Dahl salt-sensitive (DS) hypertensive rats, which may contribute to hypertension.[4,5]

Tissue angiotensinogen is known as an important effector system for the regulation of BP. The overexpression of the angiotensinogen gene in the heart increases BP and cardiac hypertrophy[6] and young spontaneously hypertensive rats show an elevation of tissue angiotensinogen expression. Angiotensin-converting enzyme (ACE) 2, through the generation of the vasodilator Ang-(1-7) and by hydrolyzing part of Ang II, counterbalances the vasopressor effect of ACE that is mediated by Ang II. Genetic inactivation of ACE2 in mice resulted in severe cardiac dysfunction and mild elevation of BP.[7] We hypothesized that the beneficial effects of an MR antagonist or a type 1 Ang II receptor (AT1R) blocker in the treatment of salt-sensitive hypertension could be mediated by reduced activation of the local RAAS and changes in the balance between the opposing activities of ACE and ACE2.

Methods

Animal Experiments

All experiments were performed according to the guidelines for the use of experimental animals of the Animal Research Committee of Kanazawa University. Male Dahl salt-sensitive (DS) rats and Dahl salt-resistant (DR) rats (Seac Yoshitomi, Yoshitomi cho, Japan), 4 to 5 weeks old, were initially fed a standard chow purchased from Nippon Charles River (Kanagawa, Japan). Both DS and DR rats were fed low sodium chow (0.45%) or high sodium chow (7%) for 8 weeks (n = 20 in each group) with or without the addition of eplerenone (100 mg/kg/d; Pharmacia/Pfizer Groton, CT). Eplerenone was synthesized at Pharmacia/Pfizer and incorporated into the Teklad 22/5 rodent diet at a concentration of 1.0 mg/g of chow as previously reported.[3] The DS rats were treated orally for 8 weeks with candesartan cilexetil alone (10 mg/kg/d; Takeda Chemical Industries, Osaka, Japan) or with the combination of eplerenone and candesartan cilexetil. Candesartan was given orally by a gastric tube. All rats were housed in metabolic cages and daily urinary excretions were collected.

Blood pressure was determined by the tail–cuff method using photoelectric volume oscillometry (BP-98A, Softron, Tokyo, Japan). In several rats, intra-arterial BP was measured as previously reported.[8] The BP data measured by the tail–cuff method agreed with the data obtained from direct intra-arterial measurements. Blood was collected from the tail vein as previously reported.[8] Plasma aldosterone concentrations (PAC) were estimated with radioimmunoassay (RIA) after extraction with a Sep-Pak C18 cartridge (Waters associates, Milfold, MA) as previously reported.[8] Plasma renin activity (PRA) was measured using a commercial RIA kit.

Perfusion Experiments

Eight rats from each group were used for experiments involving mesenteric arterial perfusion.[9] After the rat was anesthetized with pentobarbital, the superior mesenteric artery was immediately cannulated and perfused with Krebs–Ringer solution (pH 7.4) at a temperature of 37°C and oxygenated with a 95% O_2–5% CO_2 gas mixture at a constant flow rate of 3 mL/min. All connections of the mesenteric vascular bed to the small intestine were carefully dissected as previously reported.[9] Noradrenalin was added to a scaled reservoir that was continuously gassed with 95% O_2–5% CO_2 and kept at 37°C, and from which Krebs solution was continuously perfused to the mesenteric vascular bed. Acetylcholine was injected in a volume of 10 μL into the perfusate in the silicone rubber close to the vascular bed. The perfusion pressure was constantly monitored and recorded by means of a pressure transducer connected to a polygraph (RM 600; Nihon-Koden, Tokyo, Japan).

Quantification of mRNA of Type III Collagen, Angiotensinogen, ACE, and ACE2 in the Heart

Before the animals were killed, they were anesthetized intraperitoneally with pentobarbital (100 mg/kg), intubated, and mechanically ventilated. The chest was opened by a median sternotomy, and the heart, aorta, and mesenteric artery were removed. The right and left ventricles (plus the interventricular septum) were weighed. Total RNA was extracted from the heart by using TRIzol (Invitrogen Japan, Tokyo, Japan) according to the manufacturer's protocol. Real-time quantitative reverse transcription–polymerase chain reaction was done using the TaqMan One-Step RT-PCR Master Mix Reagent Kit with an ABI Prism 7000 HT Detection System (Applied Biosystems Japan, Tokyo, Japan) according to the manufacturer's protocol. The sequences of sense and antisense primers and probes for angiotensinogen, ACE, and ACE2 were designed as previously reported.[10,11] To obtain a calibration curve, serial dilutions of stock standard RNA were used. The relative amount of each mRNA was normalized to the housekeeping gene, 18 ribosome mRNA. Northern blot analysis of mRNA of type III collagen was done as previously reported.[12]

Western Blot Analysis of Cardiac ACE and ACE2

Membrane proteins from hearts were isolated and subjected to Western blot analysis as previously reported by Crackower et al.[7] For detection of ACE or ACE2, nitrocellulose membranes were incubated with mouse ACE monoclonal antibody (Chemicon, Temecula, CA) or an affinity-purified rabbit ACE2 antibody.[7] Signals on Western blots were quantified by densitometry and corrected for β-actin.

Data are expressed as the mean ± SEM. Data were compared by a two-way ANOVA or Friedman's test and Fisher's protected least significance or Scheffe's F test was

Table 1. Body weight, the ratio of heart weight to body weight, plasma renin activity, plasma aldosterone concentration, and relative amount of mRNA levels of type III collagen in each experimental group

Parameter	BW (g)	HW/BW ($\times 10^{-3}$)	PRA (ng/mL/h)	PAC (pg/mL)	Collagen III/18S mRNA
DR rats					
LS (n = 20)	455 ± 9	2.9 ± 0.07	3.1 ± 0.52	197 ± 9.8	1.1 ± 0.07
HS (n = 20)	442 ± 10	3.0 ± 0.06	0.52 ± 0.23†	73 ± 6.6†	1.2 ± 0.05
DS rats					
LS (n = 20)	449 ± 3	3.1 ± 0.03	3.3 ± 0.99	175 ± 7.8	1.3 ± 0.03
HS (n = 20)	431 ± 8	4.1 ± 0.04†	0.48 ± 0.12†	68 ± 5.1†	3.2 ± 0.12†
MRB (n = 20)	405 ± 6	3.5 ± 0.1*	1.4 ± 0.34*	59 ± 5.8	1.9 ± 0.03*
ARB (n = 20)	405 ± 6	3.8 ± 0.1*	2.1 ± 0.67*	48 ± 6.0	2.1 ± 0.06*
MRB + ARB (n = 20)	394 ± 3	3.4 ± 0.09*	0.73 ± 0.42*	51 ± 5.3	1.5 ± 0.03*

LS = low sodium diet; HS = high sodium diet; MRB = DS rats treated with eplerenone in the presence of a high sodium diet; ARB = DS rats treated with candesartan in the presence of a high sodium diet; MRB + ARB = DS rats treated with eplerenone plus candesartan in the presence of a high sodium diet; BW = body weight; HW/BW = the ratio of heart weight to body weight; PRA = plasma renin activity; PAC = plasma aldosterone concentration.

* $P < .05$ v HS; † $P < .05$ v LS.

performed when each ANOVA indicated significance. Statistical significance was accepted for $P < .05$.

Results

Table 1 summarizes the data on body weight, PRA, and PAC, and parameters of cardiac hypertrophy and fibrosis. After 4 or 8 weeks of salt-loading, BP increased significantly in DS rats (183 ± 4.3 mm Hg , 240 ± 5.4 mm Hg, respectively) (Fig. 1). High sodium diet did not increase BP in DR rats. Treatment with eplerenone or candesartan for 4 or 8 weeks blunted the increase in BP (eplerenone, 146 ± 3.9 mm Hg; candesartan, 152 ± 9.1 mm Hg at 4 weeks; eplerenone, 164 ± 4.0 mm Hg; candesartan, 178 ± 8.6 mm Hg at 8 weeks). The DS rats treated with eplerenone and candesartan for 4 or 8 weeks did not show any differences in BP compared with DS rats fed a low sodium diet or DR rats fed a high sodium diet. A high sodium diet significantly decreased PRA and PAC in DS and DR rats. Treatment with eplerenone slightly increased PRA but did not influence PAC. A high sodium diet increased the heart-to-body weight ratio and type III collagen mRNA levels in the heart ($P < .05$). Treatment with eplerenone or candesartan significantly decreased both the heart-to-body weight ratio and type III collagen mRNA levels in the heart ($P < .05$). Treatment with eplerenone and candesartan normalized the heart-to-body weight ratio and cardiac type III collagen mRNA levels in DS rats given a high sodium diet.

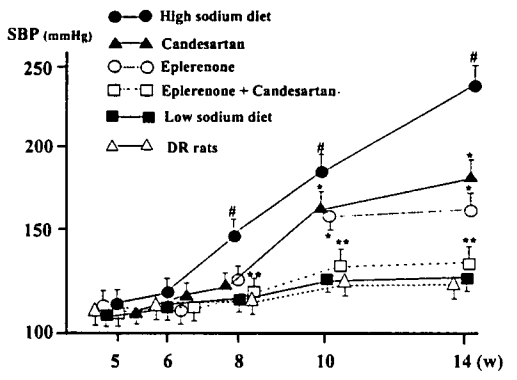

FIG. 1. Six-week-old rats were placed on high sodium diet. Systolic blood pressure (SBP) of 20 Dahl salt-sensitive hypertensive (DS) rats fed a high sodium diet (closed circle), 20 high sodium DS rats treated with candesartan cilexetil (10 mg/kg/d) (closed triangle), 20 high sodium DS rats treated with eplerenone (100 mg/kg/d) (open circle), 20 high sodium DS rats treated with candesartan plus eplerenone (open square), 20 rats fed a low sodium diet (closed square), and 6 Dahl salt-resistant (DR) rats fed a high sodium diet (open triangle). *$P < .05$ v DS rats fed a high sodium diet ; **$P < .01$ v DS rats fed a high sodium diet; #$P < .01$ v DS rats fed a low sodium diet or DR rats fed a high sodium diet.

Acetylcholine-induced relaxation was blunted in DS rats given a high sodium diet compared with those given a low sodium diet (Fig. 2). Treatment with eplerenone or candesartan for 8 weeks improved EDR and no significant difference was seen between the two groups. The combination of candesartan and eplerenone normalized EDR.

Cardiac angiotensinogen mRNA levels were elevated in DS rats given a high sodium diet compared with DS rats given a low sodium diet or DR rats (Fig. 3). Treatment with eplerenone or candesartan decreased angiotensinogen mRNA expression in the heart of DS rats. Cardiac angiotensinogen mRNA levels in DR rats on a high sodium diet were not influenced by eplerenone or candesartan (data not shown). The high sodium diet did not affect expression of ACE mRNA in the heart of DS or DR rats. However, treatment with eplerenone or candesartan significantly decreased cardiac ACE mRNA levels in DS rats ($P < .05$) (Fig. 3). The ACE mRNA/18 ribosome mRNA expression ratio (2.5 ± 0.6) in the heart of DR rats was decreased by the treatment with eplerenone (1.6 ± 0.2) or candesartan (1.5 ± 0.1). Although cardiac ACE2 mRNA levels were significantly reduced in DS rats fed a high sodium diet, the mRNA levels in the heart of DR rats were not decreased

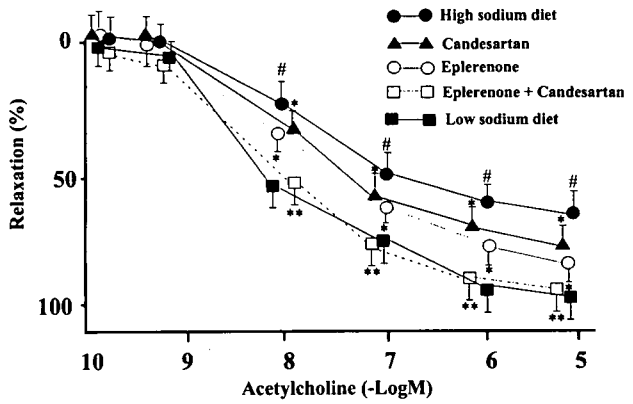

FIG. 2. Endotheliun-dependent relaxation to acetylcholine in the mesenteric arteries of eplerenone-, candesartan-, and eplerenone plus candesartan-treated Dahl salt-sensitive hypertensive (DS) rats. High sodium diet significantly attenuated vascular relaxation to acetylcholine. Eplerenone or candesartan in the presence of a high sodium diet improved vascular relaxation to acetylcholine. Combination therapy with eplerenone and candesartan normalized vascular relaxation to acetylcholine. *$P < .05$ *v* DS rats fed a high sodium diet ; **$P < .01$ *v* DS rats fed a high sodium diet; #$P < .01$ *v* DS rats fed a low sodium diet.

(high salt, 2.8 ± 0.6; low salt, 2.5 ± 0.3). Candesartan but not eplerenone increased the expression of ACE2 mRNA in DS rats (Fig. 3). Both drugs did not influence ACE2 mRNA expression in DR rats (eplerenone, 2.4 ± 0.4; candesartan, 2.7 ± 0.6). The relative protein expression levels of ACE or ACE2 paralleled the relative mRNA levels of each gene. The high sodium diet did not influence

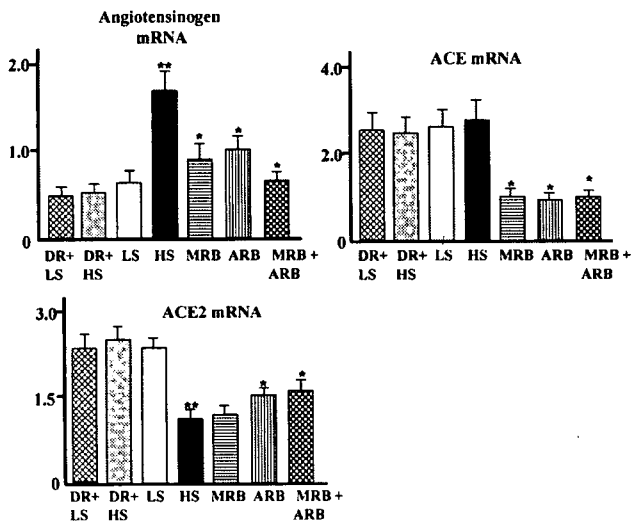

FIG. 3. Concentrations of mRNA of angiotensinogen, angiotensin-converting enzyme (ACE), and angiotensin-converting enzyme 2 (ACE2) in the hearts of Dahl salt-resistant (DR) rats fed a high sodium diet (n = 6), Dahl salt-sensitive hypertensive (DS) rats fed a low sodium diet (LS) (n = 6), DS rats fed a high sodium diet (n = 6), DS rats treated with eplerenone in the presence of a high sodium diet (MRB) (n = 6), DS rats treated with candesartan in the presence of a high sodium diet (ARB) (n = 6), and DS rats treated with eplerenone plus candesartan in the presence of a high sodium diet (MRB + ARB) (n = 6). *$P < .05$ *v* DS fed a high sodium diet (HS); **$P < .01$ *v* DS rats fed a low sodium diet (LS) or DR.

ACE protein expression in DS rats (0.38 ± 0.02) and in DR rats (0.32 ± 0.07) (Fig. 4). Treatment with eplerenone (0.14 ± 0.01) or candesartan (0.17 ± 0.04) decreased ACE protein levels in DS rats fed a high sodium diet. The decreased ACE2 protein levels (0.052 ± 0.003) was seen in DS rats fed a high sodium diet compared with DS rats fed a low sodium diet (0.14 ± 0.04) or DR rats (0.21 ± 0.08). Eplerenone or candesartan did not influence the cardiac ACE2 protein expression in DR rats (data not shown). Although candesartan increased cardiac ACE2 protein expression (0.070 ± 0.01), eplerenone did not influence protein levels in DS rats fed a high-sodium diet (0.047 ± 0.002 (Fig. 4).

Discussion

In the present study, treatment with eplerenone or candesartan partially decreased BP and combination therapy with the two drugs normalized BP and improved cardiac hypertrophy and fibrosis in DS rats and restored endothelium-dependent relaxation. Nagase et al[13] reported that eplerenone lowered BP by in DS rats. However, Nakata et al[14] found that eplerenone did not decrease BP in the same animal model. We and Nagase et al[13] started eplerenone at the age of 5 weeks (prehypertensive stage) but Nakata et al[14] treated rats with eplerenone at the age of 12 weeks (hypertensive stage). The antihypertensive effect of eplerenone in several kinds of hypertensive rat models was summarized in Table 2. Both eplerenone and AT1R blocker were reported to be efficacious in reducing BP in hypertensive blacks and whites.[15] Pitt et al[16] reported that the combination of eplerenone and an ACE inhibitor was more effective in reducing left ventricular mass and systolic BP than eplerenone alone in essential hypertensive patients. Griffin et al[17] reported that the renal protection by ACE inhibition or aldosterone blockade in spontaneously hypertensive rats was BP dependent. However, several investigators have suggested that the pathogenesis of cardiovascular and renal damage is, at least in part, independent of BP and

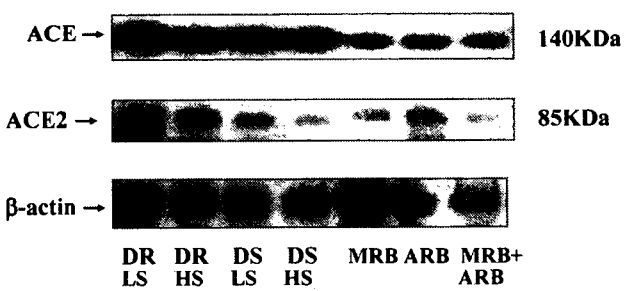

FIG. 4. The protein expression of angiotensin-converting enzyme (ACE), angiotensin-converting enzyme 2 (ACE2), and β-actin in heart in each experimental group. DR = Dahl salt-resistant rat; DS = Dahl salt-sensitive hypertensive rat; LS = rats fed a low sodium diet; HS = rats fed a high sodium diet; MRB = DS rats treated with eplerenone in the presence of a high sodium diet; ARB = DS rats treated with candesartan in the presence of a high sodium diet; MRB + ARB = DS rats treated with eplerenone plus candesartan in the presence of a high sodium diet.

Table 2. Effect of eplerenone on blood pressure in several models of hypertensive rats

Rats	Age (week-old)	Dose (mg/kg)	Duration of the treatment	Result	Reference
DS	6	125	5 weeks	Decrease	(13)
DS	12	100	7 weeks	No change	(14)
DS	11	100	7 weeks	No change	(27)
SHRSP	9	100	5 weeks	Decrease	(3)
Licorice-treated	13	182	2 weeks	Decrease	(28)
Aldosterone-treated	4	100	4 weeks	Decrease	(29)

DS = Dahl salt-sensitive rat; SHRSP = stroke-prone spontaneously hypertensive rat.

mediated by the direct tissue damage-promoting effects of the RAAS.[18,19]

A high sodium intake markedly suppressed circulating RAAS in DS rats and treatment with eplerenone did not increase PAC. Treatment with eplerenone in patients with hypertension increases both PRA and PAC. We found that in DS rats fed a low sodium diet, eplerenone increased PRA and PAC (data not shown). A high sodium diet may be more effective in decreasing activation of the circulating RAAS compared with the diuretic effect of eplerenone, which activates the RAAS.

Angiotensinogen is synthesized locally in the heart, and its expression is augmented in pressure overload-induced hypertrophy. Local overexpression of angiotensinogen in the heart of mice induces hypertrophy without hypertension.[6] Kobori et al[5] reported that a high sodium diet decreased circulating RAS in both DS rats and DR rats. However, intrarenal angiotensinogen was enhanced in DS rats but not in DR rats, and these investigators suggested that paradoxical responses of tissue RAS by a high sodium diet contribute to the development of hypertension. Salt loading reduces tissue ACE2 expression in salt-sensitive hypertensive rats and has no effect on its expression in control salt-resistant normotensive rats. In this study, a high sodium diet increased angiotensinogen mRNA and decreased ACE2 mRNA in the hearts of DS rats. Treatment with eplerenone decreased both angiotensinogen and ACE mRNA levels, but did not affect ACE2 mRNA expression. These data suggest a protective role for eplerenone by possibly decreasing formation of Ang II in the heart. Harada et al[20] reported that aldosterone induced ACE gene expression in cultured neonatal rat cardiocytes. Sun et al[21] also reported that aldosterone/NaCl-treated rat hearts showed high density ACE binding and histochemical evidence of fibrillar collagen accumulation at sites of microscopic scarring and perivascular fibrosis in intramyocardial coronary arteries. However, there are several reports that high salt intake did not increase cardiac or renal ACE activity.[4,22] In this study, eplerenone decreased cardiac ACE mRNA levels, not only in DS rats but also in DR rats fed a high salt diet. The pathophysiologic role of tissue ACE should be further studied.

Our data also showed that treatment with candesartan increased ACE2 mRNA and decreased angiotensinogen mRNA in the heart. Several lines of experimental evidence suggest a beneficial role for ACE2 in cardiovascular function. Yamamoto et al[23] reported that deletion of ACE2 accelerates pressure overload-induced cardiac dysfunction by increasing local Ang II. In ACE2−/y mice, administration of candesartan improved cardiac hypertrophy. Cardiac ACE2 mRNA expression was increased after administration of the AT1R blocker losartan, and the effect was independent of BP.[24] Ishiyama et al[25] reported that candesartan upregulated ACE2 mRNA after myocardial infarction. Huentelman et al[26] reported that overexpression of the mouse ACE2 gene in the hearts of Sprague-Dawley rats protected against Ang II-induced cardiac hypertrophy and fibrosis. Thus, ACE2 may be responsible for the beneficial actions of treatment with candesartan on cardiac hypertrophy and fibrosis in DS rats.

In conclusion, the blockade of aldosterone or angiotensin II improved vascular endothelial function and decreased cardiac mRNA expression of angiotensinogen and ACE and increased ACE2 mRNA. Dual blockade of aldosterone and Ang II further improved endothelial function and inactivated the cardiac RAAS. These changes were concomitant with improvement of cardiac hypertrophy and fibrosis in salt-sensitive hypertensive rats. These results suggest that the local RAAS is an important contributing factor to the progression of hypertension, cardiac hypertrophy, and fibrosis in salt-sensitive hypertension.[27–29]

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Original Article

Association of Genetic Variation of the Adiponectin gene with Body Fat Distribution and Carotid Atherosclerosis in Japanese Obese Subjects

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Aim: The aim of this study was to investigate the effect of SNP45 of the adiponectin gene on body fat distribution and carotid atherosclerosis in Japanese obese subjects.
Methods: A total of 64 obese subjects were investigated. Genotypes of SNP45 were assayed by polymerase chain reaction-restriction fragment length polymorphism. Visceral fat area (VFA) and subcutaneous fat area (SFA) were measured using computed tomography. The progression of atherosclerosis was evaluated by plaque score (PS) of carotid artery using B-mode ultrasonography.
Results: Men carrying the G allele of SNP45 showed higher VFA (172.8 ± 50.8 vs. 147.1 ± 58.7, $p = 0.005$), lower SFA (209.9 ± 101.8 vs. 273.4 ± 142.2, $p = 0.007$), higher VFA/SFA (V/S) ratio (1.00 ± 0.46 vs. 0.60 ± 0.26, $p < 0.001$) and higher PS (9.5 ± 3.7 vs. 6.8 ± 4.2, $p = 0.012$) than those with TT genotype. Multivariate analysis showed that SNP45 was an independent determinant of V/S ratio and PS in men. In subgroup analysis, PS tended to be associated with V/S ratio only in the carrier of 45G allele.
Conclusion: These results suggest that the G allele could be a risk factor of metabolic syndrome and the development of atherosclerosis in Japanese obese subjects.

J Atheroscler Thromb, 2007; 14:19-26.

Key words; Visceral obesity, Plaque score, Polymorphism, PCR-RFLP

Introduction

Adiponectin is a 244-amino acid protein synthesized and secreted exclusively by adipose tissue[1, 2] and plays an important role in the regulation of energy homeostasis and insulin sensitivity[3-5]. Adiponectin also has anti-atherogenic effects. This protein has been shown to suppress the expression of class A scavenger receptors in macrophages, affect the nuclear factor (NF)-κB pathway and inhibit monocyte adhesion to aortic endothelial cells[6-8].

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Genetic variations in the human adiponectin gene, especially two single nucleotide polymorphisms (SNPs) (+45T>G and +276G>T), have been reported to be associated with obesity, insulin resistance[9], type 2 diabetes[10, 11], and coronary artery disease[12]. Hara *et al.* reported that these two SNPs were associated with insulin resistance, indicating the pathogenesis of type 2 diabetes[11]. The mechanism underlying insulin resistance in type 2 diabetes is not fully understood, but many studies in nondiabetic populations have addressed the importance of upper body fat distribution. However, the association between these SNPs and body fat distribution has not been investigated. Based on these previous findings, it has recently been reported that the G allele of SNP45 was associated with susceptibility to coronary artery disease independent of conventional risk factors[12]. Although, the mechanism is not clear, we hypothesized that SNP45 could modify body

fat distribution and lead to more accumulation of visceral adipose tissue, resulting in metabolic abnormalities and the development of atherosclerosis in the process of increasing adipose tissue. To determine the validity of this hypothesis, we investigated the association of SNP45 with (1) various clinical and metabolic parameters, (2) body fat distribution, and (3) the progression of atherosclerosis using the plaque score of the carotid artery and maximum IMT in a group of Japanese obese patients.

Material and Methods

Subjects

Sixty-four Japanese obese subjects (40 men and 24 women, aged 54.2 ± 16.6 years, BMI 30.3 ± 5.3 kg/m^2), receiving medical checkups in our institute from 2002 to 2004, were recruited for this study. These included 49 patients with type 2 diabetes, among whom 24 were treated with oral hypoglycemic agents, 13 with insulin, and 12 with diet alone. Subjects with other endocrine diseases or significant renal or hepatic disease were excluded.

Obesity was defined as a body mass index (BMI) ≧25 kg/m^2, based on the criteria of the Japan Society for the Study of Obesity[13]. Diabetes mellitus was diagnosed according to World Health Organization criteria[14] and/or receiving treatment for diabetes mellitus. Informed consent was obtained from all subjects. This study was approved by the Ethics Committee of Kanazawa University.

Screening of Mutations in the Adiponectin gene

Genomic DNA was extracted from peripheral blood leukocytes using the standard procedure. Genotypes were determined at position 45 relative to the translation start site (corresponding to GenBank AB012163S1, 2, 3) by PCR, followed by allele-specific hybridization.

DNA fragments containing SNP45 (372 bp) were amplified by PCR from genomic DNA using primers 5'-GCAGCTCCTAGAAGTAGACTCTGCTG-3' and 5'-GGAGGTCTGTGATGAAAGAGGCC-3'. PCR products were incubated at 25℃ for 2 hours using *SmaI* (New England BioLabs Inc. UK). Digested products were separated by size on 3% agarose gel with ethidium bromide staining. The DNA segment from the G/G homozygote of SNP45 was digested into 209 and 163 bp fragments.

Laboratory Measurements

BMI was calculated as weight (in kilograms) divided by height (in meters) squared. Waist circumference at the umbilical level was measured in the exhalation phase of respiration while standing.

Venous blood samples were obtained after a 12-hour overnight fast. Serum total cholesterol (TC) and triglyceride (TG) were determined by enzymatic methods, and high-density lipoprotein cholesterol (HDL-C) levels were measured by a polyanion-polymer/detergent method. Serum immunoreactive insulin (IRI) was measured by enzyme-linked immunosorbent assay, blood glucose with the glucose oxidase method, and HbA_{1c} by high-pressure liquid chromatography. The insulin resistance index was calculated based on homeostasis model assessment (HOMA) [fasting glucose (mmol/L) × fasting insulin (μU/mL)/22.5][15]. Plasma adiponectin levels were measured with an enzyme-linked immunosorbent assay kit (Otsuka Pharmaceutical Co., Tokushima, Japan), and leptin was measured by radioimmunoassay.

Body Fat Distribution

All subjects underwent computed tomography (CT) at the umbilical level to measure the cross-sectional abdominal subcutaneous fat area (SFA) and visceral fat area (VFA) using Fat Scan (N2 System Corp, Osaka, Japan)[16]. The VFA/SFA ratio was calculated as visceral fat area divided by subcutaneous fat area.

Determination of Plaque Score and Max IMT

A high resolution B-mode ultrasonography unit (SS-A 370A; Toshiba; Tokyo) with a 7.5 MHz transducer was used to determine the plaque score of the carotid artery[17]. Carotid Intima-Media Thickness (IMT) was measured at each common carotid, carotid bulb, and internal carotid artery.

The maximum IMT (Max-IMT) was defined as the highest IMT value at any location in the near and far walls of the carotid arteries, including atheromatous plaques on both sides. We defined a plaque, focal IMT thickening, as an area where IMT ≧ 1.1 mm, and calculated the plaque score by totaling the maximum thickness of all plaques on the near and far walls of vessels in the scanned area[17].

Statistical Analysis

All data are shown as the mean ± SD. A chi-square test was used to confirm that the genotype frequency was in Hardy-Weinberg equilibrium and to compare differences. Continuous variables were compared by ANOVA after being adjusted for age, BMI, and sex. Univariate and stepwise regression analyses were employed to examine the association between the plaque score and clinical parameters. All statistical analyses were conducted with StatView 5.0 for Macintosh

Table 1. Genotype distribution and allele frequencies for the adiponectin gene SNP45

	SNP45 genotypes			Allele frequencie	
	T/T	T/G	G/G	T	G
n (%)	34 (53.1)	25 (39.1)	5 (7.8)	0.72	0.28
male (n = 40)	23 (57.5)	13 (32.5)	4 (10.0)	0.74	0.26
female (n = 24)	11 (45.8)	12 (50.0)	1 (4.2)	0.71	0.29

Table 2. Clinical characteristics according to adiponectin genotypes at position 45

	T/T	T/G + G/G	P
n (%)	34 (53.1%)	30 (46.9%)	
M/F	23/11	17/13	0.36
Age (years)	53 ± 15	56 ± 18	0.39
Type 2 diabetes (%)	73.5	73.3	0.98
BMI (kg/m^2)	30.7 ± 6.3	29.4 ± 3.6	0.56
Waist (cm)	103.3 ± 14.2	103.2 ± 13.0	0.37
HOMA-R	3.6 ± 2.3	3.3 ± 1.9	0.80
HbA_{1c} (%)	7.1 ± 1.9	6.7 ± 1.6	0.32
Total cholesterol (mg/dL)	211 ± 35	204 ± 43	0.44
Triglycerides (mg/dL)	152 ± 104	151 ± 82	0.80
HDL cholesterol (mg/dL)	46 ± 10	45 ± 13	0.35
Adiponectin (μg/mL)	5.5 ± 2.3	6.8 ± 4.5	0.26
Leptin (ng/mL)	10.8 ± 6.4	14.1 ± 12.3	0.07
Systolic blood pressure (mmHg)	131 ± 19	135 ± 20	0.34
Diastolic blood pressure (mmHg)	79 ± 11	79 ± 14	0.65
Subcutaneous fat area (cm^2)	275.4 ± 127.5	246.5 ± 94.6	0.32
Visceral fat area (cm^2)	140.5 ± 56.6	151.1 ± 51.0	0.06
V/S ratio	0.56 ± 0.24	0.76 ± 0.45	0.009
Max IMT	1.89 ± 0.81	2.21 ± 0.91	0.27
Plaque score	6.1 ± 4.1	9.7 ± 3.9	<0.001

NOTE. Values are the means ± SD. Heterozygotes and homozygotes for minor alleles were combined for presentation.
Abbreviations: BMI = body mass index, HOMA-R = homeostasis model assessment of insulin resistance
* P values adjusted for age, sex, and body mass index.

(Abacus Concepts, Berkeley, CA). A P value of less than 0.05 was considered statistically significant. In stepwise analysis, an F value greater than 4 was significant.

Results

Genotypes and Allele Distribution of SNP45 of the Adiponectin gene

The genotype and allele frequencies of study subjects are shown in **Table 1**. Genotype distributions were in Hardy-Weinberg equilibrium at both loci, with T being the major allele. The frequency of the T allele of SNP45 was 72%, and the frequencies of the T/T genotype, T/G genotype, and G/G genotype were 53.1%, 39.1%, and 7.8%, respectively.

Clinical and Metabolic Characteristics of this Study According to SNP45 of the Adiponectin gene

Table 2 shows a comparison of clinical characteristics and body composition according to adiponectin genotypes. Subjects were divided into 45T/T homozygote and those carrying the G allele (45T/G and 45G/G).

No differences in sex, age, or the proportion with diabetes were observed between any groups. Plasma leptin levels tended to be higher in carriers of the 45G allele (10.8 ± 6.4 vs. 14.1 ± 12.3, P = 0.07). Other variables (HbA_{1c}, plasma lipid, and plasma adiponectin levels) did not differ between these genotypes.

Table 3. Clinical characteristics according to gender

	men	women	*P*
n	40	24	
Age (years)	51 ± 16	60 ± 14	0.02
BMI (kg/m^2)	30.7 ± 6.3	29.4 ± 3.6	0.56
Waist (cm)	102.7 ± 13.7	104.2 ± 13.5	0.03
HOMA-R	3.6 ± 2.4	3.1 ± 1.3	0.76
HbA_{1c} (%)	6.7 ± 1.9	7.4 ± 1.5	0.14
Total cholesterol (mg/dL)	204 ± 40	216 ± 36	0.15
Triglycerides (mg/dL)	166 ± 108	132 ± 59	0.37
HDL cholesterol (mg/dL)	42 ± 9	53 ± 12	0.02
Adiponectin (μg/mL)	5.2 ± 2.5	7.6 ± 4.4	0.03
Leptin (ng/mL)	9.6 ± 9.2	16.8 ± 9.2	0.03
Systolic blood pressure (mmHg)	132 ± 19	135 ± 19	0.27
Diastolic blood pressure (mmHg)	80 ± 13	77 ± 9	0.65
Subcutaneous fat area (cm^2)	246.4 ± 129.1	287.6 ± 76.3	<0.001
Visceral fat area (cm^2)	158.0 ± 56.3	124.5 ± 43.1	0.002
V/S ratio	0.77 ± 0.41	0.47 ± 0.17	<0.001
Max IMT	2.09 ± 1.00	1.96 ± 0.61	0.18
Plaque score	8.1 ± 4.1	7.9 ± 5.0	0.41

NOTE. Values are the means ± SD. Heterozygotes and homozygotes for minor alleles were combined for presentation.
Abbreviations: BMI=body mass index, HOMA-R=homeostasis model assessment of insulin resistance
* *P* values adjusted for age and body mass index.

Table 4. Body fat distribution and PS according to adiponectin genotypes at position 45 in men and women

	men			women		
	T/T	T/G+G/G	*P*	T/T	T/G+G/G	*P*
VFA	147.1 ± 58.7	172.8 ± 50.8	0.005	126.7 ± 52.0	122.7 ± 36.1	0.763
SFA	273.4 ± 142.2	209.9 ± 101.8	0.007	279.8 ± 95.6	294.3 ± 58.8	0.091
V/S ratio	0.60 ± 0.26	1.00 ± 0.46	<0.001	0.49 ± 0.18	0.44 ± 0.16	0.262
PS	6.8 ± 4.2	9.5 ± 3.7	0.012	4.4 ± 4.0	10.8 ± 3.8	0.135

NOTE. Values are the means ± SD. Heterozygotes and homozygotes for minor alleles were combined for presentation.
Abbreviations: VFA=visceral fat area, SFA=subcutaneous fat area

Relationship between Genotypes and Body Fat Distribution

When we considered the VFA/SFA ratio as a marker of body fat distribution, it was significantly higher in carriers of the 45G allele (0.76 ± 0.45 vs. 0.56 ± 0.24, $P=0.009$), whereas there were no associations between SFA and SNP45. VFA tended to be higher in carriers of the 45G allele than TT homozygote (151.1 ± 51.0 vs. 140.5 ± 56.6 cm^2, $P=0.06$). Neither BMI nor waist circumference significantly differed between the two groups. Since there were sex differences in body fat distribution in this study (**Table 3**: men vs. women; VFA: 158.0 ± 56.3 vs. 124.5 ± 43.1, $P=0.002$; SFA: 246.4 ± 129.1 vs. 287.6 ± 76.3, $P<0.001$; V/S ratio: 0.773 ± 0.410 vs. 0.470 ± 0.176, $P<0.001$), we performed separate analyses of the association between SNP45 and body fat distribution by sex (**Table 4, Fig. 1**). In men, SNP45 was associated with the VFA, SFA, and V/S ratio, whereas in women SNP45 was not associated with body fat distribution.

Furthermore, to evaluate the contribution of SNP45 to the V/S ratio in men, stepwise regression analysis was used (**Table 5**). Selected variables were age, BMI, SNP45, and adiponectin. The data showed that age, SNP45, and plasma adiponectin levels were independent determinants of the V/S ratio in men ($R^2=0.588$, $P<0.0001$).

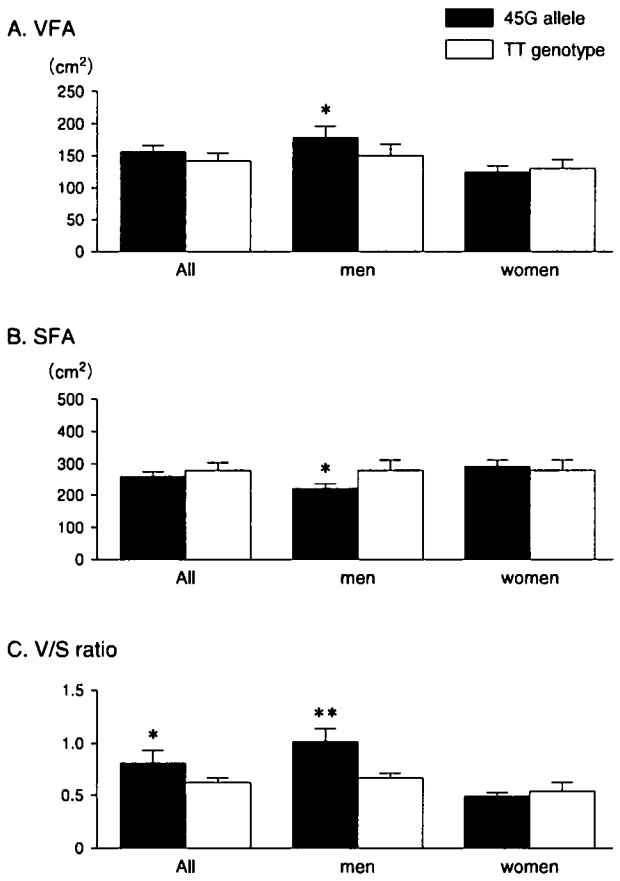

Fig. 1. Effect of SNP45 of the adiponectin gene on body fat distribution in all subjects, men and women

A. Effect of SNP45 on visceral fat area (VFA)
B. Effect of SNP45 on subcutaneous fat area (SFA)
C. Effect of SNP45 on VFA/SFA (V/F) ratio
Data are the means ± SE. $^{*}P<0.05$, $^{**}P<0.001$

Table 5. Stepwise regression analysis for determinant of V/S ratio in men

Factor	β	F-value
Age	0.017	35.763
SNP45*	0.340	24.945
Adiponectin	−0.073	13.688

$R^2=0.588$
*TT genotype=0, TG genotype=1, GG genotype=2

Table 6. Stepwise regression analysis for determinant of PS in men

Factor	β	F-value
Age	0.143	13.362
SNP45*	2.509	7.563

$R^2=0.372$
*TT genotype=0, TG genotype=1, GG genotype=2

Table 7. Correlation of PS to V/S ratio according to the genotype in men and women

	equation	r	p
men			
all	y=4.05x+4.71	0.39	0.02
G allele	y=3.22x+6.17	0.38	0.14
TT genotype	y=2.91x+4.93	0.17	0.49
women			
all	y=4.67x+5.85	0.17	0.47
G allele	y=12.00x+5.41	0.60	0.06
TT genotype	y=−2.57x+5.59	0.13	0.75

Relationship between Genotypes and Plaque Score of Carotid Artery

We investigated the effect of SNP45 on the plaque score and max IMT of carotid arteries. Carriers of the G allele had significantly greater PS than TT genotype after adjusting for age, sex, and BMI (10.0 ± 3.7 vs. 6.4 ± 4.2, $P<0.001$).

As shown in **Table 4**, SNP45 was associated with PS in men, and PS of the G allele tended to be higher than the TT genotype in women. To analyze the independent contribution of SNP45 to PS in men, stepwise regression analysis was applied (**Table 6**). Selected variables were age, BMI, V/S ratio, adiponectin, TC, HDL-C, systolic BP, and SNP45. The data showed that age and SNP45 were independent determinant of PS in men ($R^2=0.372$, $P=0.0007$).

Relationship between V/S Ratio and Plaque Score of Carotid Artery

To examine the effect of the V/S ratio on PS, we performed univariate analysis (**Table 7**).

There was a significant positive correlation between the V/S ratio and PS in men ($r=0.39$, $P=0.02$), whereas in women that correlation were not statistically significant ($r=0.17$, $P=0.47$).

Next, to investigate the impact of SNP45 on the association between the V/S ratio and PS, we subdivided into two groups according to the genotype of SNP45 in men and women. The V/S ratio tended to be associated with PS in the G allele in both men and women (men: $r=0.38$, $p=0.14$; women: $r=0.60$, $p=0.06$, respectively). In contrast, in subjects with the TT genotype, there was no relationship between the V/S ratio and PS.

Discussion

Our study had three major findings in Japanese obese subjects. First, SNP45 in the adiponectin gene was associated with body fat distribution. Second, SNP45 was associated with the development of carotid atherosclerosis. Moreover, SNP45 had an impact on the effect of visceral obesity for the progression of atherosclerosis. Third, there was a gender difference in the effect of SNP45.

First, we demonstrated that the G allele had higher VFA, lower SFA, and a significantly higher V/S ratio compared to the TT genotype in men. Multivariate regression analysis showed that SNP45 was an independent determinant of the V/S ratio. These results indicated that the G allele of SNP45 is a risky genotype of visceral adiposity, resulting in metabolic syndrome. To our knowledge, this is the first study to demonstrate the association of SNP45 with body fat distribution. Some reports have shown that SNP45 contributes to obesity, insulin resistance, or dyslipidemia[10, 18, 19]. In contrast, Ukkola *et al.* reported that SNP45 was found in equal frequency among obese and non-obese Swedish subjects[20]. In French Caucasians, the 45G allele frequency was similar in morbidly obese adults and control subjects[21]. The inconsistency between these reports suggested that SNP45 could not be associated simply with weight or prevalence of obesity, but might contribute to body fat distribution in the process of becoming obese. Since visceral adipose tissue is widely believed to play a key role in the pathogenesis of metabolic abnormalities, the G allele of SNP45 could be an independent risk factor for metabolic syndrome.

Second, another important finding of the present study was the significant association between SNP45 and carotid artery PS in men. A similar trend was observed in women. Multivariate regression analysis showed that SNP45 was an independent determinant of PS. These findings suggest that SNP45 may affect the development of carotid atherosclerosis not only by modulating visceral obesity but also by other pathways.

To the best of our knowledge, PS tends to be associated with the V/S ratio only in the G allele in both men and women. In a previous study, we described a strong association between the V/S ratio and carotid artery PS in Japanese males with metabolic syndrome[22], but patients with the TT genotype were protected from the atherogenic effect of visceral obesity. We hypothesized that visceral obesity might exaggerate the dysregulation of adiponectin properties of the G allele. The mechanism was unclear, but this hypothesis needs confirmation by expression studies.

Third, in this study the degree of the effect of SNP45 on body fat distribution and PS was different between men and women. Adipose tissue is sexually dimorphic in humans, with gender-specific differences in body fat distribution[23, 24]. Gonadal steroids are the major mediator of sex dimorphism of body composition in adults[25, 26]. Estrogen regulates both the metabolism and location of adipose tissue and plays a role in adipogenesis, adipose deposition, lipogenesis, lipolysis, and adipocyte proliferation[27]. Furthermore, in recent studies, Clegg *et al.* reported that gonadal steroids mediate body fat distribution and interact with the integrated adiposity messages conveyed to the brain[28]. Taken together with previous studies, our findings suggest that estrogen may interact with the adiponectin gene in adipocyte and modulate the effect of SNP45.

In addition, estrogen is known to have a cardioprotective effect. *In vivo* evidence suggests that the effect of estrogen on adhesion molecules is mediated by the inhibition of nuclear factor (NF)-κB DNA binding[29, 30]. As adiponectin has been shown to suppress the expression of class A scavenger receptors in macrophages, to affect the NF-κB pathway and to inhibit monocyte adhesion to aortic endothelial cells[6-8], atherogenic properties of the G allele may be suppressed by the effect of estrogen. Estrogen could interact with SNP45 and modulate the atherogenic function of adiponectin, but further large studies are needed to confirm the mechanism of gender-specific differences in the effect of SNP45.

The mechanistic relationship between SNP45 and both body fat distribution and the progression of atherosclerosis is unclear. SNP45 is located in exon 2 of the adiponectin gene and does not cause an amino acid change (GGT to GGG, Gly15Gly). One possibility is that SNP45 may have linkage disequilibrium with other undiscovered SNPs of the adiponectin gene having an effect on adiponectin expression, secretion, structure, or action. Another possibility is that SNP45 located in exon 2 is relatively close to the exon-intron boundary which may affect splicing machinery and effect adiponectin expression. The G allele of SNP45 may act through decreased adiponectin expression, which may cause increased visceral adipose tissue. Indeed, in Japanese type 2 diabetes, SNP45 is reported to be associated with reduced adiponectin levels[11]. Similar findings have been shown in an other study[31]. Furthermore, recent studies have reported various adiponectin functions as an adipocyte differentiation factor, helping to maintain equilibrium adipocyte size, as an autocrine/paracrine factor in adipose tissue and as a participating factor in the regulation of adipocyte metabolism and adipose tissue mass. In 3T3-L1 preadipocytes,

adiponectin overexpression accelerates cell proliferation and differentiation, while in mature adipocytes, autocrine adiponectin increases glucose uptake and lipid accumulation[32]. Transgenic overexpression of adiponectin in the physiological range induced morbid obesity without insulin resistance in ob/ob mice[21]. These reports indicated that hyperadiponectinemia may induce simple obesity with more subcutaneous fat accumulation, while decreased adiponectin levels may induce visceral obesity. Interestingly, the present study showed that hypoadiponectinemia was the third independent determinant of the V/S ratio. Due to these previous findings combined with our present study, the G allele might be genetically determined to have hypoadiponectinemia, contributing to the progression of visceral obesity. In contrast, the TT genotype might favor the accumulation of subcutaneous adipose tissue through hyperadiponectinemia, preventing insulin resistance, and eventually metabolic syndrome.

Adiponectin exists largely as low molecular weight (LMW) hexamers and high molecular weight (HMW) multimers[32, 33]. Recent article showed that the ratio of HMW to total adiponectin was responsible for metabolic effects[34]. Another study showed that HMW adiponectin was an important factor in metabolic syndrome[35]. Therefore, the alternative possibility of the atherogenic effect of SNP45 is that the proportion of HMW adiponectin might decrease in the G allele of SNP45, leading to atherosclerosis. As we measured total adiponectin and did not assess multimeric forms of adiponectin, further study is needed.

In conclusion, we demonstrated that SNP45 was associated with body fat distribution and PS of carotid arteries. The TT genotype is a protective genotype from metabolic syndrome and atherosclerosis progression in Japanese obese subjects. The mechanism by which SNP45 affects body fat distribution and the development of atherosclerosis has not been clarified at present. Further investigations will be needed to elucidate the functional mechanism of this polymorphism.

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High Frequency of a Retinoid X Receptor γ Gene Variant in Familial Combined Hyperlipidemia That Associates With Atherogenic Dyslipidemia

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Objective—The genetic background of familial combined hyperlipidemia (FCHL) has not been fully clarified. Because several nuclear receptors play pivotal roles in lipid metabolism, we tested the hypothesis that genetic variants of nuclear receptors contribute to FCHL.

Methods and Results—We screened all the coding regions of the PPARα, PPARγ2, PPARδ, FXR, LXRα, and RXRγ genes in 180 hyperlipidemic patients including 60 FCHL probands. Clinical characteristics of the identified variants were evaluated in other 175 patients suspected of coronary disease. We identified PPARα Asp140Asn and Gly395Glu, PPARγ2 Pro12Ala, RXRγ Gly14Ser, and FXR −1g−>t variants. Only RXRγ Ser14 was more frequent in FCHL (15%, $P<0.05$) than in other primary hyperlipidemia (4%) and in controls (5%). Among patients suspected of coronary disease, we identified 9 RXRγ Ser14 carriers, who showed increased triglycerides (1.62±0.82 versus 1.91±0.42 [mean±SD] mmol/L, $P<0.05$), decreased HDL-cholesterol (1.32±0.41 versus 1.04±0.26, $P<0.05$), and decreased post-heparin plasma lipoprotein lipase protein levels (222±85 versus 149±38 ng/mL, $P<0.01$). In vitro, RXRγ Ser14 showed significantly stronger repression of the lipoprotein lipase promoter than RXRγ Gly14.

Conclusion—These findings suggest that RXRγ contributes to the genetic background of FCHL. (***Arterioscler Thromb Vasc Biol.* 2007;27:923-928.**)

Key Words: apolipoproteins ■ gene mutations ■ lipoprotein lipase ■ familial combined hyperlipidemia ■ nuclear receptors

Familial combined hyperlipidemia (FCHL) is the most common form of inherited hyperlipidemia. FCHL shows strong genetic susceptibility resembling an autosomal dominant disease,[1–3] but most of the underlying causal mechanisms remain to be elucidated. Lipoprotein lipase (LPL) has been implicated as one of the genes that modify the lipid phenotype in FCHL.[4,5] "Intra-individual variability" of the lipoprotein phenotype is often included as a criterion in diagnosis.[6] However, a recent prospective study of FCHL families suggests that this variability may even include normolipidemic periods in affected subjects.[7] This feature indicates that FCHL could be a "disease of regulation" rather than a genetic defect in certain peripheral components of lipid metabolism.

Nuclear receptors are transcription factors that can be activated by specific ligands. Recent studies have shown that nuclear receptors, especially retinoid X receptor (RXR) and its heterodimerization partners,[8] play important roles in maintenance of lipid homeostasis on their activation by a variety of ligands derived from dietary cholesterol and fatty acids.[9] The peroxisome proliferator-activated receptors (PPARs) family, the oxysterol sensor liver X receptor (LXR), and the bile acid sensor farnesoid X receptor (FXR) are all involved in control of plasma lipid concentrations.[10] Thus, we tested the hypothesis that variants of these nuclear receptors, ie, PPARα, PPARγ2, PPARδ, LXRα, FXR, and RXRγ, could constitute part of the genetic background of atherogenic dyslipidemia, particularly of FCHL.

Methods

Subjects

The study design consists of 2 parts. First, we screened for frequent variants in the nuclear receptor candidate genes among 180 patients with primary hyperlipidemia, including 60 unrelated patients with FCHL (clinical characteristics are presented in supplemental Table I, available online at http://atvb.ahajounals.org). Patients with familial

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hypercholesterolemia and secondary hyperlipidemia were excluded. Diagnosis of FCHL was based on the fulfillment of all of the following three criteria: (1) Phenotype IIb, IIa, or IV hyperlipidemia according to the Fredrickson classification; (2) Presence of phenotype IIb, IIa, or IV hyperlipidemia in a first-degree relative and at least one family member with phenotype IIb; (3) Exclusion of familial hypercholesterolemia. Two hundred ninety-eight anonymous samples from healthy males were used as controls for frequency analysis of identified mutations. All blood samples in this study were obtained after an overnight fast.

Second, we evaluated the clinical impact of potentially relevant variants in another 175 patients who were suspected of having coronary artery disease based on any of the following reasons: ECG abnormalities, cumulative coronary risk factors, and/or chest symptoms. The group included 105 patients who had undergone coronary angiography. Patients with familial hypercholesterolemia were excluded because of their clear genetic background for hyperlipidemia. The extent and severity of atherosclerotic changes in coronary angiography were assessed by assigning scores to each of the 15 segments, according to the classification of the American Heart Association Grading Committee. The coronary stenosis index (CSI) was defined as the sum of the following scores[11]: A normal coronary angiogram was graded 0, stenosis of less than 25% was graded 1, 25% to 50% stenosis was graded 2, 50% to 75% stenosis was graded 3, and more than 75% stenosis was graded 4. CSI is a useful index for evaluating mild-moderate coronary atherosclerotic changes.

All the subjects and controls enrolled were inhabitants of the Hokuriku district of Japan. Written informed consent was obtained from each of the subjects. The study protocol was approved by the ethics committee of the Graduate School of Medical Science, Kanazawa University.

Laboratory Analyses

Total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL)-cholesterol, apolipoproteins, glucose, and thyroid hormones were measured according to standard clinical laboratory techniques. HDL-cholesterol fractions were obtained by dextran sulfate-magnesium chloride precipitation and assayed using a commercial kit (Daiichi, Tokyo, Japan).[12] Separation of lipoproteins by ultracentrifugation was performed as described by Havel et al.[13] Plasma remnant-like particle (RLP)-cholesterol was determined by immunoabsorption using the commercial RLP-C JIMRO kit.[14] Plasma cholesteryl ester transfer protein (CETP) concentrations were determined by enzyme-linked immunosorbent assay using the monoclonal antibody TP2 and a rabbit polyclonal antibody raised against recombinant human CETP.[15] For LPL assessment, blood samples were obtained 10 minutes after an intravenous injection of 30 IU heparin/kg body weight. LPL activity was measured using radiolabeled triolein emulsion after hepatic lipase (HL) inhibition by SDS as previously described.[16] LPL mass was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using specific monoclonal antibody against LPL (Daiichi Pure Chemicals Co Ltd, Tokyo, Japan).[17]

Genetic Analyses of Candidate Genes

Genomic DNA was isolated from peripheral white blood cells using standard phenol-chloroform extraction techniques. We screened all the coding regions of PPARα (NM_032644), PPARδ (NM_006238), PPARγ2 (NM_015869), LXRα (NM_005693), FXR (NM_005123), and RXRγ (NM_006917) genes with flanking exon-intron boundaries by polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) using the DCode system (Bio-Rad), which is highly accurate in detecting changes in nucleic acids.[18] The structural organization and nucleotide sequences of these genes were retrieved from the gene database of NCBI. Lists of all GC-clamped primers used in DGGE analysis are available online (supplemental Table II). Samples with a variant detected by DGGE analysis were directly sequenced on an ABI310 analyzer (Applied Biosystems). PCR-restriction-fragment-length polymorphisms analysis on the RXRγ Ser14 variant was performed with the primers 5′-AGCCGAGAGAGGCGGTAATA-3′ (forward) and 5′-TACAGGTCCACGCAGTGAAG-3′ (reverse) in patients suspected of coronary artery disease. Digestion with *Alu*I resulted in a 76-bp fragment for Ser allele and a 120-bp fragment for Gly allele.

Cell Culture and Transfection Assays

Cos7 cells were grown in DMEM supplemented with 10% FCS, penicillin/streptomycin, sodium pyruvate, glutamine, and nonessential amino acids (Gibco BRL, Invitrogen). The medium was changed every 48 hours. Cos7 cells were transfected using FuGENE 6 reagent (Roche): 150 ng of the indicated LPL firefly luciferase reporter plasmid (a generous gift of Dr B. Staels, Institut Pasteur de Lille, France), that contains the proximal 466-bp of the human LPL promoter in front of the ATG cloned into the *Hind*III site of the pGL3 plasmid, was cotransfected with or without 100 ng of the human RXRγ expressing vector (a generous gift of Dr W. Lamph, Ligand Pharmaceuticals Inc, San Diego, Calif). After an overnight incubation, cells were incubated with medium containing 10% FCS with or without the retinoid LGD1069, (1 μmol/L, Sigma) and luciferase activity was assayed 48 hours later using an Orion luminometer (Berthold). Transfection studies were performed at least 3 times in triplicate. Transfection efficiency was monitored by cotransfection of 150 ng of a SV40-driven β-galactosidase expression plasmid. A positive RXRE TKpGL3 construct was made by cloning 3 copies of the direct repeat AGGTCA spaced by 5 nucleotides in the TKpGL3 plasmid.

Plasmid Site-Directed Mutagenesis

Nucleotide substitution was introduced in the plasmid expressing human RXRγ using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, The Netherlands) and the primer 5′-CATGAAGTTTCCCGCAAGCTATGGAGGCTCCCCTGG C-3′ in which the nucleotide in bold indicates the mutation.

Statistical Analysis

The frequency distribution of genotypes was compared using standard χ^2 tests. Student t test was used for normally distributed parameters and the Kruskal–Wallis test was used for non-normally distributed parameters: triglycerides levels, LPL levels, and CSI. JMP 5.1.2 software (SAS Institute Inc) was used for statistical calculation.

Results

Identified Variants in Nuclear Receptor Genes

With PCR-DGGE analysis, we identified 4 variants with amino acid changes, ie, Asp140Asn and Gly395Glu in the PPARα gene, Pro12Ala in the PPARγ2 gene, Gly14Ser in the

TABLE 1. Frequencies of Nuclear Receptor Genes Variants Identified in This Study

	FCHL n=60	Other Hyperlipidemia n=120	General Population n=298	*P* Value
PPARαGly395Glu				
Glu395	3 (5%)	1 (0.8%)	6 (2%)	ns
PPARαAsp140Asn				
Asn140	2 (3%)	1 (0.8%)	2 (0.6%)	ns
PPARγ2Pro12Ala				
Ala12	5 (8%)	10 (8%)	20 (7%)	ns
FXR −1g−>t				
−1g/t	19 (32%)	34 (28%)	108 (36%)	ns
−1t/t	2 (3%)	6 (5%)	27 (9%)	ns
RXRγGly14Ser				
Ser14	9 (15%)	5 (4%)	15 (5%)	0.03

TABLE 2. Clinical Characteristics of Patients With RXRγ Variant

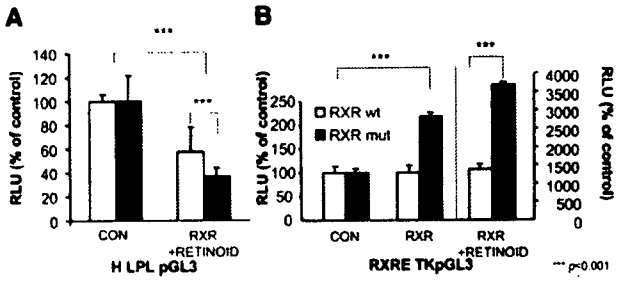
	RXRγ Gly14Ser		
	Gly/Gly	Gly/Ser	*P* Value
Number (M/F)	166 (78/88)	9 (5/4)	
Age, y	58±15	58±7	ns
BMI, kg/m²	23.4±5	23.9±2	ns
Smoking, %	36	33	ns
Total cholesterol, mmol/L	5.98±1.4	5.96±1.55	ns
Triglycerides, mmol/L	1.62±0.82	1.91±0.42	*P*<0.05
HDL cholesterol, mmol/L	1.32±0.41	1.04±0.26	*P*<0.05
LDL cholesterol, mmol/L	3.94±1.27	4.07±1.45	ns
HDL2 cholesterol, mmol/L	0.78±0.28	0.54±0.10	*P*<0.05
HDL3 cholesterol, mmol/L	0.44±0.10	0.39±0.08	ns
ApoA-I, g/L	1.38±0.31	1.18±0.18	ns
ApoA-II, g/L	0.32±0.06	0.28±0.05	*P*<0.05
ApoB, g/L	1.31±0.38	1.35±0.31	ns
ApoC-II, g/L	0.06±0.02	0.05±0.02	ns
ApoC-III, g/L	0.11±0.05	0.10±0.03	ns
ApoE, g/L	0.06±0.02	0.05±0.01	ns
RLP cholesterol, mmol/L	0.15±0.10	0.21±0.10	*P*<0.01
CETP, mg/L	2.52±0.82	2.48±0.73	ns
Intraindividual lipoprotein phenotype variability, %	27	88	*P*<0.01
Fasting glucose, mmol/L	5.72±1.39	5.33±0.72	ns
HbA1c, %	5.6±1.0	5.8±1.0	ns
Fasting insulin, pmol/L	70.8±90.3	52.1±1.0	ns
HOMA-IR	2.28±2.1	2.19±1.7	ns
Diabetes, %	28	33	ns
HL activity, U/L	0.24±0.09	0.26±0.07	ns
LPL activiy, U/L	0.11±0.06	0.08±0.03	*P*<0.05
LPL mass, ng/mL	222±85	149±38	*P*<0.01
FT3, pmol/L	0.42±0.01	0.044±0.01	ns
FT4, pmol/L	15.2±5.15	13.3±2.57	ns
TSH, μU/mL	2.31±2.8	2.53±0.9	ns
Number (M/F)	100 (50/50)	5 (4/1)	
CSI	12.3±10	21.4±6	*P*<0.05
			mean±SD

BMI indicates body mass index; HOMA-IR, homeostasis model assessment; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid stimulating hormone.

RXRγ gene, and 1 nucleotide substitution in a flanking coding region, ie, FXR −1g−>t variant. The PPARγ2 Pro12Ala polymorphism has already been well-described,[19] whereas the others represent novel variants identified in this study. In humans, variants in the RXRγ gene have been associated with elevated triglyceride levels in familial type 2 diabetes, but none of these variants showed an altered coding sequence.[20] Therefore, this is the first description of a RXRγ variant with an amino acid substitution. In the PPARα gene, the Leu162Val variant has been reported in Western countries,[21] but this variant was not identified in this study. We also identified some silent nucleotide substitutions, ie, 891C−>G (rs13306747) and 1431C−>T (rs1724155) in the PPARγ2 gene, 1233C−>T (rs9658166) in the PPARδ gene, and 1134A−>G (rs1131379) in the LXRα gene. We did not identify variants with amino acid changes in the PPARδ and LXRα genes. We further investigated the variants with amino acid substitutions and the −1g−>t FXR variant, because of the likelihood that these induced altered physiological function.

Higher Frequency of RXRγ Variant in FCHL

We evaluated the frequencies of the 5 identified polymorphisms in subjects with FCHL, subjects with other forms of primary hyperlipidemia and in the general population (Table 1). Only the RXRγ Ser14 variant was found to be significantly more frequent in FCHL patients (15%) compared with that in other forms of primary hyperlipidemia (4%) or the general population (5%).



A, Cos7 cells were cotransfected with RXRγ wild-type or the Ser14 variant and activated with retinoid in presence of the LPL promoter. B, Cos7 cells were cotransfected with RXRγ wild-type or the Ser14 variant and activated with retinoid in presence of a positive RXRE cloned in the TKpGL3 plasmid.

Atherogenic Plasma Lipids Profiles and Coronary Atherosclerosis Associated With the RXRγ Ser14 Variant

To establish the impact of the identified RXRγ variant on metabolic parameters and on coronary atherosclerosis, we evaluated anthropometric parameters and laboratory data from 175 patients suspected of coronary disease. The RXRγ Ser14 variant was identified in 9 patients, all of whom were heterozygotes. Eight of the RXRγ Ser14 carriers had hyperlipidemia, while the remaining 1 demonstrated an isolated low HDL cholesterol level. Clinical characteristics of patients with or without the RXRγ Ser14 allele are shown in Table 2. There was no difference in age or body mass index between the two groups. In their lipid profiles, RXRγ Ser14 carriers had higher TG, lower HDL cholesterol especially in the HDL2 subfraction, and lower apolipoprotein A-II levels. There was no difference in CETP protein levels between the groups. Furthermore, we found that the RLP cholesterol level was significantly higher in the RXRγ Ser14 carriers than in the wild-type. Subjects with this variant also showed significantly lower LPL activities and protein levels in post-heparin plasma. Separation of lipoproteins demonstrated that the Ser14 carriers had higher TG levels in very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) fractions, higher cholesterol levels in VLDL, and lower cholesterol levels in HDL (supplemental Table III).

Two RXRγ Ser14 carriers were diagnosed as FCHL (22%), and 2 additional carriers were suspected of FCHL with hyperlipidemic siblings without information on first-degree relatives. Among non-carriers, 22 of 166 patients were diagnosed as FCHL (13%). One hundred twenty-five patients suspected of coronary disease showed hyperlipidemia and the intraindividual variability of lipoprotein phenotype was significantly more frequent in RXRγ Ser14 carriers (7 of 8 hyperlipidemic patients; 88%) than in wild-type (32 of 117 hyperlipidemic patients; 27%, Table 2).

There was no significant difference in the thyroid hormone levels between the two groups.

Four males and 1 female were identified as RXRγ variant carriers among 105 patients who underwent coronary angiography. The carriers of RXRγ Ser14 demonstrated significantly higher CSI than those with the wild-type (Table 2).

RXRγ Variant Represses More Efficiently the LPL Promoter Activity

Because RXRγ Ser14 carriers showed significantly lower LPL activities and protein levels in post-heparin plasma, we hypothesized that activated-RXRγ downregulates LPL gene expression by a transcriptional mechanism and that RXRγ variant is more effective in repressing the LPL promoter activity. Therefore, transfection assays were performed using the LPL promoter cotransfected with either wild-type RXRγ or the variant (Figure). Interestingly, RXRγ Gly14 significantly repressed (−40%) the LPL promoter activity, whereas the RXRγ Ser14 repressed even more strongly (−60%, $P<0.001$, Figure A). Moreover, the RXRγSer14 was a more potent activator of a positive RXRE cloned in front of a TKpGL3 plasmid (note the different scales in Figure B). Taken together, our results indicate that RXRγ downregulates human LPL gene expression, at least partially by a transcriptional mechanism, and that the newly identified RXRγ variant is a more potent repressor than the wild-type in this respect, as well as a more potent transactivator of a positive RXR response element.

Gain of Function Variant of PPARα and Increased LDL-C Levels

The carriers of the PPARα variant Gly395Glu tended to have higher frequency in the FCHL population, although not statistically significant. Four subjects were identified as PPARα Glu395 carriers in the coronary artery disease-suspected group and showed significantly higher LDL-cholesterol levels (supplemental Table IV). On in vitro functional analysis, Glu395 showed a moderately but significantly increased transcriptional activity compared with wild-type PPARα (supplemental Figure I, available online at http://atvb.ahajournals.org). The previously described Leu162Val variant of the PPARα gene has been shown to give gain of function in in vitro,[24] has been associated with raised LDL-cholesterol levels.[21,22] Our results appear to be in accordance with these previous reports.

Discussion

The main findings of the present study are the following: (1) identification of novel polymorphisms in plasma lipid levels-associated nuclear receptor genes, (2) a higher frequency of the RXRγ gene variant Gly14Ser in subjects with FCHL, (3) RXRγ Ser14 variant carriers showed more atherogenic dyslipidemia associated with coronary atherosclerosis, (4) the RXRγ variant showed a stronger response to its ligand in repression of the LPL promoter than the wild-type RXRγ.

RXRs are major heterodimerization partners of nuclear receptors such as PPARs, LXRs, and FXR. Three RXR isotypes have been identified: RXRα, RXRβ, and RXRγ. Synthetic RXR ligands induce hypertriglyceridemia through decreased clearance of VLDL by LPL-dependent pathways,[23,24] except in 1 study.[25] In contrast to the embryonic lethality observed in RXRα- and RXRβ-deficient mice, RXRγ-deficient mice develop apparently normal.[26] Yet, RXRγ-deficient mice showed reduced fasting plasma TG levels and increased skeletal muscle LPL activity when fed a high fat diet.[27] The human RXRγ gene is located on chro-

mosome 1q21-q23, ie, the so-called "FCHL locus",[28] and both linkage analysis and a twin study have indicated that the RXRγ gene is linked with dyslipidemia in Chinese and German families.[29,30]

To our knowledge, there are only few data concerning the physiological roles and targets of RXRγ in humans. The RXRγ gene is mainly expressed in skeletal muscles, central nervous system, skin, intestine, and lung. In the present study, LPL protein mass and activity were significantly decreased in RXRγ variant carriers. Because LPL is mainly expressed in adipose tissues and in skeletal muscles, we assume that this is attributable to the fact that the presence of the RXRγ variant affects LPL expression in skeletal muscles. RXRγ mRNA is detectable in adipose tissue only at a low level,[31] but it has been reported that RXRγ could replace RXRα in heterodimerization with PPARγ in adipose tissue.[32] Therefore, there is a possibility that RXRγ variant expression in adipose tissue contributes to the changes in LPL.

It has been reported that RXRγ-deficient mice show a 17% increase in serum thyroid hormone (T4) and a 20% increase in thyroid-stimulating hormone (TSH) levels.[33] In the present study, thyroid hormone levels did not appear to differ sufficiently between variant carriers and non-carriers to explain the differences observed in lipid levels.

It has been shown that low LPL levels contribute to disorders associated with TG-rich lipoprotein catabolism with low HDL, especially in HDL2,[34,35] and are associated with increased risk for future coronary disease.[36] Thus, the low LPL could well contribute to the increase in TG and the decrease in HDL-cholesterol levels in subjects with the RXRγ variant.

We assessed the functional consequence of the RXRγ Ser14 variant in vitro. The activation function-1 (AF-1) domain of RXRγ is located between amino acids 1 and 103, and is required for optimal ligand-dependent transactivation of RXR response element.[37] Fourteen amino acids are located within the AF-1 domain and are conserved among humans, mice, and chickens. In a transfection assay, RXRγ Ser14 repressed LPL promoter activity more strongly than the wild-type RXRγ. In addition, the Ser14 variant was a more potent inducer of a positive RXR response element. Therefore, we speculate that the Ser14 variant induces a better recruitment and/or stabilization of RXR cofactors. Further studies will be required to understand the precise molecular mechanism(s) involved in the LPL regulation by RXRγ Ser14.

Within the so-called FCHL locus, on chromosome 1q21-q23, several genes have been reported to be associated with the FCHL phenotype[28,30,38] and with type 2 diabetes.[39] First, the thioredoxin interacting protein gene was shown to be associated with combined hyperlipidemia in mice, but no disease-causing mutation has been found in humans so far.[40,41] Currently upstream stimulatory factor 1 (USF1) is considered the most promising candidate gene of FCHL.[42] In the USF1 gene, no amino acid substitution has been identified in the coding regions, but single nucleotide polymorphisms in the 3′untranslated region and in intron 7 have been reported to be associated with FCHL, metabolic syndrome, or type 2 diabetes mellitus quite reproducibly.[43–45] However, populations did not show any such association have also been reported.[46–48] These reports emphasize the complexity of phenotypic expression in multi-factorial diseases such as FCHL. RXRγ had been reported to show an association with TG and cholesterol levels on linkage analysis,[29,30] and we identified novel RXRγ variant that associated with atherogenic dyslipidemia. However, the changes in lipid levels attributable to the RXRγ variant alone were not sufficient to cause FCHL. Thus, we suggest the RXRγ gene variant to be a strong modifier rather than a causative gene in development of the FCHL phenotype.

In conclusion, the present study suggests that a variant of RXRγ gene contributes to genetic dyslipidemia, including FCHL, based on the increased frequency of this variant in FCHL, its association with an atherogenic lipid profile, and initial functional studies.

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Disclosures

None.

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Differences in the diagnostic value of various criteria of negative T waves for hypertrophic cardiomyopathy based on a molecular genetic diagnosis

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ABSTRACT

Differences in the diagnostic value of a variety of definitions of negative T waves for HCM (hypertrophic cardiomyopathy) have not yet been clarified, resulting in a number of definitions being applied in previous studies. The aim of the present study was to determine the most accurate diagnostic definition of negative T waves for HCM in genotyped populations. Electrocardiographic and echocardiographic findings were analysed in 161 genotyped subjects (97 carriers and 64 non-carriers). We applied three different criteria that have been used in previous studies: Criterion 1, negative T wave > 10 mm in depth in any leads; Criterion 2, negative T wave > 3 mm in depth in at least two leads; and Criterion 3, negative T wave > 1 mm in depth in at least two leads. Of the three criteria, Criterion 3 had the highest sensitivity (43% compared with 5 and 26% in Criterion 1 and Criterion 2 respectively; $P < 0.0001$) and retained a specificity of 95%, resulting in the highest accuracy. In comparison with abnormal Q waves, negative T waves for Criterion 3 had a lower sensitivity in detecting carriers without LVH (left ventricular hypertrophy) (12.9% for negative T waves compared with 22.6% for abnormal Q waves). On the other hand, in detecting carriers with LVH, the sensitivity of negative T waves increased in a stepwise direction with the increasing extent of LVH ($P < 0.001$), whereas there was less association between the sensitivity of abnormal Q waves and the extent of LVH. In conclusion, Criterion 3 for negative T waves may be the most accurate definition of HCM based on genetic diagnoses. Negative T waves may show different diagnostic value according to the different criteria and phenotypes in genotyped populations with HCM.

INTRODUCTION

HCM (hypertrophic cardiomyopathy) is a primary cardiac disorder, often transmitted genetically, with a heterogeneous clinical and morphological expression [1]. Although echocardiography has become the standard in the diagnosis of HCM [2], electrocardiographic criteria remain widely used, due to its simplicity and accessibility. Negative T waves are one of the major electrocardiographic abnormalities in HCM and have been of

Key words: echocardiography, electrocardiography, genotyping, hypertrophic cardiomyopathy (HCM), left ventricular hypertrophy (LVH), molecular diagnosis, negative T wave.

Abbreviations: HCM, hypertrophic cardiomyopathy; LV, left ventricular; LVH, LV hypertrophy; MWT, maximum wall thickness; NPV, negative predictive value; PPV, positive predictive value.

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