The evidence of polymorphisms of the liver X receptor gene as a DNA-based biomarker for susceptibility to coronary artery disease in a Japanese population

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Coronary artery disease (CAD) is a multifactorial and polygenic disorder, which arises due to atherosclerosis of the coronary arteries. Both numerous genetic factors and environmental risk factors may contribute to the pathogenesis of atherosclerosis. Thus, in order to identify the genetic determinants of CAD, an association of genetic polymorphisms of the liver X receptor-alpha (*NR1H3*) and -beta (*NR1H2*) genes with susceptibility to CAD was examined in a Japanese population. Eight tag single nucleotide polymorphisms (SNPs) in *NR1H3* and *NR1H2* were analyzed by PCR-restriction fragment length polymorphism or PCR-direct DNA sequencing method in 143 Japanese patients with CAD and 164 healthy control subjects with normal coronary arteries. Subsequently, haplotypes composed of the two tag SNPs in *NR1H2* were constructed. Significant differences in the clinical risk factors, dyscholesteremia and diabetes mellitus, were observed between CAD patients and controls (P = 0.040 and P = 0.005, respectively). The frequencies of a C allele in the multiplicative model and its homozygous C/C genotype in the recessive model at rs2279238 in *NR1H3* were significantly higher in CAD patients as compared to those in controls (P = 0.039 and P = 0.016, respectively). Furthermore, the frequency of a Hap 4/any diplotype of *NR1H2* was significantly higher in CAD patients in comparison to controls (P = <0.0001, OR = 17.16). Multivariate logistic regression analysis revealed that these polymorphisms, dyscholesteremia, and diabetes mellitus independently contributed to susceptibility to CAD. In conclusion, *NR1H3* and *NR1H2* appears to be the genetic determinants of CAD. Furthermore, the genetic polymorphisms of *NR1H3* and *NR1H2* may be useful as new DNA-based diagnostic biomarkers for identifying high-risk individuals susceptible to CAD.

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Introduction

Coronary artery disease (CAD) is a multifactorial and polygenic disease and is one of the most frequent causes of the morbidity and mortality worldwide.[1] CAD arises due to atherosclerosis of the coronary arteries, which is characterized by progressive deposition of lipids and fibrous matrix in the arterial walls.[1][2] In addition to several environmental risk factors, such as aging, obesity, smoking, hypertension, dyscholesteremia, and diabetes mellitus, numerous genetic factors may contribute to the pathogenesis of CAD.[1-3] Genome-wide linkage-based family studies, candidate gene-based association studies, and large-scale genome-wide association studies using single nucleotide polymorphisms (SNPs) have identified several genetic factors that underlie the genetic determinants of the onset and/or development of CAD.[3-7] These include possible CAD-susceptibility genes such as CD14,[8][9] toll-like receptor 4,[9][10] lymphotoxin- ,[11] 5-lipoxygenase

activating protein,[12] and leukotriene A4 hydrolase,[13] as well as chromosomal loci on numerous chromosomes (1, 2, 3, 6, 9, 10, 15, 16, 17, and X)[5 6 14-19] for Caucasian populations. In particular, with regard to Japanese populations, platelet-activating factor acetylhydrolase,[20] NADH/NADPH oxidase p22 phox,[20] apolipoprotein C-III,[20 21] connexin 37,[21] fatty acid-binding protein 2,[21] tumor necrosis factor- ,[21] apolipoprotein E,[21] adiponectin,[22] *BRCA1*-associated protein,[23] and megakaryoblastic leukemia factor-1[24] confer susceptibility to CAD in the Japanese populations.

Both cholesterol transporters and the cholesterol metabolic pathways are subject to a highly complex regulatory system for whole-body cholesterol homeostasis.[25-27] Several transcription factors serve as key regulators of ATP-binding cassette (ABC) transporters and signaling molecules in the cholesterol metabolic pathways, which are involved in the pathogenesis of CAD. For example, the liver X receptors (LXRs), LXR-alpha (LXRA coded by *NR1H3*[28]) and LXR-beta (LXRB coded by *NR1H2*[29]), are members of the nuclear hormone receptor superfamily of transcription factors and respond to oxidized cholesterol derivatives (oxysterols) such as 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24(S), 25-epoxycholesterol.[30 31] Ligand-activated LXRs form obligate heterodimers with retinoid X receptors and transcriptionally regulate the expression of target genes containing the LXR response elements. LXR-mediated gene activation decreases whole-body cholesterol accumulation, eventually leading to protection against atherosclerosis and hypercholesterolemia.[25-27] Therefore, the dysregulation of the LXR signaling pathways may result in atherosclerosis and subsequent CAD.

In this study, a candidate gene-based association study was carried out by selecting LXRA (*NR1H3*) and LXRB (*NR1H2*) as candidate CAD-susceptibility genes. The purpose of this study was to investigate whether SNPs and their combination polymorphisms, which are referred to as haplotypes, in *NR1H3* and *NR1H2* are associated with susceptibility to the onset and/or development of CAD in a Japanese population, and whether such polymorphisms can be used as new genetic biomarkers for identifying high-risk individuals susceptible to CAD.

Methods

Subjects

All study subjects were Japanese who were unrelated to one another, and were enrolled from three general hospitals in Nagasaki, Japan, between October 2007 and October 2008.

The subjects included 143 patients with CAD and 164 age- and gender-matched healthy individuals as control subjects. The clinical characteristics, including the clinical risk factors for CAD, at the end point of this study are shown in Table 1. The study protocol was approved by the Committee for Ethical Issues dealing with the Human Genome and Gene Analysis at Nagasaki University, and written informed consent was obtained from each subject.

Table 1. Clinical characteristics of CAD patients and control subjects

Characteristics	CAD patients	Control subjects	P value*
Number	143	164	
Age, mean± SD (years)	67.9± 10.2	67.2± 9.9	0.458
Male/female (%)	98/45(68.5/31.5)	114/50(69.5/30.5)	0.853
BMI, mean± SD (kg/m^2)	23.9± 3.2	23.6± 3.2	0.688
Hypertension(present/absent)	101/42	113/51	0.743
dyscholesteremia(present/absent)	55/88	45/119	0.040
Smoking(present/absent)	53/90	46/118	0.092
Diabetes mellitus (present/absent)	36/107	21/143	0.005

*Characteristics were statistically compared by Mann-Whitney *U* test or chi-square test.
BMI was calculated on following formula: body weight (kg) / height X height (m).
Abbreviation: CAD, coronary artery disease; SD, standard deviation; BMI, body mass index.

The definite diagnosis of CAD was made on the basis of the presence of 75% stenosis in at least one of the three major coronary arteries revealed by coronary angiography (CAG). Control subjects were defined as having angiographically normal coronary arteries by CAG in spite of symptoms, e.g., chest oppressive feeling. Hypertension was defined as being under current treatment with antihypertensive agents, or as having systolic blood pressure of $\geq$ 140 mmHg and/or diastolic blood pressure of $\geq$ 90 mmHg. Dyscholesteremia was defined as being under current lipid-lowering therapy, or as having serum low-density lipoprotein cholesterol levels of $\geq$ 140 mg/dl or serum high density lipoprotein cholesterol levels of < 40 mg/dl. Current smokers were defined as smoking $\geq$ 10 cigarettes daily and regular smoking during the past 6 months. Diabetes mellitus was defined as currently undergoing treatment with insulin or oral antidiabetic agents, or as having the blood glucose levels of $\geq$ 126 mg/dl after an overnight fast.

Preparation of genomic DNA

Genomic DNA was extracted from whole blood samples using a QuickGene DNA Whole Blood Kit S (Fujifilm, Tokyo, Japan) with a QuickGene-800 (Fujifilm) according to the manufacturer's protocol.

Selection of tag SNPs in NR1H3 and NR1H2

All of SNPs in *NR1H3* (GenBank accession number: U22662; MIM 602423) located on chromosome 11p11.2[28] and *NR1H2* (GenBank accession number: U14534; MIM 600380) located on chromosome 19q13.3[29] were obtained using the Japanese data in Tokyo (Rel 24/phaseII Nov08, on NCBI B36 assembly, dbSNP b126) available on the International HapMap Web site.[32] Candidate tag SNPs were selected from all SNPs in the each chromosomal region including 2-kb upstream with priority in minor allele frequency of more than 10% in the International HapMap data. Subsequently, genotyped tag SNPs among the candidate tag SNPs were determined using the Haploview 4.1 software program.[33] The gene structure and positions of genotyped tag SNPs in *NR1H3* and *NR1H2* are shown in Figure 1.
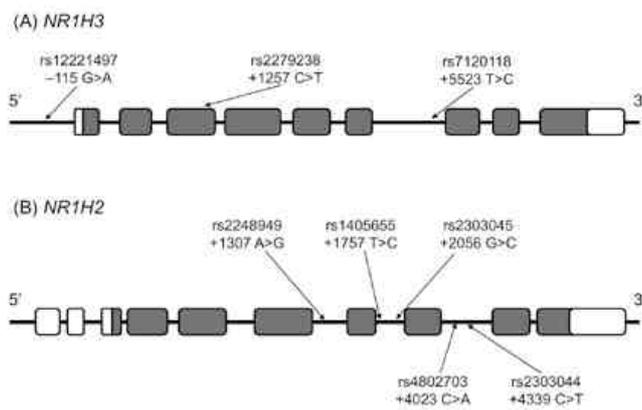

Fig. 1. Locations of genotyped tag SNPs in *NR1H3* and *NR1H2* The horizontal bars indicate the genomic sequences of *NR1H3* (A) and of *NR1H2* (B). Full boxes represent exons in each gene, and open boxes show the untranslated regions. The arrows indicate the positions of the genotyped tag SNP sites in this study and their names are presented above each site.

Genotyping of tag SNPs in NR1H3

Three SNPs, rs12221497 (the promoter region), rs2279238 (exon 3), and rs7120118 (intron 6) in *NR1H3* were selected as genotyped tag SNPs for this study (Figure 1). The two tag SNPs, rs12221497 and rs2279238, were analyzed by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP). The polymorphic region was amplified by PCR with a GeneAmp System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) using 25 ng genomic DNA in a 25-μ l reaction mixture containing 0.8X GoTaq Green master mix (Promega, Madison, WI, USA) and 15 pmol each of forward and reverse primers (Table 2). The amplification protocol consisted of initial denaturation at 95℃ for 2 minutes, followed by 35 cycles of denaturation at 95℃ for 30 seconds, annealing for 30 seconds at each temperature of selected primer pair (Table 2), and extension at 72℃ for 30 seconds, and final extension at 72℃ for 5 minutes. The PCR products were digested with each restriction enzyme (Table 2), separated by electrophoresis on a 6% polyacrylamide gel (Nacalai Tesque, Kyoto, Japan) for rs12221497 or a 2% ME-agarose gel (Nacalai Teque) for rs2279238, and subsequently visualized with an ultraviolet transilluminator (Alpha Innotech Co., San Leandro, CA, USA) after ethidium bromide staining.

The remaining rs7120118 SNP was analyzed by PCR-direct DNA sequencing method. The polymorphic region was amplified by PCR using 15 pmol each of forward and reverse primers (Table 2). The other constituents of the PCR mixture and the amplification protocol were the same as described above (Table 2). The PCR products were treated with ExoSAP-IT (Amersham Pharmacia Biotech, Plscataway, NJ, USA) and cycle sequenced using a BigDye Terminator v3.1 Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems). The cycle sequencing was hot-started at 96℃ for 30 seconds, followed by 25 cycles of denaturation

Table 2. The sequences of primer pairs and annealing temperature for PCR and restriciton enzyme for RFLP at each tag SNP

Gene	SNP	Analytic method	Sequence of forward primer (5' to 3')	Sequence of reverse primer (5' to 3')	Annealing temperature (℃)	restriction enzyme
LXRA	rs12221497	PCR-RFLP	GAGGATCACTTGAGCCCAGG	GCCACAAGGACATCTCTTCC	64	*Mnl* I
(*NR1H3*)	rs2279238	PCR-RFLP	CTTTCTGAGCCTCACTTTCC	CGCAGCTCAGAACATTGTAG	62	*Fnu*4H I
	rs7120118	Sequencing	TCCCCTCCTTCAGAATATCC	TTTTGAGCCCCAAAGTGTGG	60	
LXRB	rs2248949	PCR-RFLP	GCTAACAGCGGCTCAAGAAC	GTGGAAGTCGTCCTTGCTGT	63	*Bam* HI
(*NR1H2*)	rs1405655	PCR-RFLP	ATGAGCCAAGGAGAGAAAGG	TGTTCTCTCAGCCACACCAG	61	*Xsp* I
	rs2303045	PCR-RFLP	GCTAACAGCGGCTCAAGAAC	GTGGAAGTCGTCCTTGCTGT	63	*Msp*A1 I
	rs4802703	PCR-RFLP	CTGGGCCTCTTCCTAATGTG	AGAACTCGAAGATGGGGTTG	60	*Xsp* I
	rs2303044	PCR-RFLP	AGGGAGGGTGGTGATAGGAT	GAACTCGAAGATGGGGTTGA	61	*Hae* III

Abbreviation: PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

at 96℃ for 10 seconds, annealing at 50℃ for 5 seconds, and extension at 60℃ for 4 minutes using 1 pmol PCR forward or reverse primer. After the sequencing reaction solutions were purified using Sephadex G-50 superfine columns (Amersham Pharmacia Biotech), the samples were dried and sequenced with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Genotyping of tag SNPs in NR1H2

Five SNPs, rs2248949 (intron 6), rs1405655 (intron 7), rs2303045 (intron 7), rs4802703 (intron 8), and rs2303044 (intron 8) in *NR1H2* were selected as genotyped tag SNPs (Figure 1) and subsequently analyzed by PCR-RFLP using 15 pmol each of forward and reverse primers (Table 2). The other constituents of the PCR mixture and the amplification protocol were the same as described above. The PCR products were digested with each restriction enzyme (Table 2). The digests were then separated on a 2% agarose gel as described above.

Haplotype structures of NR1H2

The two tag SNPs in *NR1H2*, which showed a close association with susceptibility to CAD, were utilized to infer the haplotype structure and to analyze the haplotype frequency in an expectation-maximization algorithm using the SNP Alyze 7.0 standard software package (Dynacom Inc., Yokohama, Japan) in order to emphasize the variability and to enhance the power of detecting allelic association of rare variants.[34 35]

Statistical analysis

Differences in age and body mass index between CAD patients and control subjects were evaluated by Mann-Whitney *U* test using the SPSS 17 statistical software package (SPSS Japan Inc., Tokyo, Japan). Likewise, differences in gender and clinical risk factors between CAD patients and control subjects were compared by chi-square test using the Prism 5 statistical software package (GraphPad Software Inc., San Diego, CA, USA). In order to determine whether each SNP was in the Hardy-Weinberg equilibrium, a chi-square test with Yates' correction was performed using the SNP Alyze 7.0 standard software package. The frequencies of alleles and genotypes between CAD patients and control subjects were compared by chi-square test or logistic regression analysis in three different genetic models: multiplicative model, dominant model, and recessive model using the SNP Alyze 7.0

standard software package. The frequencies and distributions of haplotypes of *NR1H2* were compared by logistic regression analysis using SPSS 17. Diplotype comparison of *NR1H2* was analyzed by Fisher's exact test using Prism 5. Subsequently, a comparison of the genetic and clinical risk factors for susceptibility to CAD, which showed a statistically significant association with susceptibility to CAD by univariate analyses and were thereby selected as variables, between CAD patients and control subjects was carried out by multivariate logistic regression analysis using SPSS 17. The odds ratio (OR) with 95% confidence interval (CI) was calculated using SPSS 17. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Comparison of the clinical characteristics between CAD patients and control subjects

The clinical characteristics of CAD patients were compared with those of control subjects (Table 1). Significant differences in two clinical risk factors, dyscholesteremia and diabetes mellitus, were observed between CAD patients and control subjects ($P = 0.040$ and $P = 0.005$, respectively). However, there were no significant differences in the other characteristics, such as the mean age, gender, mean body mass index, hypertension, and smoking (Table 1).

Association between tag SNPs in NR1H3 and susceptibility to CAD

The frequencies and distributions of genotypes at three tag SNPs in *NR1H3* were identified (Table 3) and compared in three different genetic models (multiplicative, dominant, and recessive) between CAD patients and control subjects (Table 4). The distributions of these tag SNPs in *NR1H3* among CAD patients and control subjects corresponded well to the Hardy-Weinberg equilibrium, thus implying that the subject base has a homogeneous genetic background.

One tag SNP, rs2279238, was significantly associated with susceptibility to CAD in two genetic models. The frequencies of the C minor allele in the multiplicative model and its homozygous C/C genotype in the recessive model at rs2279238 in CAD patients were significantly higher as compared to those in control subjects ($P = 0.039$ and $P = 0.016$, respectively; Table 4). No significant differences were observed in frequencies of other alleles and genotypes between CAD patients and control subjects.

Association between tag SNPs in NR1H2 and susceptibility to CAD

The frequencies and distributions of genotypes at five tag SNPs in *NR1H2* were identified (Table 3) and compared in three genetic models between CAD patients and control subjects (Table 4). The distributions of these tag SNPs in *NR1H2* among CAD patients and control subjects corresponded well to the Hardy-Weinberg equilibrium.

One tag SNP, rs1405655, were significantly associated with the lack of susceptibility to CAD in two genetic models. Whereas, another tag SNP, rs2303044, showed susceptibility to CAD in two genetic models. The frequencies of the homozygous T/C genotype and minor homozygous C/C genotype in the dominant model at rs1405655 were significantly decreased in CAD patients as compared to those in control subjects ($P = 0.007$; Table 4). In contrast, the frequencies of the homozygous C/T genotype and minor homozygous T/T genotype in the dominant model at rs2303044 were significantly increased in CAD patients in comparison to those in control subjects ($P = 0.006$; Table 4).

Association between haplotypes and diplotypes of NR1H2 and susceptibility to CAD

Four haplotypes composed of the two tag SNPs (rs1405655 and rs2303044), which displayed a significant association with CAD susceptibility and whose linkage disequilibrium is with $r^2 = 0.499$, D' = 0.887 in CAD patients and with $r^2 = 0.558$, D' = 0.952 in control subjects, were hypothesized and identified using the SNP Alyze 7.0 standard software package (Table 5). Logistic regression analysis revealed that the frequency of a Hap 4 haplotype (T alleles at both rs1405655 and rs2303044) was significantly increased in CAD patients as compared to that in control subjects ($P = 0.00012$, OR = 16.992, 95% CI = 4.004 - 72.119). In contrast, the frequency of another Hap 3 haplotype (C alleles at both rs1405655 and rs2303044) was significantly decreased in CAD patients in comparison to that in control subjects ($P = 0.00021$, OR = 0.136, 95% CI = 0.047 - 0.390).

Furthermore, seven diplotypes composed of the four haplotypes were identified. Subsequently, CAD patients and

Table 3. Distrbutions of genotypes at tag SNPs in *NR1H3* and *NR1H2* between CAD patients and control subjects

Gene	SNP	Genotype	Number (%) of genotypes	
			CAD patients	Control subjects
LXRA (*NR1H3*)	rs12221497	G/G	112(78.3)	142(86.6)
		G/A	30(21.0)	22(13.4)
		A/A	1(0.7)	0
	rs2279238	T/T	52(36.3)	71(43.3)
		C/T	63(44.1)	76(46.3)
		C/C	28(19.6)	17(10.4)
	rs7120118	C/C	71(49.7)	90(54.9)
		T/C	56(39.2)	65(39.6)
		T/T	16(11.2)	9(5.5)
LXRB (*NR1H2*)	rs2248949	G/G	113(79.0)	132(80.5)
		A/G	28(19.6)	29(17.7)
		A/A	2(1.4)	3(1.8)
	rs1405655	T/T	90(62.9)	78(47.6)
		C/T	51(35.7)	78(47.6)
		C/C	2(1.4)	8(4.9)
	rs2303045	G/G	96(67.1)	111(67.7)
		G/C	44(30.8)	44(26.8)
		C/C	3(2.1)	9(6.5)
	rs4802703	C/C	106(74.1)	109(66.5)
		C/A	36(25.2)	50(30.5)
		A/A	1(0.7)	5(3.0)
	rs2303044	C/C	71(49.7)	108(65.8)
		C/T	66(46.1)	48(29.3)
		T/T	6(4.2)	8(4.9)
Total number of participants			143	164

Abbreviation: CAD, coronary artery disease.

Table 4. Allele and genotype comparison in three genetic models between CAD patients and control subjects

Gene	SNP	Multiplicative model		Dominant model		Recessive model	
		OR (95% CI)	*P* value*	OR (95% CI)	*P* value*	OR (95% CI)	*P* value*
LXRA (*NR1H3*)	rs12221497	1.751(0.993-3.096)	0.051	1.786(0.980-3.257)	0.058		
	rs2279238	1.412(1.017-1.961)	0.039	1.337(0.843-2.114)	0.217	2.252(1.163-4.361)	0.016
	rs7120118	1.312(0.921-1.869)	0.132	1.233(0.787-1.934)	0.360	2.170(0.928-5.075)	0.074
LXRB (*NR1H2*)	rs2248949	1.055(0.635-1.751)	0.837	1.095(0.627-1.912)	0.750	0.761(0.125-4.621)	0.767
	rs1405655	0.593(0.406-0.866)	0.007	0.534(0.338-0.844)	0.007	0.277(0.058-1.324)	0.108
	rs2303045	0.909(0.602-1.372)	0.650	1.026(0.635-1.656)	0.918	0.369(0.098-1.390)	0.141
	rs4802703	0.684(0.440-1.064)	0.091	0.692(0.422-1.135)	0.145	0.224(0.026-1.940)	0.174
	rs2303044	1.565(1.582-2.283)	0.020	1.905(1.203-3.012)	0.006	0.854(0.289-2.523)	0.775

*Alleles and genotypes in three genetic models were compared by chi-square test or logistic regression analysis.
Abbreviation: CAD, coronary artery disease; OR, odds ratio; CI, confidence interval.

Table 5. Distributions of haplotypes of *NR1H2* between CAD patients and control subjects

Gene	Haplotype	SNP		Number (%) of haplotypes		Haplotype comparison*	
		rs1405655	rs2303044	CAD patients	Control subjects	OR (95% CI)	*P* value
LXRB	Hap 1	T	C	204(71.3)	232(70.7)	1.029(0.726-1.460)	0.871
(*NR1H2*)	Hap 2	C	T	51(17.8)	63(19.2)	0.913(0.607-1.374)	0.662
	Hap 3	C	C	4(1.4)	31(9.5)	0.136(0.047-0.390)	0.00021
	Hap 4	T	T	27(9.4)	2(0.6)	16.992(4.004-72.119)	0.00012
Total number of haplotypes				286	328		

*Each haplotype was compared with other haplotypes combined by logistic regression analysis.
Abbreviation: CAD, coronary artery disease; OR, odds ratio; CI, confidence interval.

Table 6. Diplotype comparison of susceptibility to CAD between CAD patients and control subjects

Gene	Diplotype	Number (%) of diplotypes		Diplotype comparison*	
		CAD patients	Control subjects	OR (95% CI)	*P* value
LXRB	Hap 4/any	25(17.5)	2(1.2)		
(*NR1H2*)				17.16(3.985-73.90)	< 0.0001
	Other diplotypes	118(82.5)	162(98.8)		
Total number of participants		143	164		

*Diplotype comparison was statistically analyzed by Fisher's exact test.
Abbreviation: CAD, coronary artery disease; OR, odds ratio; CI, confidence interval.

control subjects were divided into two subgroups according to the possession of a Hap 4 risk haplotype as an individual (Table 6). Fisher's exact test revealed that the possession of the Hap 4/any diplotype as an individual increased susceptibility to CAD in Japanese individuals ($P < 0.0001$, OR = 17.16, 95% CI = 3.985 - 73.90).

Gene-gene and gene-environment interactions among genetic and clinical risk factors for susceptibility to CAD

The two clinical risk factors, dyscholesteremia and diabetes mellitus, were selected as environmental variables by univariate analysis using chi-square test (Table 1). The two genetic factors, the C/C genotype at rs2279238 in *NR1H3* and the Hap 4/any diplotype of *NR1H2*, were selected as genetic variables by chi-square test or Fisher's exact test (Tables 4 and 6). Thus, the gene-gene and gene-environment interactions were analyzed by multivariate logistic regression analysis between CAD patients and control subjects (Table 7). Multivariate analysis indicated that four variable risk factors, dyscholesteremia, diabetes mellitus, the C/C genotype at rs2279238 in *NR1H3*, and the Hap 4/any diplotype of *NR1H2*, independently contributed to susceptibility to CAD ($P = 0.036$, $P = 0.008$, $P = 0.022$, and $P < 0.0001$, respectively; Table 7).

Table 7. The gene-gene and gene-environment interactions among *NR1H3* genotype, *NR1H2* diplotype, dyscholesteremia, and diabetes mellitus for susceptibility to CAD

Factor	Factor comparison*	
	OR (95% CI)	*P* value
dyscholesteremia	1.719(1.036-2.852)	0.036
Diabetes mellitus	2.316(1.248-4.299)	0.008
C/C genotype at rs2279238 in *NR1H3*	2.231(1.121-4.440)	0.022
Hap 4/any diplotype of *NR1H2*	19.267(4.258-87.176)	<0.0001

*Factors were statistically analyzed by multivariate logistic regression analysis.
Abbreviation: CAD, coronary artery disease; OR, odds ratio; CI, confidence interval.

Discussion

This study is the first to demonstrate that an association between *NR1H3* and *NR1H2* polymorphisms and susceptibility to CAD in the Japanese population. The possession of the C/C genotype at rs2279238 in *NR1H3* or the Hap 4/any diplotype of *NR1H2* conferred increased susceptibility to CAD. Furthermore, the genetic polymorphisms, the C/C genotype at rs2279238 in *NR1H3* and the Hap 4/any diplotype of *NR1H2*, as well as co-morbidities of dyscholesteremia and/or diabetes mellitus, independently contributed to the onset and/or development of CAD. In particular, the possession of the Hap 4/any diplotype of *NR1H2* indicated an

approximately 19-fold increase in the odds ratio for CAD in this study (Table 7), although such CAD patients account for only approximately 17.5% (25/143 = 17.5% in Table 6) of the genetic variance observed in CAD. These findings suggest that *NR1H3* and *NR1H2* are genetic determinants of predisposition to the onset and/or development of CAD in Japanese individuals. However, it remains to be confirmed whether this association is reproducible in a large number of Japanese subjects as well as other ethnic populations because this study population was very small. Additional studies are needed because different populations will often have different allele frequencies and haplotype structures.

Ligand-activated LXRs (LXRA and LXRB) regulate a set of target genes related to a decrease in whole-body cholesterol accumulation by inhibiting intestinal cholesterol absorption through ABC transporters, ABCG5 and ABCG8, and by inhibiting cholesterol transfer from peripheral tissues.[36-38] In addition, the activation of LXR-medicated signals leads to an increase in cholesterol efflux from macrophages through ABCA1 and ABCG1,[39][40] uptake from serum into the liver by apolipoprotein E,[41] catabolism from cholesterol into bile acids by cholesterol 7-alpha-hydroxylase in the hepatocytes,[42] and biliary secretion of cholesterol into the bile ducts through ABCG5 and ABCG8 in the hepatocytes.[36-38] Taken together, the LXR signaling pathways are endogenous inhibitors of atherosclerosis. Therefore, it seems likely to speculate that the polymorphisms of *NR1H3* and *NR1H2*, especially the C/C genotype at rs2279238 in *NR1H3* and the Hap 4/any diplotype of *NR1H2*, may diminish the function of LXRs by reducing the expression of these target genes, decreasing their affinity for the DNA binding site, and/or altering their interactions with other nuclear receptors such as retinoid X receptor.[26][27][43] This process may alter the efficiency of the LXR-medicated signals, thus indicating that a decrease in anti-atherogenic factors can lead to the progression to coronary atherosclerosis, although the potential mechanisms of action of the *NR1H3* and *NR1H2* polymorphisms identified in this study remain unknown.

In particular, atherosclerosis of the coronary arteries is focused on macrophage. Atherosclerosis is attributed to progressive plaque formation, finally resulting in the thickening and hardening of the coronary arteries.[1][2] Blood monocytes migrate into the subendothelial layer of the intima, where they differentiate into macrophages. Most macrophages phagocytose and accumulate oxidized low-density lipoprotein in their cytoplasm, thereby leading to foam cell formation. Foam cells not only mediate the release of reactive oxygen species and inflammatory cytokines,[44] but also induce the proliferation of vascular smooth muscle cells,

which play an important role in growth of atherosclerotic plaques.[45] Subsequently, foam cells undergo apoptosis in the intima.[46] During the process of atherosclerosis, LXRs suppress blood monocyte transmigration and the release of reactive oxygen species and inflammatory cytokinesis in the intima by inhibiting the NF- B-induced signalings.[47][48] In addition to LXRs inhibit apoptosis of foam cells,[49][50] synthetic LXR agonists, GW3965 and T0901317, suppress the proliferation of vascular smooth muscle cells by inhibiting cell cycle progression from G_1 to S phase,[45] thereby resulting in a reduction of atherosclerosis in mouse models.[51] That is why the polymorphisms of *NR1H3* and *NR1H2* may diminish anti-atherosclerotic actions of LXRs in macrophages/foam cells in the intima, leading to the thickening and hardening of coronary arteries and eventually developing the predisposition to CAD.

Conclusions

NR1H3 and *NR1H2* appear to be genetic determinants for susceptibility to CAD in the Japanese population. These genetic polymorphisms of *NR1H3* and *NR1H2* may therefore be useful as new DNA-based diagnostic biomarkers for identifying high-risk individuals susceptible to CAD. Finally, LXRs, especially their agonists, may be good target molecules for the development of novel drugs in the future.

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