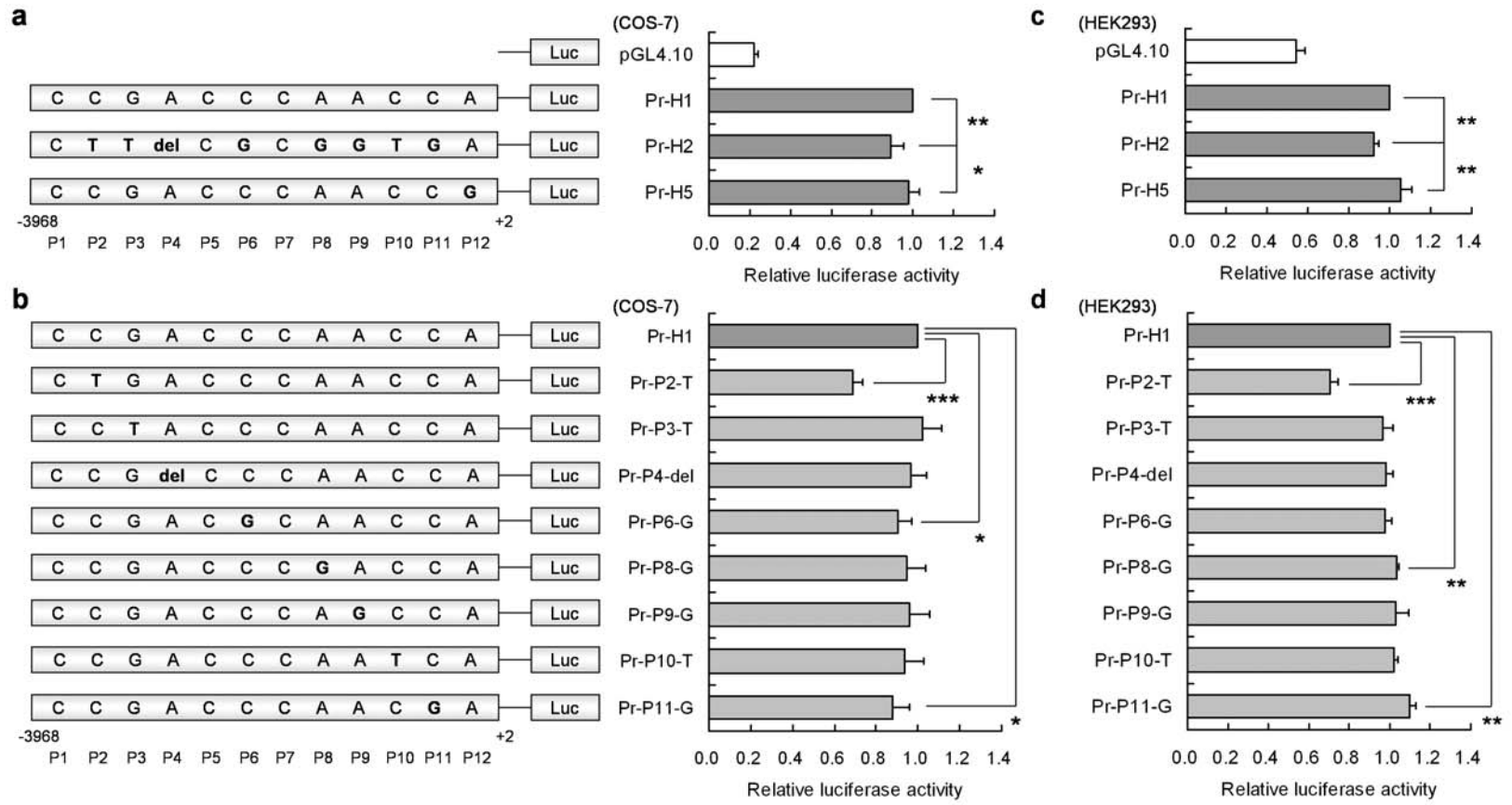

a
(COS-7)
Luc
pGL4.10
C C G A C C C A A C C A
Pr-H1
C T T del C G C G G T G A
Pr-H2
C C G A C C C A A C C G
Pr-H5
-3968
+2
P1 P2 P3 P4 P5 P6 P7 P8 P9 P10 P11 P12
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
Relative luciferase activity
c
(HEK293)
b
(COS-7)
C T G A C C C A A C C A
Pr-P2-T
C C T A C C C A A C C A
Pr-P3-T
C C G del C C C A A C C A
Pr-P4-del
C C G A C G C A A C C A
Pr-P6-G
C C G A C C C G A C C A
Pr-P8-G
C C G A C C C A G C C A
Pr-P9-G
C C G A C C C A A T C A
Pr-P10-T
C C G A C C C A A C G A
Pr-P11-G
d
(HEK293)

**Figure Legends**

(Fig. 1)
Haplotype block structure of the *TNFRSF4* gene. *Top* Organization of the *TNFRSF4* gene. Exons are indicated by boxes (black, coding sequences; white, untranslated sequences). *Bottom* Linkage disequilibrium structure of polymorphisms across the *TNFRSF4* gene region using data from 32 Japanese controls. Haplotype blocks were defined by the solid spine of LD method in Haploview. The number in each cell represents the LD parameter $r^2$ ($\times$ 100), blank cells denote $r^2 = 1$. Each cell is painted with graduated color relative to the strength of LD between markers, which is defined by the $r^2$ value.

(Fig. 2)
Effect of haplotypes (a, c) and each polymorphism (b, d) on the transcriptional activity of the *TNFRSF4* promoter. Relative luciferase activities after transient transfection in COS-7 (a, b) and HEK293 (c, d) cell lines are shown. Activities of the Pr-H1 constructs were considered as 100%. Each experiment was conducted in triplicate for each sample, and the results are expressed as mean ± SD for six (COS-7) or five (HEK293) independent experiments. ***** $P<0.05$, ****** $P<0.01$, ******* $P<0.001$.

**Supplemental Tables**

(Supplemental Table 1) A total 121 candidate genes for essential hypertention

| Gene name | Gene symbol |
|---|---|
| Acrosin | *ACR* |
| Adducin-1, alpha | *ADD1* |
| Adducin-2, beta | *ADD2* |
| Adrenomedullin | *ADM* |
| Angiopoietin-like 2 | *ANGPTL2* |
| Angiotensin converting enzyme 2 | *ACE2* |
| Aquaporin-1(proximal) | *AQP1* |
| Aquaporin-2(collecting duct) | *AQP2* |
| Aquaporin-3(collecting duct) | *AQP3* |
| Aquaporin-4(medulally collecting duct) | *AQP4* |
| Aquaporin-6(podocyte) | *AQP6* |
| Aromatic L-amino acid decarboxylase | *DDC* |
| ATPase, H+ transporting, lysosomal (vacuolar proton pump), subunit 1 | *ATP6AP1* |
| ATPase, Na+K+ transporting, alpha-1 polypeptide | *ATP1A1* |
| Beta-2-adrenergic receptor | *ADRB2* |
| Calcium channel, voltage-dependent, T type, alpha 1G subunit | *CACNA1G* |
| Calcium/calmodulin-dependent serine protein kinase | *CASK* |
| Calpain 10 | *CAPN10* |
| Carbonic anhydrase 3 | *CA3* |
| Catepsin | *CAT* |
| CD36 antigen | *CD36* |
| CD97 antigen | *CD97* |
| CDC-like kinase | *CLK1* |
| Chloride channel, kidney, B | *CLCNKB* |
| Chloride channel-5 | *CLCN5* |
| Claudin 4 | *CLDN4* |
| Cytochrome c oxidase, subunit VIa, polypeptide 1 | *COX6A1* |
| Cytochrome P450, 4a11 | *CYP4A11* |
| Death-associated protein kinase 3 | *DAPK3* |
| Dopamin receptor D1 | *DRD1* |
| Dopamin receptor D1B | *DRD1B* |
| Dopamine receptor D3 | *DRD3* |
| Dopamine-beta-hydroxylase | *DBH* |
| Estrogen receptor 1 | *ESR1* |

| | |
|---|---|
| Estrogen receptor 2 | *ESR2* |
| Fatty acid binding protein 4, adipocyte | *FABP4* |
| FXYD domain-containing ion transport regulator 2 | *FXYD2* |
| Gap junction protein, beta 2 | *GJB2* |
| Glutathione S-transferase, alpha 2 (Yc2) | *GSTA2* |
| Glycogen synthase 1 | *GYS1* |
| Glycogen synthase 2 | *GYS2* |
| Granzyme B | *GZMB* |
| Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2 | *GNGT2* |
| Heat shock 70kD protein 5 (glucose-regulated protein, 78kD) | *HSPA5* |
| Hepatoma-derived growth factor, related protein 3 | *HDGFRP3* |
| Hyaluronidase 1 | *HYAL1* |
| Hydroxysteroid 11-beta dehydrogenase 1 | *HSD11B1* |
| Insulin-like growth factor binding protein 5 | *IGFBP5* |
| Kallikrein 1 | *KLK1* |
| Kallikrein 13 | *KLK13* |
| Kallistatin | *SERPINA4* |
| Kinesin family member 21B | *KIF21B* |
| Kininogen | *KKG* |
| Kit ligand | *KITLG* |
| Latent transforming growth factor beta binding protein 2 | *LTBP2* |
| Leucine rich repeat (in FLII) interacting protein 1 | *LRRFIP1* |
| Maternally expressed gene 3 | *MEG3* |
| Molybdenum cofactor synthesis 2 | *MOCS2* |
| Natriuretic peptide precursor B | *NPPB* |
| Natriuretic peptide precursor C | *NPR3* |
| Natriuretic peptide receptor A/guanylate cyclase A | *NPR1* |
| Natriuretic peptide receptor C | *NPR3* |
| Nephrin | *NPHN* |
| Nephrocystin | *NPHP1* |
| Nitric oxide synthase 3 | *NOS3* |
| Nuclear receptor subfamily 2, group F, member 6 | *NR2F6* |
| Parvalbumin | *PVALB* |
| Phosphodiesterase 1A, calmodulin-dependent | *PDE1A* |
| Placental growth factor | *PGF* |
| Platelet derived growth factor receptor, beta polypeptide | *PDGFRB* |
| Potassium inwardly-rectifying channel, subfamily J, member 1 | *KCNJ1* |

| | |
|---|---|
| Potassium inwardly-rectifying channel, subfamily J, member 11 | *KCNJ11* |
| Potassium inwardly-rectifying channel, subfamily J, member 6 | *KCNJ6* |
| Potassium large conductance calcium-activated channel, subfamily M,beta member 1 | *KCNMB1* |
| Proline-serine-threonine phosphatase-interacting protein 1 | *PSTPIP1* |
| Prostaglandin E receptor EP4 subtype | *PTGER4* |
| Protein C receptor, endothelial | *PROCR* |
| Receptor (calcitonin) activity modifying protein 2 | *RAMP2* |
| Ribosomal protein S4, X-linked | *RPS4X* |
| SAPK/ERK kinase-1 | *SEK1* |
| Serine/threonine kinase 19 | *STK19* |
| Serine/threonine kinase 6 | *AURKA* |
| SFFV proviral integration 1 | *SPI1* |
| Sodium channel, nonvoltage-gated 1, alpha | *SCNN1A* |
| Sodium channel, nonvoltage-gated 1, beta | *SCNN1B* |
| Sodium channel, nonvoltage-gated 1, gamma | *SCNN1G* |
| Sodium channel, voltage-gated, type I, delta polypeptide | *SCNN1D* |
| Solute carrier family 1 (glial high affinity glutamate transporter),member 2 | *SLC1A2* |
| Solute carrier family 12 member 1 | *SLC12A1* |
| Solute carrier family 12 sodium/potassium/chloride transporters) | *SLC12A3* |
| Solute carrier family 14 (monocarboxylic acid transporters), member 2 | *SLC14A2* |
| Solute carrier family 16, member 1 | *SLC16A1* |
| Solute carrier family16,member 7 | *SLC16A7* |
| Solute carrier family 18 (vesicular monoamine), member 1 | *SLC18A1* |
| Solute carrier family 19 (folate transporter), member 1 | *SLC19A1* |
| Solute carrier family 21, member 3 (organic anion transporter) | *SLCO1A2* |
| Solute carrier family 22 (organic cation transporter), member 1-like | *SLC22A1L* |
| Solute carrier family 26, member 4 | *SLC26A4* |
| Solute carrier family 4, anion exchanger, member 1 (erythrocytemembrane protein band 3, Diego blood group) | *SLC4A1* |
| Solute carrier family 6 (neurotransmitter transporter, noradrenalin),member 2 | *SLC6A2* |
| Solute carrier family 8, member 1 (sodium-calcium exchanger-1) | *SLC8A1* |
| Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 | *SLC9A3* |
| Solute carrier family 9 (sodium/hydrogen exhanger), isoform 1 | *SLC9A1* |
| Splicing factor, arginine/serine-rich 5 (SRp40, HRS) | *SFRS5* |
| Steroidogenic acute regulatory protein | *STAR* |

| | |
|---|---|
| Syntaxin binding protein 1 | *STXBP1* |
| Synuclein, alpha | *SNCA* |
| Thyroid hormone responsive SPOT14 homolog | *THRSP* |
| TNF superfamily member 11 | *TNFSF11* |
| Ttk protein kinase | *TTK* |
| TNF receptor superfamily, member 11b (osteoprotegerin) | *TNFRSF11B* |
| TNF receptor superfamily, member 18 | *TNFRSF18* |
| TNF receptor superfamily, member 4 | *TNFRSF4* |
| Uncoupling protein, mitochondrial | *UCP1* |
| Vaccinia related kinase 1 | *VRK1* |
| Vascular endothelial growth factor C | *VEGFC* |
| Vesicle-associated membrane protein 3 | *VAMP3* |
| Villin 2 | *VIL2* |
| WNK lysine deficient protein kinase 1 | *PRKWNK1* |
| WNK lysine deficient protein kinase 4 | *PRKWNK4* |
| WW domain binding protein 1 | *WBP1* |

(Supplemental Table 2) List of genes downregulated in the kidneys of four-copy (Agt 2/2) mice versus one-copy (Agt 0/1) mice

| Accession Number | GeneName | Fold change |
|---|---|---|
| AA521869 | steroidogenic acute regulatory protein | -3.1 |
| AI386062 | carbonic anhydrase 3 | -2.5 |
| AA080270 | fatty acid binding protein 4, adipocyte | -2.4 |
| AA980357 | gelsolin | -1.9 |
| AA458072 | adipocyte complement related protein of 30 kDa | -1.8 |
| W41372 | uncoupling protein, mitochondrial | -1.8 |
| AI120315 | splicing factor, arginine/serine-rich 5 (SRp40, HRS) | -1.6 |
| AA276616 | steroidogenic acute regulatory protein | -1.6 |
| W10293 | androgen regulated vas deferens protein | -1.5 |
| W15725 | cell death-inducing DNA fragmentation factor, alpha subunit-like effector A | -1.5 |
| AA437572 | inner centromere protein | -1.5 |
| W34620 | kallikrein 26 | -1.5 |
| AI892379 | aminolevulinic acid synthase 1 | -1.4 |
| AA066458 | cadherin 16 | -1.4 |
| AA061218 | DNA segment, Chr 17, human D6S81E 1 | -1.4 |
| AA760070 | esterase 1 | -1.4 |
| AA624690 | fibroblast growth factor regulated protein | -1.4 |
| AA546842 | hypothetical protein MGC7720 | -1.4 |
| AA413767 | interferon regulatory factor 3 | -1.4 |
| AA003609 | ISL1 transcription factor, LIM/homeodomain, (islet-1) | -1.4 |
| W36421 | Moloney leukemia virus 10 | -1.4 |
| AI451780 | prostate specific ets transcription factor | -1.4 |
| AA387891 | RAD1 homolog (S. pombe) | -1.4 |
| AA712003 | resistin like alpha | -1.4 |
| AA244388 | retinoic acid early transcript gamma | -1.4 |
| AA065574 | solute carrier family 12, member 1 | -1.4 |
| AA437805 | SWAP complex protein, 70 kDa | -1.4 |
| AA756672 | thyroid hormone responsive SPOT14 homolog (Rattus) | -1.4 |
| AI553108 | a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2) | -1.3 |
| AA413485 | A kinase anchor protein | -1.3 |
| W82672 | aconitase 1 | -1.3 |
| AA606587 | adenylosuccinate lyase | -1.3 |

| | | |
|---|---|---|
| AA794074 | aldo-keto reductase family 1, member B1 (aldose reductase) | -1.3 |
| AI386037 | alpha-2-HS-glycoprotein | -1.3 |
| AA755981 | angiopoietin-like 2 | -1.3 |
| W40608 | anterior gradient 2 (Xenepus laevis) | -1.3 |
| AI325603 | apolipoprotein E | -1.3 |
| AA239727 | ATP-binding cassette, sub-family B (MDR/TAP), member 1 | -1.3 |
| AA967857 | brain protein | -1.3 |
| AA546660 | calpain 10 | -1.3 |
| AI325851 | CD97 antigen | -1.3 |
| AA684191 | CDC-like kinase | -1.3 |
| AI894016 | complement component 1, q subcomponent, c polypeptide | -1.3 |
| AA117547 | cyclin D1 | -1.3 |
| W12527 | cytochrome P450, 2b19 | -1.3 |
| AA117605 | cytotoxic granule-associated RNA-binding protein 1 | -1.3 |
| W89290 | dante | -1.3 |
| AI510645 | DEAD (aspartate-glutamate-alanine-aspartate) box polypeptide, Y chromosome | -1.3 |
| AI120685 | decay accelerating factor 1 | -1.3 |
| AA123152 | diacylglycerol acyltransferase | -1.3 |
| AA017823 | dishevelled 2, dsh homolog (Drosophila) | -1.3 |
| AA215144 | DNA segment, Chr 16, ERATO Doi 36, expressed | -1.3 |
| AA624262 | DNA segment, Chr 2, Brigham & Women's Genetics 0886 expressed | -1.3 |
| AA863502 | embryonic stem cell phosphatase | -1.3 |
| AA681602 | enolase 3, beta muscle | -1.3 |
| AI326499 | epidermal growth factor | -1.3 |
| AI509558 | formin-like | -1.3 |
| AA177916 | fusion 2 (human) | -1.3 |
| AA458241 | G0/G1 switch gene 2 | -1.3 |
| AI386181 | gamma-glutamyl carboxylase | -1.3 |
| AA930013 | gasdermin | -1.3 |
| AI385860 | GLI-Kruppel family member GLI | -1.3 |
| AI325384 | glucokinase activity | -1.3 |
| AI326575 | glucose-6-phosphatase, catalytic | -1.3 |
| AI154009 | guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2 | -1.3 |
| AA450922 | heat shock 70kD protein 5 (glucose-regulated protein, 78kD) | -1.3 |
| AI661389 | hepatoma-derived growth factor, related protein 3 | -1.3 |

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|---|---|---|
| AA473972 | heterogeneous nuclear ribonucleoprotein H1 | -1.3 |
| AA688635 | hyaluronidase 1 | -1.3 |
| AI789916 | hydroxysteroid 11-beta dehydrogenase 1 | -1.3 |
| AA432994 | hypothetical protein, MNCb-2032 | -1.3 |
| W15610 | integrin beta 4 | -1.3 |
| AI430631 | just another zinc finger protein | -1.3 |
| AA760344 | keratin complex 1, acidic, gene 15 | -1.3 |
| AI645902 | kinesin family member 21B | -1.3 |
| AI099009 | latent transforming growth factor beta binding protein 2 | -1.3 |
| AI614021 | leucine rich repeat (in FLII) interacting protein 1 | -1.3 |
| W97303 | maternally expressed gene 3 | -1.3 |
| W34188 | Moloney leukemia virus 10 | -1.3 |
| AI592114 | molybdenum cofactor synthesis 2 | -1.3 |
| AI036466 | Mus musculus adult male testis cDNA, RIKEN full-length enriched library, clone:4921528E07, full insert sequence | -1.3 |
| AA611779 | Mus musculus adult male xiphoid cartilage cDNA, RIKEN full-length enriched library, clone:5230400J22, full insert sequence | -1.3 |
| AA672784 | Mus musculus mRNA for erythroid differentiation regulator, partial | -1.3 |
| AA606711 | myeloid-associated differentiation marker | -1.3 |
| AA276726 | natural killer cell BY55 precursor | -1.3 |
| AA619774 | next to the Brca1 | -1.3 |
| AA512708 | nitric oxide synthase 2, inducible, macrophage | -1.3 |
| AA517353 | nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha | -1.3 |
| AI117296 | Paneth cell enhanced expression | -1.3 |
| AA106128 | phosphoenolpyruvate carboxykinase 1, cytosolic | -1.3 |
| AI327450 | phospholipase A2, group IB, pancreas | -1.3 |
| AA271866 | phospholipase A2, group VI | -1.3 |
| AA982549 | placental growth factor | -1.3 |
| AA624552 | platelet derived growth factor receptor, beta polypeptide | -1.3 |
| AA517595 | polymerase (DNA directed), delta 2, regulatory subunit (50 kDa) | -1.3 |
| AA466979 | pre B-cell leukemia transcription factor 1 | -1.3 |
| AI326527 | preproacrosin | -1.3 |
| AA980931 | proline-serine-threonine phosphatase-interacting protein 1 | -1.3 |
| AA016910 | protein C receptor, endothelial | -1.3 |
| AA221322 | protein tyrosine phosphatase, non-receptor type 5 | -1.3 |

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|---|---|---|
| AA051479 | Public domain EST | -1.3 |
| AI614454 | reduced in osteosclerosis transporter | -1.3 |
| AA681171 | RNA polymerase 1-2 (128 kDa subunit) | -1.3 |
| AA238294 | sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4G | -1.3 |
| AI605734 | septin 3 | -1.3 |
| AI180767 | serine/threonine kinase 19 | -1.3 |
| W47914 | sialyltransferase 8 (alpha-2, 8-sialytransferase) B | -1.3 |
| AI120278 | solute carrier family 16 (monocarboxylic acid transporters), member 2 | -1.3 |
| AA066386 | solute carrier family 22 (organic cation transporter), member 1-like | -1.3 |
| AI156032 | solute carrier family 26, member 4 | -1.3 |
| AI894071 | sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1 | -1.3 |
| AA414787 | t-complex testis-expressed 3 | -1.3 |
| AI466119 | transducin-like enhancer of split 2, homolog of Drosophila E(spl) | -1.3 |
| AI385796 | transketolase | -1.3 |
| AA274169 | tumor necrosis factor receptor superfamily, member 18 | -1.3 |
| **AI323199** | **tumor necrosis factor receptor superfamily, member 4** | **-1.3** |
| AW210120 | vesicle-associated membrane protein 3 | -1.3 |
| AA066685 | villin 2 | -1.3 |
| W53568 | WW domain binding protein 1 | -1.3 |

(Supplemental Table 3) List of genes upregulated in the kidneys of four-copy (Agt 2/2) mice versus one-copy (Agt 0/1) mice

| Accession Number | GeneName | Fold change |
|---|---|---|
| AA498760 | syntaxin binding protein 1 | 1.9 |
| AA638765 | metallothionein 1 | 1.8 |
| W15809 | hemoglobin, beta adult major chain | 1.7 |
| W36474 | metallothionein 2 | 1.7 |
| AA517682 | repeat family 3 gene | 1.7 |
| AA106071 | hemoglobin, beta adult major chain | 1.6 |
| AA066763 | hemoglobin, beta adult major chain | 1.6 |
| AA183239 | insulin-like growth factor binding protein 5 | 1.5 |
| AA106365 | cytochrome P450, 4a14 | 1.5 |
| AI182724 | suppressor of initiator codon mutations, related sequence 1 (S. cerevisiae) | 1.5 |
| AI481911 | IG ALPHA CHAIN C REGION | 1.5 |
| AA432457 | ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B) | 1.5 |
| AI892243 | ferritin heavy chain | 1.5 |
| AI553078 | CD36 antigen | 1.5 |
| W08923 | cytochrome c oxidase, subunit VI a, polypeptide 1 | 1.5 |
| AA108723 | FXYD domain-containing ion transport regulator 2 | 1.5 |
| AA763351 | death-associated kinase 3 | 1.4 |
| AA273270 | ribosomal protein S4, X-linked | 1.4 |
| AA244820 | retinol dehydrogenase type 6 | 1.4 |
| AI390326 | ribosomal protein S6 | 1.4 |
| AA003877 | nel-like 2 homolog (chicken) | 1.4 |
| AA461886 | tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin) | 1.4 |
| AI048040 | claudin 4 | 1.4 |
| AI604245 | protocadherin 7 | 1.4 |
| AI642307 | ribosomal protein S11 | 1.4 |
| AA065880 | nuclear factor I/B | 1.4 |
| AA106894 | carbonic anhydrase 4 | 1.4 |
| AA067971 | ral guanine nucleotide dissociation stimulator | 1.4 |
| AA066072 | phosphatidylcholine transfer protein | 1.4 |
| AA709984 | RAB17, member RAS oncogene family | 1.4 |
| AA619950 | cyclin I | 1.4 |

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|---|---|---|
| AA437994 | baculoviral IAP repeat-containing 4 | 1.4 |
| AA414425 | translocase of inner mitochondrial membrane 23 homolog (yeast) | 1.4 |
| AA146478 | IG ALPHA CHAIN C REGION | 1.4 |
| AA606908 | ring finger protein 10 | 1.4 |
| AA544212 | cellular repressor of E1A-stimulated genes | 1.4 |
| AA727017 | immediate early response 2 | 1.4 |
| AA596753 | latexin | 1.4 |
| AA469630 | choline kinase | 1.4 |
| AA265490 | bone morphogenetic protein receptor, type 1A | 1.4 |
| AA183318 | ATPase, H+ transporting, lysosomal (vacuolar proton pump), subunit 1 | 1.4 |
| AA624897 | ribosomal protein S16 | 1.4 |
| AA795558 | Ttk protein kinase | 1.4 |
| AA673731 | chitinase 3-like 3 | 1.4 |
| AI510034 | synuclein, alpha | 1.4 |
| AI892747 | glutathione S-transferase, alpha 2 (Yc2) | 1.4 |
| AA450910 | brachyury | 1.4 |
| AI597531 | gap junction membrane channel protein beta 2 | 1.4 |
| AA474223 | centrin 2 | 1.4 |
| AI550227 | Mus musculus unknown protein mRNA, partial cds | 1.4 |
| AI390129 | proteasome (prosome, macropain) subunit, alpha type 2 | 1.3 |
| AA027530 | placental lactogen 2 | 1.3 |
| AI020130 | granzyme B | 1.3 |
| AA675376 | DNA segment, Chr 7, ERATO Doi 373, expressed | 1.3 |
| AA674571 | radixin | 1.3 |
| AI552258 | reversion-inducing-cysteine-rich protein with kazal motifs | 1.3 |
| AA423123 | histocompatibility 2, T region locus 17 | 1.3 |
| AA290362 | origin recognition complex, subunit 5 homolog (S. cerevisiae) | 1.3 |
| AI390207 | calcium/calmodulin-dependent serine protein kinase | 1.3 |
| AA221832 | four jointed box 1 (Drosophila) | 1.3 |
| AI390473 | T-cell acute lymphocytic leukemia 1 | 1.3 |
| AA230517 | retinoic acid receptor, gamma | 1.3 |
| AA182058 | zinc finger protein 318 | 1.3 |
| AA189288 | etoile | 1.3 |
| AI324355 | mesenchyme homeobox 2 | 1.3 |
| AA014972 | granzyme F | 1.3 |
| AA165899 | LIM-domain containing, protein kinase | 1.3 |
| W17640 | aplysia ras-related homolog D (RhoD) | 1.3 |

| | | |
|---|---|---|
| W16027 | phosphodiesterase 1A, calmodulin-dependent | 1.3 |
| W14436 | matrix metalloproteinase 9 | 1.3 |
| AA174428 | Unc-51 like kinase 2 (C. elegans) | 1.3 |
| AA067153 | platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit | 1.3 |
| AA104976 | staufen (RNA-binding protein) homolog 2 (Drosophila) | 1.3 |
| AA666910 | membrane protein, palmitoylated 6 (MAGUK p55 subfamily member 6) | 1.3 |
| AA617414 | nucleosome binding protein 1 | 1.3 |
| AA763056 | suppressor of clear, C. elegans, homolog of | 1.3 |
| AA798766 | selectin, lymphocyte | 1.3 |
| AA716921 | Mus musculus adult male testis cDNA, RIKEN full-length enriched library, clone:4932441D06, full insert sequence | 1.3 |
| AA645995 | TG interacting factor | 1.3 |
| AA755273 | protein related to DAC and cerberus | 1.3 |
| AA672766 | Mouse mRNA for TI-227 | 1.3 |
| AI385493 | crystallin, gamma B | 1.3 |
| AA570965 | Mouse complement factor H-related protein mRNA, complete cds, clone 9C4 | 1.3 |
| AI385457 | retinol binding protein 2, cellular | 1.3 |
| AI323047 | splicing factor 3a, subunit 2, 66kD | 1.3 |
| AA671019 | procollagen, type IX, alpha 3 | 1.3 |
| AI549654 | programmed cell death 10 | 1.3 |
| AA210568 | DNA methyltransferase 3B | 1.3 |
| AA438152 | guanine nucleotide binding protein, alpha inhibiting 3 | 1.3 |
| AA212042 | RAB9, member RAS oncogene family | 1.3 |
| AA450855 | peroxiredoxin 5 | 1.3 |
| AA437837 | core binding factor beta | 1.3 |
| AI550200 | SEC61, alpha subunit 2 (S. cerevisiae) | 1.3 |
| AI121593 | X-linked myotubular myopathy gene 1 | 1.3 |
| AI390848 | aplysia ras-related homolog G (RhoG) | 1.3 |
| AI120332 | ribosomal protein L41 | 1.3 |
| AI159330 | globin inducing factor, fetal | 1.3 |
| W13748 | ribosomal protein L12 | 1.3 |
| AI158823 | immunoglobulin heavy chain 3 (serum IgG2b) | 1.3 |
| AI156457 | cytochrome P450, 7b1 | 1.3 |
| AI158565 | guanine nucleotide binding protein (G protein), gamma 10 | 1.3 |
| AA771678 | protein kinase, cGMP-dependent, type II | 1.3 |

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|---|---|---|
| AI550872 | RAB11a, member RAS oncogene family | 1.3 |
| AA510654 | SEC22 vesicle trafficking protein-like 1 (S. cerevisiae) | 1.3 |
| AA497627 | intersectin (SH3 domain protein 1A) | 1.3 |
| AA466550 | poliovirus receptor-related 3 | 1.3 |
| AA415862 | SRY-box containing gene 2 | 1.3 |
| W35084 | hematopoietic zinc finger | 1.3 |
| AA474336 | synaptonemal complex protein 3 | 1.3 |
| AA414670 | mitogen activated protein kinase kinase 4 | 1.3 |
| AA437876 | degenerative spermatocyte homolog (Drosophila) | 1.3 |
| AA437878 | Rho-associated coiled-coil forming kinase 1 | 1.3 |
| AA444231 | ribosomal protein S5 | 1.3 |
| AA259281 | LIM homeobox protein 2 | 1.3 |
| AA245183 | mab-21-like 2 (C. elegans) | 1.3 |
| AA161823 | lymphocyte antigen 116 | 1.3 |
| AA671424 | fibroblast growth factor inducible 14 | 1.3 |
| AA638960 | casein alpha | 1.3 |
| AA178447 | plexin 6 | 1.3 |
| AA562552 | glutathione peroxidase 1 | 1.3 |
| AA543948 | phosphatidylcholine transfer protein-like | 1.3 |
| AA474438 | profilin 2 | 1.3 |
| AA880295 | immunoglobulin joining chain | 1.3 |
| AI536384 | differential display and activated by p53 | 1.3 |
| AI390811 | glutamine fructose-6-phosphate transaminase 2 | 1.3 |
| AA726944 | vascular endothelial growth factor C | 1.3 |
| AA727051 | myeloid leukemia factor 1 | 1.3 |
| AA866934 | vesicle transport through interaction with t-SNAREs 1b homolog | 1.3 |
| AA596456 | prostaglandin E receptor EP4 subtype | 1.3 |
| AA596742 | testis derived transcript | 1.3 |
| AA068464 | ect2 oncogene | 1.3 |
| AA068624 | hydroxyacid oxidase (glycolate oxidase) 3 | 1.3 |
| AA065684 | helicase, lymphoid specific | 1.3 |
| AI326554 | aldo-keto reductase family 1, member C1 (aldehyde reductase) | 1.3 |
| AA066598 | Kruppel-like factor 9 | 1.3 |
| AI597336 | tripartite motif protein TRIM2 | 1.3 |
| AA450785 | Y box protein 1 | 1.3 |
| AI390104 | destrin | 1.3 |
| W16222 | septin 1 | 1.3 |
| W16059 | glutathione S-transferase like | 1.3 |

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|---|---|---|
| AA967493 | mitogen activated protein kinase kinase kinase 7 | 1.3 |
| AA473417 | cytochrome b-245, alpha polypeptide | 1.3 |
| AA237845 | coagulation factor XIII, beta subunit | 1.3 |
| AA250679 | aldo-keto reductase AKR1C12 | 1.3 |
| AA239479 | hydroxyacid oxidase 1, liver | 1.3 |
| AA270885 | parvalbumin | 1.3 |
| AA198237 | nuclear receptor subfamily 2, group F, member 6 | 1.3 |
| AI644821 | vaccinia related kinase 1 | 1.3 |
| AA220400 | integrin binding sialoprotein | 1.3 |
| AI893749 | serine/threonine kinase 6 | 1.3 |
| AA118767 | guanine nucleotide binding protein (G protein), gamma 12 | 1.3 |
| AI893970 | platelet derived growth factor, alpha | 1.3 |
| AA118183 | origin of replication 3 homolog (S. cerevisiae) | 1.3 |
| AA114742 | Mus musculus E-cadherin binding protein E7 mRNA, complete cds | 1.3 |
| W89951 | insulin-like growth factor binding protein 3 | 1.3 |
| AA833460 | histocompatibility-2 complex class 1-like sequence | 1.3 |
| AA739464 | killer cell lectin-like receptor, subfamily A, member 3 | 1.3 |
| AI893936 | hepatic nuclear factor 4 | 1.3 |
| AA870247 | mitogen regulated protein, proliferin 3 | 1.3 |
| AA546971 | membrane-spanning 4-domains, subfamily A, member 2 | 1.3 |
| AA267725 | DNA segment, Chr 1, ERATO Doi 622, expressed | 1.3 |
| AA198236 | kit ligand | 1.3 |
| AA254942 | carbonic anhydrase-like sequence 1 | 1.3 |
| AA239990 | SFFV proviral integration 1 | 1.3 |
| AA242256 | zinc finger protein of the cerebellum 1 | 1.3 |
| AI509951 | calcium channel, voltage-dependent, T type, alpha 1G subunit | 1.3 |
| AW209707 | hemoglobin Z, beta-like embryonic chain | 1.3 |
| AA623561 | retinoblastoma binding protein 4 | 1.3 |
| AA623498 | X-linked lymphocyte-regulated complex | 1.3 |
| AA624429 | mannosidase 1, beta | 1.3 |
| AA624421 | cornichon homolog (Drosophila) | 1.3 |
| AA607085 | polymerase, gamma | 1.3 |
| AA606561 | PFTAIRE protein kinase 1 | 1.3 |
| AI594243 | CD8beta opposite strand | 1.3 |
| AA606973 | ribosomal protein, mitochondrial, L7 | 1.3 |
| AA575501 | calmodulin 2 | 1.3 |
| AA437942 | cytokine inducible SH2-containing protein 1 | 1.3 |

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|---|---|---|
| AA517509 | transcription factor Dp 1 | 1.3 |
| AI596527 | ribosomal protein L26 | 1.3 |
| AA981355 | Mus musculus DNA cytosine methyltransferase mRNA | 1.3 |
| AI324371 | interleukin 4 | 1.3 |
| AA655648 | neuronal protein 15.6 | 1.3 |
| AA717170 | BCR downstream signaling 1 | 1.3 |
| AA795621 | lymphocyte antigen 84 | 1.3 |
| AA822050 | NAD(P) dependent steroid dehydrogenase-like | 1.3 |
| AI323028 | thymidylate synthase | 1.3 |
| AI552765 | S-adenosylmethionine decarboxylase 1 | 1.3 |
| AI386059 | acidic ribosomal phosphoprotein PO | 1.3 |
| AA080175 | claudin 8 | 1.3 |
| AA080181 | myelin protein zero | 1.3 |
| AI326538 | integrin, alpha E, epithelial-associated | 1.3 |
| AI595008 | Sin3-associated polypeptide 18 | 1.3 |

(Supplemental Table 4) Genotyped SNPs and *P* values for overall distribution of haplotypes in initial screening of candidate genes

| Gene name | Gene symbol | SNP ID[a] | Male + Female *P* value[b] | Male *P* value[b] | Female *P* value[b] |
|---|---|---|---|---|---|
| Acrosin | *ACR* | IMS-JST33261 | 0.667 | 0.651 | 1.000 |
| Adducin-1, alpha | *ADD1* | IMS-JST032023<br>IMS-JST005769 | 0.554 | 0.20 | 0.506 |
| Adducin-2, beta | *ADD2* | IMS-JST126484<br>IMS-JST108417<br>IMS-JST024418<br>IMS-JST024420 | 0.53 | 0.387 | 0.135 |
| Angiopoietin-like 2 | *ANGPTL2* | rs1281157<br>IMS-JST052573 | 0.131 | 0.062 | 0.138 |
| Aquaporin-1 (proximal) | *AQP1* | IMS-JST003211<br>IMS-JST003212<br>IMS-JST021256<br>IMS-JST008162<br>IMS-JST008162 | 0.311 | 0.455 | 0.090 |
| **Aquaporin-2 (collecting duct)** | ***AQP2*** | IMS-JST138705<br>IMS-JST092583<br>IMS-JST138708<br>IMS-JST138709 | **0.038*** | 0.4 | 0.081 |
| Aquaporin-3 (collecting duct) | *AQP3* | IMS-JST111570<br>IMS-JST111570<br>IMS-JST111569<br>IMS-JST016827 | 0.980 | 0.970 | 0.931 |
| Aquaporin-4 (medulally collecting duct) | *AQP4* | IMS-JST116766<br>IMS-JST116767<br>IMS-JST116771<br>IMS-JST003213<br>IMS-JST003214<br>IMS-JST116776 | 0.108 | 0.634 | 0.984 |
| Aromatic L-amino acid decarboxylase | *DDC* | rs2044859<br>rs1470750<br>rs2060761 | 0.818 | 0.971 | 0.323 |
| ATPase, H+ transporting, | *ATP6AP1* | IMS-JST082807 | 0.265 | 0.119 | 0.391 |

| | | | | | |
|---|---|---|---|---|---|
| lysosomal (vacuolar proton pump), subunit 1 | | IMS-JST098943 | | | |
| Beta-2-adrenergic receptor | *ADRB2* | IMS-JST087866<br>IMS-JST132959<br>IMS-JST132960 | 0.91 | 0.948 | 0.208 |
| Calcium channel, voltage-dependent, T type, alpha 1G subunit | *CACNA1G* | IMS-JST011704<br>IMS-JST095890 | 0.359 | 0.504 | 0.519 |
| Calpain 10 | *CAPN10* | IMS-JST149659<br>IMS-JST100982 | 0.762 | 0.987 | 0.072 |
| Carbonic anhydrase 3 | *CA3* | IMS-JST111349<br>IMS-JST111350<br>IMS-JST111351<br>IMS-JST111352<br>IMS-JST064895 | 0.75 | 0.884 | 0.13 |
| Catepsin | *CAT* | IMS-JST055760<br>IMS-JST009272<br>IMS-JST009271 | 0.615 | 0.466 | 0.968 |
| CD36 antigen | *CD36* | IMS-JST005702 | 0.332 | 0.901 | 0.086 |
| CD97 antigen | *CD97* | IMS-JST059524<br>IMS-JST059522 | 0.856 | 0.781 | 0.557 |
| Chloride channel, kidney, B | *CLCNKB* | IMS-JST052374 | 0.947 | 0.521 | 0.080 |
| Dopamine receptor D3 | *DRD3* | rs1385884<br>rs1872575 | 0.257 | 0.240 | 0.428 |
| Dopamine-beta-hydroxylase | *DBH* | IMS-JST090738<br>IMS-JST000449 | 0.920 | 0.296 | 0.083 |
| **Estrogen receptor 2** | ***ESR2*** | IMS-JST051491<br>IMS-JST051808<br>IMS-JST030988<br>IMS-JST030987 | **0.016*** | **0.018*** | 0.415 |
| FXYD domain-containing ion transport regulator 2 | *FXYD2* | IMS-JST053456<br>IMS-JST072429 | 0.135 | 0.107 | 0.616 |
| **Glycogen synthase 1** | ***GYS1*** | IMS-JST065629<br>IMS-JST097169 | **0.021*** | 0.127 | 0.071 |
| Granzyme B | *GZMB* | IMS-JST069533 | 0.679 | 0.902 | 0.083 |

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| | | IMS-JST069532<br>IMS-JST016601 | | | |
| Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2 | *GNGT2* | rs634370<br>rs850526 | 0.343 | 0.504 | 0.236 |
| Heat shock 70kD protein 5 (glucose-regulated protein, 78kD) | *HSPA5* | IMS-JST119759 | 0.08 | 0.104 | 0.597 |
| Hydroxysteroid 11-beta dehydrogenase 1 | *HSD11B1* | IMS-JST017377<br>IMS-JST031982<br>IMS-JST031983<br>IMS-JST017380<br>IMS-JST017380 | 0.495 | 0.881 | 0.201 |
| Insulin-like growth factor binding protein 5 | *IGFBP5* | IMS-JST012990<br>IMS-JST108187<br>IMS-JST012991 | 0.126 | 0.119 | 0.688 |
| **Kallikrein 1** | ***KLK1*** | IMS-JST060444<br>IMS-JST179918 | 0.084 | 0.449 | **0.029*** |
| Kallistatin | *SERPINA4* | IMS-JST115784<br>IMS-JST005765<br>IMS-JST005765 | 0.571 | 0.663 | 0.375 |
| Kininogen | *KKG* | IMS-JST061447<br>IMS-JST061448<br>IMS-JST061450 | 0.432 | 0.61 | 0.069 |
| Kit ligand | *KITLG* | IMS-JST138554<br>IMS-JST138552 | 0.192 | 0.086 | 0.792 |
| Latent transforming growth factor beta binding protein 2 | *LTBP2* | IMS-JST093976<br>IMS-JST093978<br>IMS-JST093979<br>IMS-JST093980 | 0.123 | 0.828 | 0.075 |
| Maternally expressed gene 3 | *MEG3* | IMS-JST139888<br>IMS-JST139889 | 0.415 | 0.341 | 0.776 |
| Natriuretic peptide precursor C | *NPR3* | IMS-JST150214<br>IMS-JST150204 | 0.131 | 0.062 | 0.234 |
| **Nephrin** | ***NPHN*** | IMS-JST175323<br>IMS-JST000541 | 0.078 | **0.048*** | 0.148 |

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| Nephrocystin | *NPHP1* | IMS-JST066065 | 0.055 | 0.093 | 0.421 |
| Nitric oxide synthase 3 | *NOS3* | IMS-JST117271<br>IMS-JST117269 | 0.181 | 0.313 | 0.281 |
| Parvalbumin | *PVALB* | IMS-JST033721<br>IMS-JST033718<br>IMS-JST033714 | 0.361 | 0.399 | 0.374 |
| Placental growth factor | *PGF* | IMS-JST139888<br>IMS-JST139889 | 0.415 | 0.341 | 0.776 |
| Platelet derived growth factor receptor, beta polypeptide | *PDGFRB* | IMS-JST109456<br>IMS-JST109453<br>IMS-JST012450<br>IMS-JST087370 | 0.079 | 0.070 | 0.428 |
| Potassium inwardly-rectifying channel, subfamily J, member 6 | *KCNJ6* | IMS-JST144710<br>IMS-JST144706<br>IMS-JST144687 | 0.083 | 0.065 | 0.081 |
| Potassium large conductance calcium-activated channel, subfamily M,beta member 1 | *KCNMB1* | IMS-JST006470<br>IMS-JST057105 | 0.19 | 0.111 | 0.983 |
| Protein C receptor, endothelial | *PROCR* | IMS-JST047041 | 0.445 | 0.731 | 0.132 |
| Serine/threonine kinase 19 | *STK19* | rs474534<br>IMS-JST033921<br>rs377370<br>rs389883 | 0.168 | 0.522 | 0.193 |
| Serine/threonine kinase 6 | *AURKA* | IMS-JST114769<br>IMS-JST144216 | 0.615 | 0.818 | 0.156 |
| Sodium channel, nonvoltage-gated 1, alpha | *SCNN1A* | IMS-JST006608<br>IMS-JST092986<br>IMS-JST092985 | 0.571 | 0.663 | 0.375 |
| Sodium channel, nonvoltage-gated 1, beta | *SCNN1B* | IMS-JST059724<br>IMS-JST059723<br>IMS-JST059722<br>IMS-JST095265 | 0.561 | 0.81 | 0.305 |
| **Solute carrier family 1 (glial high affinity** | ***SLC1A2*** | IMS-JST069326<br>IMS-JST069329 | **0.017*** | **0.04*** | **0.014*** |

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| **glutamate transporter), member 2** | | IMS-JST117720 | | | |
| Solute carrier family 12 member 1 | *SLC12A1* | IMS-JST027033<br>IMS-JST027035<br>IMS-JST043660 | 0.124 | 0.338 | 0.471 |
| Solute carrier family 12 sodium/potassium/chloride transporters) | *SLC12A3* | IMS-JST040573 | 0.487 | 0.354 | 0.883 |
| Solute carrier family 14 (monocarboxylic acid transporters), member 2 | *SLC14A2* | IMS-JST082696<br>IMS-JST143078<br>IMS-JST072854 | 0.409 | 0.247 | 0.06 |
| Solute carrier family 18 (vesicular monoamine), member 1 | *SLC18A1* | IMS-JST111224<br>IMS-JST065233<br>IMS-JST065231 | 0.099 | 0.143 | 0.522 |
| Solute carrier family 22 (organic cation transporter), member 1-like | *SLC22A1L* | IMS-JST018612<br>IMS-JST018613<br>IMS-JST058628<br>IMS-JST037271 | 0.097 | 0.168 | 0.355 |
| Solute carrier family 26, member 4 | *SLC26A4* | IMS-JST046871<br>IMS-JST046872<br>IMS-JST089508<br>IMS-JST007917 | 0.628 | 0.844 | 0.312 |
| Solute carrier family 4, anion exchanger, member 1 (erythrocytemembrane protein band 3, Diego blood group) | *SLC4A1* | IMS-JST000828<br>IMS-JST035905<br>IMS-JST007939 | 0.274 | 0.218 | 0.373 |
| Solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2 | *SLC6A2* | IMS-JST113472<br>IMS-JST113474<br>IMS-JST065623<br>IMS-JST014686<br>IMS-JST014687<br>IMS-JST014688 | 0.306 | 0.358 | 0.343 |
| Solute carrier family 8, member 1 (sodium-calcium exchanger-1) | *SLC8A1* | IMS-JST008552<br>IMS-JST008552<br>IMS-JST024796<br>IMS-JST012941 | 0.608 | 0.284 | 0.449 |
| **Solute carrier family 9** | ***SLC9A3*** | IMS-JST109880 | 0.255 | 0.984 | **0.005*** |

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| **(sodium/hydrogen exchanger), isoform 3** | | IMS-JST025479 | | | |
| **Steroidogenic acute regulatory protein** | ***STAR*** | IMS-JST005138<br>IMS-JST005139 | **0.048*** | **0.02*** | 0.076 |
| **Syntaxin binding protein 1** | ***STXBP1*** | IMS-JST111612<br>IMS-JST037891<br>IMS-JST012952<br>IMS-JST012952 | **0.014*** | **0.014*** | 0.51 |
| Synuclein, alpha | *SNCA* | IMS-JST131016<br>IMS-JST131014<br>IMS-JST130997 | 0.612 | 0.432 | 0.288 |
| Thyroid hormone responsive SPOT14 homolog | *THRSP* | rs545869 | 0.684 | 0.937 | 0.110 |
| Ttk protein kinase | *TTK* | IMS-JST049829<br>IMS-JST157819 | 0.401 | 0.532 | 0.862 |
| **TNF receptor superfamily, member 4** | ***TNFRSF4*** | IMS-JST053050<br>IMS-JST053049 | 0.452 | 0.63 | **0.024*** |
| Vascular endothelial growth factor C | *VEGFC* | IMS-JST130723<br>IMS-JST130724<br>IMS-JST109095 | 0.487 | 0.607 | 0.153 |
| Vesicle-associated membrane protein 3 | *VAMP3* | IMS-JST011175<br>IMS-JST007813 | 0.088 | 0.063 | 0.123 |
| Villin 2 | *VIL2* | IMS-JST110282<br>IMS-JST088251<br>IMS-JST088249 | 0.965 | 0.836 | 0.834 |
| WNK lysine deficient protein kinase 1 | *PRKWNK1* | rs1159744<br>rs2158502<br>rs718389 | 0.726 | 0.542 | 0.294 |
| WNK lysine deficient protein kinase 4 | *PRKWNK4* | rs324075 | 0.637 | 0.953 | 0.221 |

[a] IMS-JST# number from the Japanese SNP database (http://snp.ims.u-tokyo.ac.jp/) or rs# number from dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/).

[b] P values for overall distribution of haplotypes were calculated by permutation test at 1000 iterations using SNPAlyze v4.1 Pro software (DYNACOM, Mobara, Japan).

* Significant difference.