*Original Article*

# Haplotype-Based Case-Control Study of the Human CYP4F2 Gene and Essential Hypertension in Japanese Subjects

Zhenyan FU[1),2)], Tomohiro NAKAYAMA[1),3)], Naoyuki SATO[1)], Yoichi IZUMI[3)], Yuji KASAMAKI[4)], Atsushi SHINDO[4)], Masakatsu OHTA[4)], Masayoshi SOMA[5)], Noriko AOI[1)], Mikano SATO[1)], Koichi MATSUMOTO[3)], Yukio OZAWA[4)], and Yitong MA[2)]

**CYP4F2 acts primarily as an enzyme that converts arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE), a metabolite involved in the regulation of blood pressure in humans. The aim of the present study was to assess the association between the human CYP4F2 gene and essential hypertension (EH) using a haplotype-based case-control study that included separate analysis of the two gender groups. The 249 EH patients and 238 age-matched controls were genotyped for 5 single-nucleotide polymorphisms (SNPs) of the human CYP4F2 gene (rs3093105, rs3093135, rs1558139, rs2108622, rs3093200). Data were analyzed for 3 separate groups: all subjects, and men and women separately. For the total population and for male subjects, the distribution of the dominant model of rs1558139 (CC *vs.* CT+TT) differed significantly between the EH patients and control subjects ($p=0.037$ and $p=0.005$, respectively), with a higher percentage of EH patients showing the CC genotype. Logistic regression showed that, for men, the CC genotype of rs1558139 was more prevalent in the EH patients than in the control subjects ($p=0.026$), while, for the total population, the difference disappeared ($p=0.247$). For men, the overall distribution of the haplotypes was significantly different between the EH patients and the control subjects ($p=0.042$), and the frequency of the T-T-G haplotype was also significantly lower for EH patients than for control subjects ($p=0.009$). In conclusion, the present results indicate that rs1558139 might be a genetic marker for EH and the T-T-G haplotype might be a protective genetic marker for EH in Japanese men. (*Hypertens Res* 2008; 31: 1719–1726)**

***Key Words*: CYP4F2, single-nucleotide polymorphism, haplotype, case-control study, androgen**

## Introduction

Essential hypertension (EH) is a major risk factor for many common causes of morbidity and mortality including stroke, myocardial infarction, congestive heart failure, and end-stage renal disease (*1*). The etiology and pathogenesis of EH are likely comprised of a multifactorial disorder that results from the inheritance of several susceptibility genes as well as from multiple environmental determinants. A variety of gene variants have been shown to be associated with EH (*2*).

Cytochrome P450 (CYP) is a superfamily of cysteinato-

From the [1)]Division of Molecular Diagnostics, Department of Advanced Medical Science, Nihon University School of Medicine, Tokyo, Japan; [2)]Department of Cardiovascular Medicine, First Affiliated Hospital, Xinjiang Medical University, Urumqi, P.R. China; [3)]Division of Nephrology, Hypertension and Endocrinology, [4)]Division of Cardiovascular Medicine, and [5)]Division of General Medicine, Department of Medicine, Nihon University School of Medicine, Tokyo, Japan.

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Address for Reprints: Tomohiro Nakayama, M.D., Ph.D., Division of Molecular Diagnostics, Department of Advanced Medical Science, Nihon University School of Medicine, 30–1 Ooyaguchi-kamimachi, Itabashi-ku, Tokyo 173–8610, Japan. E-mail: tnakayam@med.nihon-u.ac.jp

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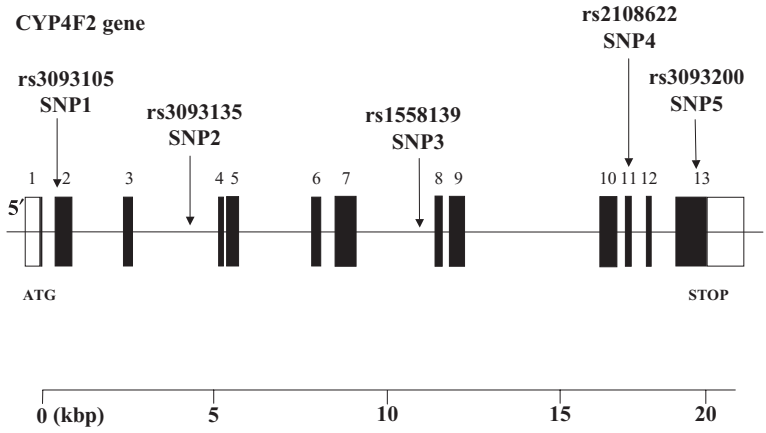


**Fig. 1.** *Structure of the human CYP4F2 gene. This gene consists of 13 exons separated by 12 introns. Boxes indicate exons, and lines indicate introns and intergenic regions. Filled boxes indicate coding regions. Arrows mark the polymorphism locations.*

heme enzymes that are not only involved in the metabolism of xenobiotics, but also metabolize a host of endobiotics. CYP enzymes are classified into families, subfamilies, and individual isoenzymes, based on similarities in their amino acid sequence (*3*). To date, the 5 human CYP4F members that have been identified are CYP4F2, CYP4F3, CYP4F8, CYP4F11, and CYP4F12, all of which are located on chromosome 19 (*4*). The CYP4F2 gene is located at 19pter–p13.11, spans approximately 20 kbp, and contains 13 exons.

The CYP4F2 family is expressed at high levels in the kidney and liver and is involved not only in the metabolism of leukotriene B4 but also arachidonic acid (AA) (*5*). CYP4F2 converts AA to 20-hydroxyeicosatetraenoic acid (20-HETE), which exhibits potent biological effects on renal tubular and vascular functions and on the long-term control of arterial pressure (*6*). It has been well established that alterations in renal 20-HETE production contribute to the development of hypertension in both rodent models and humans (*7*, *8*). Recently, Stec *et al*. identified a functional variant in the human CYP4F2 gene (rs2108622, V433M) that results in a decrease in 20-HETE production from AA (*9*). However, large, population-based studies are needed to determine whether the functional variants of the CYP4F2 gene are related to hypertension. Given the potential importance of 20-HETE in the regulation of hypertension, the aim of the present study was to assess the association between EH and the human CYP4F2 gene in Japanese subjects using a haplotype-based case-control method.

## Methods

### Subjects

Subjects diagnosed with EH were recruited at Nihon University Itabashi Hospital and other neighboring hospitals in Tokyo from 1993 to 2003. A total of 249 EH patients were enrolled in the present study, with a male/female (m/f) ratio of 1.94. EH was diagnosed based on the following criteria: seated systolic blood pressure (SBP)>160 mmHg or diastolic blood pressure (DBP)>100 mmHg on 3 occasions within 2 months after the first blood pressure (BP) reading. None of the EH patients were receiving antihypertensive medication and patients diagnosed with secondary hypertension were excluded. A total of 238 normotensive (NT) age-matched individuals (m/f ratio=1.88) were enrolled as control subjects. Controls were members of the New Elder Citizen Movement in Japan, and all subjects lived in Tokyo or the suburbs of Tokyo. All controls were confirmed to have grade 0 on the modified Rankin Scale. In this study group, participants with cancer or autoimmune diseases were excluded. None of the control subjects had a family history of hypertension, and all had a SBP of <130 mmHg and a DBP of <85 mmHg. The sample size was thought to be large enough because our previous studies successfully isolated EH susceptibility markers using a similar sample size (*10*, *11*). Informed consent was obtained from each subject in accordance with the protocol approved by the Human Studies Committee of Nihon University.

### Genotyping

There are 225 single-nucleotide polymorphisms (SNPs) for the human CYP4F2 gene listed in the National Center for Biotechnology Information SNP database Build 126 (http://www.ncbi.nlm.nih.gov/SNP). We screened the data for the Tag SNPs on the International HapMap Project website (http://www.hapmap.org/index.html.ja) and used a cut off level of $r^2 \geq 0.5$, along with a cut off level of $\leq 0.1$ for the minor allele frequency (MAF). According to the above criteria we selected rs3093135, rs1558139, and rs2108622 as SNPs for this gene. rs3093135 and rs1558139 were located in introns, and rs2108622 was located in an exon with a nonsyn-

**Table 1. Characteristics of Study Participants**

| | Total | | | Men | | | Women | | |
|---|---|---|---|---|---|---|---|---|---|
| | EH patients ($n$=249) | Control subjects ($n$=238) | $p$-value | EH patients ($n$=165) | Control subjects ($n$=155) | $p$-value | EH patients ($n$=84) | Control subjects ($n$=83) | $p$-value |
| Age (years) | 51.1±5.6 | 51.3±8.9 | 0.766 | 50.9±5.7 | 52.0±6.8 | 0.123 | 51.4±5.3 | 50.0±11.7 | 0.294 |
| BMI (kg/m$^2$) | 24.6±3.4 | 22.8±3.3 | <0.001 | 24.6±3.3 | 23.1±3.3 | <0.001 | 24.5±3.6 | 22.4±3.4 | <0.001 |
| SBP (mmHg) | 173.6±20.1 | 112.4±10.6 | <0.001 | 171.2±19.0 | 113.0±10.2 | <0.001 | 178.1±21.4 | 111.4±11.3 | <0.001 |
| DBP (mmHg) | 106.3±12.9 | 69.4±8.6 | <0.001 | 106.5±12.7 | 70.1±8.1 | <0.001 | 105.8±13.2 | 68.2±9.3 | <0.001 |
| Pulse rate (beats/min) | 77.3±15.0 | 72.9±12.6 | 0.002 | 76.6±15.6 | 72.7±14.0 | 0.045 | 78.9±13.8 | 73.2±9.5 | 0.009 |
| Creatinine (mg/dL) | 0.8±0.2 | 0.8±0.2 | 0.192 | 0.9±0.2 | 0.9±0.2 | 0.148 | 0.7±0.2 | 0.7±0.1 | 0.915 |
| Total cholesterol (mg/dL) | 210.1±35.8 | 202.8±40.9 | 0.041 | 204.3±32.7 | 198.2±37.4 | 0.132 | 220.6±38.8 | 211.2±45.6 | 0.154 |
| HDL cholesterol (mg/dL) | 56.1±17.4 | 57.4±18.1 | 0.468 | 52.9±16.6 | 55.1±16.9 | 0.261 | 61.9±17.3 | 61.6±19.6 | 0.920 |
| Uric acid (mg/dL) | 5.7±1.6 | 5.4±1.4 | 0.037 | 6.2±1.5 | 5.8±1.4 | 0.016 | 4.6±1.3 | 4.5±1.2 | 0.571 |
| Hyperlipidemia (%) | 26 | 19 | 0.055 | 22 | 15 | 0.102 | 34 | 26 | 0.263 |
| Diabetes (%) | 10 | 3 | <0.001 | 11 | 3 | 0.005 | 8 | 2 | 0.090 |
| Drinking (%) | 59 | 45 | 0.014 | 73 | 63 | 0.093 | 29 | 11 | 0.014 |
| Smoking (%) | 54 | 42 | 0.013 | 66 | 52 | 0.025 | 32 | 23 | 0.257 |

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high density lipoprotein; EH, essential hypertension. Continuous variables were expressed as mean±SD. Categorical variables were expressed as percentage. The $p$-value of continuous variables were calculated using Mann-Whitney $U$-test. The $p$-value of categorical variables were calculated using Fisher's exact test.

onymous substitution amino acid change. Variations of rs2108622 that can alter the production of 20-HETE have been identified (*9*). In the current study, we also looked at rs3093105 and rs3093200, which are located in the exons and are known to be nonsynonymous SNPs. We designated these SNPs as SNP1 (rs3093105), SNP2 (rs3093135, C_27482167_10), SNP3 (rs1558139, C_2583813_10), SNP4 (rs2108622, C_16179493_40), and SNP5 (rs3093200), in order of increasing distance from the 5′ end of the gene (Fig. 1). SNP2, SNP3 and SNP4 were genotyped using a kit from Applied Biosystems Inc. (ABI; Foster City, USA). Since SNP1 and SNP5 were not registered in the genotyping kit from ABI, we purchased and used a Custom TaqMan® SNP Genotyping Assay for these SNPs.

Blood samples were collected from all participants, and genomic DNA was extracted from the peripheral blood leukocytes by phenol and chloroform extraction.

Genotyping was performed using the TaqMan® SNP Genotyping Assay (ABI). TaqMan® SNP Genotyping Assays were performed using Taq amplification. In the 5′ nuclease assay, polymerase chain reaction (PCR) discriminates between SNPs because allele-specific fluorogenic probes that hybridize to the template are cleaved by the 5′ nuclease activity of Taq polymerase. The probes contain a 3′ minor groove–binding group (MGB) that hybridizes to single-stranded targets with greater sequence specificity than is seen with ordinary DNA probes. This reduces nonspecific probe hybridization, and results in low background fluorescence in the 5′ nuclease PCR assay (TaqMan®, ABI). Cleavage results in increased emission of the reporter dye. Each 5′ nuclease assay requires 2 unlabeled PCR primers and 2 allele-specific probes. Each probe is labeled with 2 reporter dyes at the 5′ end. In the present study, VIC and carboxyfluorescein (FAM) were used as the reporter dyes. The primers and probes used in the TaqMan® SNP Genotyping Assays (ABI) were chosen based on information available on the ABI website (http://myscience.appliedbiosystems.com).

PCR amplification was performed using 2.5 μL of TaqMan® Universal Master Mix, No AmpErase® UNG (2×) (ABI) in a 5-μL final reaction volume containing 2 ng of DNA, 0.046 μL of TaqMan® SNP Genotyping Assay Mix (40×), primers at a concentration of 331 nmol/L each, and probes at a final concentration of 73.6 nmol/L each. Thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 62°C for 1 min. Thermal cycling was performed using the GeneAmp 9700™ system.

Each 96-well plate contained 80 DNA samples of an unknown genotype and 4 reaction mixtures containing reagents but no DNA (control). The control samples without DNA are a necessary part of the Sequence Detection System (SDS) 7700™ signal processing, as outlined in the TaqMan Allelic Discrimination Guide (ABI). The plates were read on the SDS 7700 instrument using the end-point analysis mode of the SDS version 1.6.3 software package (ABI). The genotypes were determined visually based on the dye-component fluorescent emission data depicted in the $X$-$Y$ scatter-plot of the SDS software. The genotypes were also determined automatically by the software's signal processing algorithms. The results of each scoring method were saved in 2 separate

**Table 2. Genotype and Allele Distributions for the Patients with EH and the Control Subjects**

| | | Total | | | Men | | | Women | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | EH patients (*n* (%)) | Control subjects (*n* (%)) | *p*-value | EH patients (*n* (%)) | Control subjects (*n* (%)) | *p*-value | EH patients (*n* (%)) | Control subjects (*n* (%)) | *p*-value |
| rs3093105 (SNP1) | | | | | | | | | | |
| Genotype | T/T | 197 (79.1) | 190 (79.8) | | 130 (78.8) | 124 (80.0) | | 67 (79.8) | 66 (79.5) | |
| | T/G | 47 (18.9) | 43 (18.1) | 0.972 | 30 (18.2) | 27 (17.4) | 0.952 | 17 (20.2) | 16 (19.3) | 0.597 |
| | G/G | 5 (2.0) | 5 (2.1) | | 5 (3.0) | 4 (2.6) | | 0 (0.0) | 1 (1.2) | |
| dominant model | TT | 197 (79.1) | 190 (79.8) | 0.845 | 130 (78.8) | 124 (80.0) | 0.789 | 67 (79.8) | 66 (79.5) | 0.969 |
| | TG+GG | 52 (20.9) | 48 (20.2) | | 35 (21.2) | 31 (20.0) | | 17 (20.2) | 17 (20.5) | |
| recessive model | GG | 5 (2.0) | 5 (2.1) | 0.943 | 5 (3.0) | 4 (2.6) | 0.808 | 0 (0.0) | 1 (1.2) | 0.313 |
| | TG+TT | 244 (98.0) | 233 (97.9) | | 160 (97.9) | 151 (97.4) | | 84 (100.0) | 82 (98.8) | |
| Allele | T | 441 (88.6) | 423 (88.9) | 0.878 | 290 (87.9) | 275 (88.7) | 0.744 | 151 (89.9) | 148 (89.2) | 0.829 |
| | G | 57 (11.4) | 53 (11.1) | | 40 (12.1) | 35 (11.3) | | 17 (10.1) | 18 (10.8) | |
| rs3093135 (SNP2) | | | | | | | | | | |
| Genotype | T/T | 196 (78.7) | 190 (79.8) | | 129 (78.2) | 124 (80.0) | | 67 (79.8) | 66 (79.5) | |
| | T/A | 48 (19.3) | 43 (18.1) | 0.942 | 31 (18.8) | 27 (17.4) | 0.917 | 17 (20.2) | 16 (19.3) | 0.597 |
| | A/A | 5 (2.0) | 5 (2.1) | | 5 (3.0) | 4 (2.6) | | 0 (0.0) | 1 (1.2) | |
| dominant model | TT | 196 (78.7) | 190 (79.8) | 0.761 | 129 (78.2) | 124 (80.0) | 0.690 | 67 (79.8) | 66 (79.5) | 0.969 |
| | TA+AA | 53 (21.3) | 48 (20.2) | | 36 (21.8) | 31 (20.0) | | 17 (20.2) | 17 (20.5) | |
| recessive model | AA | 5 (2.0) | 5 (2.1) | 0.943 | 5 (3.0) | 4 (2.6) | 0.808 | 0 (0.0) | 1 (1.2) | 0.313 |
| | TA+TT | 244 (98.0) | 233 (97.9) | | 160 (97.0) | 151 (97.4) | | 84 (100.0) | 82 (98.8) | |
| Allele | T | 440 (88.4) | 423 (88.9) | 0.802 | 289 (87.6) | 275 (88.7) | 0.658 | 151 (89.9) | 148 (89.2) | 0.829 |
| | A | 58 (11.6) | 53 (11.1) | | 41 (12.4) | 35 (11.3) | | 17 (10.1) | 18 (10.8) | |
| rs1558139 (SNP3) | | | | | | | | | | |
| Genotype | C/C | 125 (50.2) | 97 (40.7) | | 92 (55.8) | 62 (40.0) | | 33 (39.3) | 35 (42.2) | |
| | C/T | 93 (37.3) | 113 (47.5) | 0.068 | 54 (32.7) | 74 (47.7) | 0.013 | 39 (46.4) | 39 (47.0) | 0.786 |
| | T/T | 31 (12.5) | 28 (11.8) | | 19 (11.5) | 19 (12.3) | | 12 (14.3) | 9 (10.8) | |
| dominant model | CC | 125 (50.2) | 97 (40.7) | 0.037 | 92 (55.8) | 62 (40.0) | 0.005 | 33 (39.3) | 35 (42.2) | 0.705 |
| | CT+TT | 124 (49.8) | 141 (59.3) | | 73 (44.2) | 93 (60.0) | | 51 (60.7) | 48 (57.8) | |
| recessive model | TT | 31 (12.5) | 28 (11.8) | 0.817 | 19 (11.5) | 19 (12.3) | 0.837 | 12 (14.3) | 9 (10.8) | 0.502 |
| | CT+CC | 218 (87.5) | 210 (88.2) | | 146 (88.5) | 136 (87.7) | | 72 (85.7) | 74 (89.2) | |
| Allele | C | 343 (68.9) | 307 (64.5) | 0.147 | 238 (72.1) | 198 (63.9) | 0.025 | 105 (62.5) | 109 (65.7) | 0.547 |
| | T | 155 (31.1) | 169 (35.5) | | 92 (27.9) | 112 (36.1) | | 63 (37.5) | 57 (34.3) | |
| rs2108622 (SNP4) | | | | | | | | | | |
| Genotype | G/G | 143 (57.4) | 128 (53.8) | | 95 (57.6) | 86 (55.5) | | 48 (57.1) | 42 (50.6) | |
| | G/A | 74 (29.7) | 91 (38.2) | 0.059 | 50 (30.3) | 58 (37.4) | 0.188 | 24 (28.6) | 33 (39.8) | 0.271 |
| | A/A | 32 (12.9) | 19 (8.0) | | 20 (12.1) | 11 (7.1) | | 12 (14.3) | 8 (9.6) | |
| dominant model | GG | 143 (57.4) | 128 (53.8) | 0.418 | 95 (57.6) | 86 (55.5) | 0.706 | 48 (57.1) | 42 (50.6) | 0.397 |
| | GA+AA | 106 (42.6) | 110 (46.2) | | 70 (42.4) | 69 (44.5) | | 36 (42.9) | 41 (49.4) | |
| recessive model | AA | 32 (12.9) | 19 (8.0) | 0.080 | 20 (12.1) | 11 (7.1) | 0.129 | 12 (14.3) | 8 (9.6) | 0.355 |
| | GA+GG | 217 (87.1) | 219 (92.0) | | 145 (87.9) | 144 (92.9) | | 72 (85.7) | 75 (90.4) | |
| Allele | G | 360 (72.3) | 347 (72.9) | 0.831 | 240 (72.7) | 230 (74.2) | 0.675 | 120 (71.4) | 117 (70.5) | 0.849 |
| | A | 138 (27.7) | 129 (27.1) | | 90 (27.3) | 80 (25.8) | | 48 (28.6) | 49 (29.5) | |
| rs3093200 (SNP5) | | | | | | | | | | |
| Genotype | C/C | 248 (99.6) | 238 (100.0) | | 164 (99.4) | 155 (100.0) | | 84 (100.0) | 83 (100.0) | |
| | C/A | 1 (0.4) | 0 (0.0) | — | 1 (0.6) | 0 (0.0) | — | 0 (0.0) | 0 (0.0) | — |
| | A/A | 0 (0.0) | 0 (0.0) | | 0 (0.0) | 0 (0.0) | | 0 (0.0) | 0 (0.0) | |
| dominant model | CC | 248 (99.6) | 238 (100.0) | 0.328 | 164 (99.4) | 155 (100.0) | 0.332 | 84 (100.0) | 83 (100.0) | — |
| | CA+AA | 1 (0.4) | 0 (0.0) | | 1 (0.6) | 0 (0.0) | | 0 (0.0) | 0 (0.0) | |
| recessive model | AA | 0 (0.0) | 0 (0.0) | — | 0 (0.0) | 0 (0.0) | — | 0 (0.0) | 0 (0.0) | — |
| | CA+CC | 249 (100.0) | 238 (100.0) | | 165 (100.0) | 155 (100.0) | | 84 (100.0) | 83 (100.0) | |
| Allele | C | 497 (99.8) | 476 (100.0) | 0.328 | 329 (99.7) | 310 (100.0) | 0.332 | 168 (100.0) | 166 (100.0) | — |
| | A | 1 (0.2) | 0 (0.0) | | 1 (0.3) | 0 (0.0) | | 0 (0.0) | 0 (0.0) | |

SNP, single-nucleotide polymorphism; EH, essential hypertension. The *p*-value of genotypes and alleles were calculated using Fisher's exact test.

**Table 3. Odds Ratios and 95% Confidence Intervals (CI) for Each Risk Factor and SNP Genotype Associated with Essential Hypertension**

| Risk factor | Total | | | Men | | | Women | | |
|---|---|---|---|---|---|---|---|---|---|
| | Odds ratios | 95% CI | *p*-value | Odds ratios | 95% CI | *p*-value | Odds ratios | 95% CI | *p*-value |
| rs1558139 CC genotype | 0.775 | 0.504–1.193 | 0.247 | 1.829 | 1.076–3.111 | 0.026 | 1.659 | 0.757–3.638 | 0.206 |
| BMI | 0.853 | 0.797–0.913 | 0.000 | 0.884 | 0.814–0.959 | 0.003 | 0.770 | 0.670–0.884 | 0.000 |
| DM | 1.985 | 0.810–4.866 | 0.134 | 2.426 | 0.839–7.017 | 0.102 | 1.029 | 0.173–6.126 | 0.975 |
| Smoking | 0.582 | 0.378–0.896 | 0.014 | 1.885 | 1.101–3.230 | 0.021 | 0.713 | 0.295–1.725 | 0.453 |

SNP, single-nucleotide polymorphism; BMI, body mass index; DM, diabetes mellitus.

output files for later comparison (*12*).

### Biochemical Analysis

We measured the plasma concentration of total cholesterol and high-density lipoprotein (HDL) cholesterol and the serum concentration of creatinine and uric acid using standard methods employed by the Clinical Laboratory Department of Nihon University Hospital (*13*).

### Statistical Analysis

All continuous variables are expressed as mean±SD. Differences in continuous variables between EH patients and control subjects were analyzed using the Mann-Whitney *U*-test. Differences in categorical variables were analyzed using Fisher's exact test. Hardy-Weinberg equilibrium was assessed by $\chi^2$ analysis. Differences in distributions of genotypes and alleles between EH patients and control subjects were analyzed using Fisher's exact test. Based on the genotype data of the genetic variations, we performed linkage disequilibrium (LD) analysis and haplotype-based case-control analysis, using the expectation maximization (EM) algorithm (*14*) and the software SNPAlyze version 3.2 (Dynacom Co., Ltd., Yokohama, Japan) (*15*). The SNP5 showed little heterogeneity as only one heterozygosity was found in the EH patients, therefore, SNP5 was not available for the haplotype-based case-control study. Pairwise LD analysis was performed using SNP1, SNP2, SNP3, and SNP4. We used $|D'|$ values of $\geq 0.5$ to assign SNP locations to 1 haplotype block. SNPs with an $r^2$ value of $<0.5$ were selected as tagged, which means they were available for the haplotype. In the haplotype-based case-control analysis, haplotypes with a frequency of $<0.01$ were excluded. With regard to this method, the general consensus is that the effect of less frequent haplotypes should be excluded (*10*, *12*). The analysis was also performed for each of the 3 groups: total population, men, and women. The frequency distribution of the haplotypes was calculated by $\chi^2$ analysis. In addition, logistic regression analysis was performed to assess the contribution of the major risk factors. Statistical significance was established at $p<0.05$. Statistical analyses were performed using SPSS software for Windows, version 12 (SPSS Inc., Chicago, USA).

## Results

Table 1 shows the clinical characteristics of the study participants. For men, women, and total subjects, the following values were significantly higher in the EH patients than in the control subjects: body mass index (BMI), SBP, DBP, and pulse rate. For men and total subjects, serum uric acid and the prevalence of diabetes mellitus and smoking were significantly higher for the EH patients than the control subjects. For women and total subjects, the prevalence of drinking was higher in the EH group than in the control group. For the total subject group, total cholesterol was higher in the EH group as compared to the control group. There was no significant difference for any of the following variables between the EH patients and the control subjects: serum creatinine and the prevalence of hyperlipidemia.

Table 2 shows the distribution of the genotypes and alleles of the 5 SNPs. The genotype distributions for each of the SNPs in the control subjects were in agreement with the predicted Hardy-Weinberg equilibrium values (data not shown). For the male subjects, the genotype distribution of SNP3 differed significantly between the EH patients and the control subjects ($p=0.013$). For the total population and the men, the distribution of the dominant model of rs1558139 (CC *vs*. CT+TT) differed significantly between the EH patients and the control subjects ($p=0.037$, $p=0.005$, respectively), with the CC genotype higher in the EH patients than in the control subjects. For the male subjects, the C allele was significantly higher in the EH patients as compared to control subjects ($p=0.025$).

Table 3 shows the results of the logistic regression analysis. For the men, the frequency of the CC genotype of rs1558139 differed significantly between the EH patients and control subjects ($p=0.026$), even after an adjustment for confounding factors such as BMI, diabetes mellitus, and smoking. However, for the total group, there were no differences noted for the frequency of the CC genotype of rs1558139 after adjustment for the aforementioned confounding factors ($p=0.247$).

Table 4 shows patterns of linkage disequilibrium for the CYP4F2 gene with $|D'|$ and $r^2$ values. All 4 SNPs were located in 1 haplotype block because all $|D'|\geq 0.5$. Because the $r^2$ value of SNP1–SNP2$\geq 0.5$, it is not very effective for a

**Table 4. Pairwise Linkage Disequilibrium (|*D′*| above Diagonal and $r^2$ below Diagonal) for the Four SNPs**

| | | |*D′*| | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | EH patients | | | | Control subjects | | | |
| | SNP | SNP1 | SNP2 | SNP3 | SNP4 | SNP1 | SNP2 | SNP3 | SNP4 |
| $r^2$ | SNP1 | | 1.000 | 1.000 | 0.972 | | 0.979 | 1.000 | 0.938 |
| | SNP2 | 0.981 | | 1.000 | 0.973 | 0.958 | | 1.000 | 0.907 |
| | SNP3 | 0.058 | 0.060 | | 0.341 | 0.069 | 0.069 | | 0.438 |
| | SNP4 | 0.319 | 0.326 | 0.099 | | 0.297 | 0.278 | 0.130 | |

SNP, single-nucleotide polymorphism; EH, essential hypertension. Underline: $|D'| \geq 0.5$ or $r^2 \geq 0.5$.

**Table 5. Haplotype Analysis for the Patients with EH and the Control Subjects**

| Haplotypes | CYP4F2 polymorphism | | | Overall *p* value | | | Frequency in total | | | Frequency in men | | | Frequency in women | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SNP1 | SNP3 | SNP4 | Total | Men | Women | EH patients | Control subjects | *p* value | EH patients | Control subjects | *p* value | EH patients | Control subjects | *p* value |
| | | | | 0.470 | 0.042 | 0.457 | | | | | | | | | |
| H1 | T Mj | C Mj | G Mj | | | | 0.578 | 0.536 | 0.192 | 0.593 | 0.529 | 0.099 | 0.524 | 0.559 | 0.509 |
| H2 | T Mj | T Mn | G Mj | | | | 0.156 | 0.191 | 0.152 | 0.132 | 0.209 | 0.009 | 0.199 | 0.146 | 0.190 |
| H3 | G Mn | C Mj | A Mn | | | | 0.109 | 0.108 | 0.978 | 0.010 | 0.000 | 0.094 | 0.096 | 0.098 | 1.000 |
| H4 | T Mj | T Mn | A Mn | | | | 0.158 | 0.165 | 0.753 | 0.119 | 0.108 | 0.661 | 0.181 | 0.187 | 0.887 |
| H5 | T Mj | C Mj | A Mn | | | | — | — | — | 0.145 | 0.154 | 0.798 | — | — | — |
| H6 | G Mn | T Mn | A Mn | | | | — | — | — | — | — | — | 0.000 | 0.011 | 0.156 |

EH, essential hypertension. Haplotypes with frequency >0.01 were estimated using SNPAlyze software. The *p* values were calculated by $\chi^2$ analysis.

haplotype-based case-control study when SNP1 and SNP2 are used simultaneously. In our haplotype-based case-control study, although the minor allele frequency of SNP1 in the controls was equal to that of SNP2, the minor allele frequency of SNP1 in the NCBI data was larger than that for SNP2. Therefore, we constructed haplotypes using SNP1, SNP3, and SNP4.

In the haplotype-based case-control study, using SNP1, SNP3, and SNP4, 4 haplotypes were established in the total group, and 5 haplotypes were established in both the groups for men and women (Table 5). For men, the overall distribution of the haplotypes was significantly different between the EH patients and the control subjects ($p=0.042$). In addition, for men, the frequency of the T-T-G haplotype was significantly lower in the EH patients as compared to that seen in the control subjects ($p=0.009$).

## Discussion

It has been demonstrated that many CYP subfamilies are associated with EH, for example, CYP2J2, CYP2C9 (EET synthesis), CYP3A5 (metabolizes cortisol into 6β-hydroxycortisol), CYP4A11 (20-HETE synthesis), CYP8A (prostacyclin synthesis), and CYP11B2 (aldosterone synthesis) (*8*, *16*–*20*). In humans, the CYP4 subfamily mainly catalyzes AA to 20-HETE, which acts either in a prohypertensive or antihypertensive manner, depending on whether it is expressed at renovascular or tubular sites. In the renal tubule, 20-HETE blocks sodium transport and acts primarily as a natriuretic, antihypertensive substance. In the renal vasculature, 20-HETE has vasoconstricting and prohypertensive effects (*6*, *21*). CYP4A11 was first described as being able to catalyze AA into 20-HETE. Additionally, it was reported that the C-to-T mutation in the CYP4A11 gene influenced the production of 20-HETE, and that this was associated with EH in large human populations (*8*). In our previous haplotype-based case-control study, we succeeded in identifying a haplotype of the CYP4A11 gene that was associated with EH in Japanese men (*10*). Recently, immunoprecipitation studies using human kidney microsomes have shown that the CYP4F2

accounts for up to 70% of the 20-HETE production in humans. Therefore, CYP4F2-catalyzed 20-HETE formation is thought to be quantitatively more important than CYP4A11-catalyzed 20-HETE formation (*5*). The major difference between CYP4A11 and CYP4F2 with regard to function is that the CYP4F2 isoform is expressed in the liver, lung, and white blood cells and is also the main enzyme responsible for the ω-hydroxylation of leukotriene B4 (LTB4) (*22*). A functional variant of the human CYP4F2 gene (rs2108622, V433M) that is capable of altering the production of 20-HETE has recently been identified (*9*). Alterations in renal 20-HETE production that might contribute to the development of hypertension have been demonstrated in both animals and humans (*7*, *8*). Large population-based studies are needed to determine whether the functional variants of the CYP4F2 gene are related to the development of hypertension in humans. In our haplotype-based case-control study in Japanese subjects, we genotyped 5 SNPs in the CYP4F2 gene (including rs2108622) and assessed the association between the CYP4F2 gene and EH.

In the present study, we did not find any significant difference in the genotypic and allele distribution of SNP4 (rs2108622) in the total population, men, and women groups, while the distribution of the dominant model of SNP3 (rs1558139, CC *vs*. CT+TT) differed significantly between the EH patients and control subjects in the total population and in men ($p=0.037$ and $p=0.005$, respectively). When the logistic regression was adjusted for the BMI, diabetes mellitus, and smoking, the same results were observed in men ($p=0.026$). However, for the total group, the difference between the EH patients and control subjects disappeared ($p=0.247$). For men, the C allele was also significantly higher in EH patients compared to the control subjects ($p=0.025$). These results indicate that the risk of EH is increased in men with the CC genotype and the C allele of SNP3. Additionally, the findings suggest that SNP3 might be a gender-specific genetic marker for EH. Only 1 CA heterozygous genotype of SNP5 (rs3093200) was found in 1 male EH patient. The clinical features for this patient were not different from the other EH patients.

Morris and Kaplan found that for genes with multiple susceptibilities, analysis based on haplotypes have advantages over analysis based on individual SNPs, particularly when linkage disequilibria between the SNPs are weak (*23*). For men, the overall distribution of the haplotypes were significantly different between the EH patients and the control subjects ($p=0.042$). In addition, the frequency of the T-T-G haplotype constructed with SNP1, SNP3, and SNP4 for men was also significantly lower for EH patients as compared to the control subjects, and thus, the T-T-G haplotype is a protective haplotype in men. In the present study, although SNP4 (rs2108622) was not associated with EH, we successfully isolated the significant haplotypes. This result suggests that there is a possible mutation that has an effect on the production of 20-HETE associated with EH rather than SNP4 because the haplotype-based case-control study is a more powerful tool than the simple case-control study that uses each SNP (*24*). This mutation may be linked to the significant haplotype.

Some case-control studies have identified gene variants associated with gender-specific susceptibility to EH (*24*, *25*). Animal experiments have revealed that the expression of the number of P450 enzymes is sex-dependent (*26*, *27*). The present haplotype-based case-control study also showed gender-specific (for men only) genotype and haplotype significant differences between the EH patients and the control subjects. This is very interesting in light of previous findings that showed that male cyp4a14$^{-/-}$ or cyp4a10$^{-/-}$ mice have higher BP than female cyp4a14$^{-/-}$ or cyp4a10$^{-/-}$ mice, respectively. The male cyp4a14$^{-/-}$ mice exhibited increases in plasma androgen, and their hypertensive phenotype was found to be androgen sensitive (*28*, *29*). Cyp4a14 and cyp4a10 do not exist in humans, but as human CYP4A11 is 72.69% identical with murine cyp4a14 and 73.02% identical with murine cyp4a10 by amino acid sequence, murine cyp4a14 and cyp4a10 can therefore be regarded as being homologous to human CYP4A11. Unfortunately, there has yet to be a knockout animal model established for the CYP4F gene that includes cyp4f2. In the current experiment, we also did not obtain plasma androgen data because we were not able to obtain informed consent to collect blood samples for the purpose of measuring the plasma androgen levels. When all of the current results are taken together, our findings suggest that SNP3 (rs1558139) and the T-T-G haplotype might be associated with the androgen-mediated activity of the CYP4F2 enzyme.

In conclusion, the CC genotype and the C allele of rs1558139 in the human CYP4F2 gene might be susceptibility markers. In addition, the T-T-G haplotype appears to be a protective marker for EH in Japanese men. However, due to the moderate size of the population examined in this study, in order to be able to conclusively discuss the statistical significance of the interaction between the genotype (or haplotype) and the phenotype, further studies based on larger populations are needed. Such studies would allow us to isolate the functional mutations of the CYP4F2 gene that regulate BP, and to evaluate the function of the CYP4F2 variants that are involved in the metabolism of sex hormones.

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## References

1. Suzuki H, Kanno Y, Efficacy of Candesartan on Outcome in Saitama Trial (E-COST) Group: Effects of candesartan on cardiovascular outcomes in Japanese hypertensive patients. *Hypertens Res* 2005; **28**: 307–314.
2. Sugimoto K, Katsuya T, Ohkubo T, *et al*: Association between angiotensin II type 1 receptor gene polymorphism

and essential hypertension: the Ohasama Study. *Hypertens Res* 2004; **27**: 551–556.
3. Capdevila JH, Falck JR, Harris RC: Cytochrome P450 and arachidonic acid bioactivation: molecular and functional properties of the arachidonate monooxygenase. *J Lipid Res* 2000; **41**: 163–181.
4. Bylund J, Bylund M, Oliw EH: cDNA cloning and expression of CYP4F12, a novel human cytochrome P450. *Biochem Biophys Res Commun* 2001; **280**: 892–897.
5. Lasker JM, Chen WB, Wolf I, Bloswick BP, Wilson PD, Powell PK: Formation of 20-hydroxyeicosatetraenoic acid, a vasoactive and natriuretic eicosanoid, in human kidney. Role of Cyp4F2 and Cyp4A11. *J Biol Chem* 2000; **275**: 4118–4126.
6. Roman RJ: P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol Rev* 2002; **82**: 131–185.
7. Kalsotra A, Cui X, Anakk S, Hinojos CA, Doris PA, Strobel HW: Renal localization, expression, and developmental regulation of P450 4F cytochromes in three substrains of spontaneously hypertensive rats. *Biochem Biophys Res Commun* 2005; **338**: 423–431.
8. Gainer JV, Bellamine A, Dawson EP, *et al*: Functional variant of CYP4A11 20-hydroxyeicosatetraenoic acid synthase is associated with essential hypertension. *Circulation* 2005; **111**: 63–69.
9. Stec DE, Roman RJ, Flasch A, Rieder MJ: Functional polymorphism in human CYP4F2 decreases 20-HETE production. *Physiol Genomics* 2007; **30**: 74–81.
10. Fu Z, Nakayama T, Sato N, *et al*: A haplotype of the CYP4A11 gene associated with essential hypertension in Japanese men. *J Hypertens* 2008; **26**: 453–461.
11. Sato M, Nakayama T, Soma M, *et al*: Association between prostaglandin E2 receptor gene and essential hypertension. *Prostaglandins Leukot Essent Fatty Acids* 2007; **77**: 15–20.
12. Morita A, Nakayama T, Doba N, Hinohara S, Soma M: Polymorphism of the C-reactive protein (CRP) gene is related to serum CRP Level and arterial pulse wave velocity in healthy elderly Japanese. *Hypertens Res* 2006; **29**: 323–331.
13. Aoi N, Soma M, Nakayama T, *et al*: Variable number of tandem repeat of the 5′-flanking region of type-C human natriuretic peptide receptor gene influences blood pressure levels in obesity-associated hypertension. *Hypertens Res* 2004; **27**: 711–716.
14. Dempster AP, Laird NM, Rubin DB: Maximum likelihood from incomplete data *via* the EM algorithm. *J R Stat Soc Ser B* 1977; **39**: 1–22.
15. Kobayashi Y, Nakayama T, Sato N, Izumi Y, Kokubun S, Soma M: Haplotype-based case-control study revealing an association between the adrenomedullin gene and proteinuria in subjects with essential hypertension. *Hypertens Res* 2005; **28**: 229–236.
16. King LM, Gainer JV, David GL, *et al*: Single nucleotide polymorphisms in the CYP2J2 and CYP2C8 genes and the risk of hypertension. *Pharmacogenet Genomics* 2005; **15**: 7–13.
17. Yu BN, Luo CH, Wang D, *et al*: CYP2C9 allele variants in Chinese hypertension patients and healthy controls. *Clin Chim Acta* 2004; **348**: 57–61.
18. Kivistö KT, Niemi M, Schaeffeler E, *et al*: CYP3A5 genotype is associated with diagnosis of hypertension in elderly patients: data from the DEBATE Study. *Am J Pharmacogenomics* 2005; **5**: 191–195.
19. Iwai N, Katsuya T, Ishikawa K, *et al*: Human prostacyclin synthase gene and hypertension: the Suita Study. *Circulation* 1999; **100**: 2231–2236.
20. Sookoian S, Gianotti TF, Gonzalez CD, Pirola CJ: Association of the C−344T aldosterone synthase gene variant with essential hypertension: a meta-analysis. *J Hypertens* 2007; **25**: 5–13.
21. McGiff JC, Quilley J: 20-Hydroxyeicosatetraenoic acid and epoxyeicosatrienoic acids and blood pressure. *Curr Opin Nephrol Hypertens* 2001; **10**: 231–237.
22. Jin R, Koop DR, Raucy JL, Lasker JM: Role of human CYP4F2 in hepatic catabolism of the proinflammatory agent leukotriene B4. *Arch Biochem Biophys* 1998; **359**: 89–98.
23. Morris RW, Kaplan NL: On the advantage of haplotype analysis in the presence of multiple disease susceptibility alleles. *Genet Epidemiol* 2002; **23**: 221–233.
24. O'Donnell CJ, Lindpaintner K, Larson MG, *et al*: Evidence for association and genetic linkage of the angiotensin-converting enzyme locus with hypertension and blood pressure in men but not women in the Framingham Heart Study. *Circulation* 1998; **97**: 1766–1772.
25. Ono K, Mannami T, Iwai N: Association of a promoter variant of the haeme oxygenase-1 gene with hypertension in women. *J Hypertens* 2003; **21**: 1497–1503.
26. Waxman DJ, Ram PA, Pampori NA, Shapiro BH: Growth hormone regulation of male-specific rat liver P450s 2A2 and 3A2: induction by intermittent growth hormone pulses in male but not female rats rendered growth hormone deficient by neonatal monosodium glutamate. *Mol Pharmacol* 1995; **48**: 790–797.
27. Muller DN, Schmidt C, Barbosa-Sicard E, *et al*: Mouse Cyp4a isoforms: enzymatic properties, gender- and strain-specific expression, and role in renal 20-hydroxyeicosatetraenoic acid formation. *Biochem J* 2007; **403**: 109–118.
28. Holla VR, Adas F, Imig JD, *et al*: Alterations in the regulation of androgen-sensitive Cyp 4a monooxygenases cause hypertension. *Proc Natl Acad Sci U S A* 2001; **98**: 5211–5216.
29. Nakagawa K, Holla VR, Wei Y, *et al*: Salt-sensitive hypertension is associated with dysfunctional Cyp4a10 gene and kidney epithelial sodium channel. *J Clin Invest* 2006; **116**: 1696–1702.