Original Article

Genotypic Effect of ABCG1 Gene Promoter – 257T>G Polymorphism on Coronary Artery Disease Severity in Japanese Men

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Aim: ATP-binding cassette transporter G1 (ABCG1) is a cholesterol transporter that plays a role in cholesterol efflux in the presence of high-density lipoprotein (HDL). Moreover, HDL-mediated cholesterol efflux is increased in cultured ABCG1 overexpressed cells; however, the physiological roles of ABCG1 and its contribution to atherosclerosis in humans remain unclear.

Methods: The effect of ABCG1 – 257T>G mutation on transcription activity was determined by a reporter assay. One hundred nine Japanese men with coronary artery disease (CAD) were analyzed. ABCG1 – 257T>G polymorphism was assessed by mutation-selective PCR methods to identify T/T, T/G, and G/G genotypes.

Results: The reporter assay showed that ABCG1 transcription activity was significantly lower ($p < 0.01$) in the G allele of – 257T>G polymorphism compared with that in the T allele. Clinically, there were no significant differences in serum triglyceride and total, LDL, and HDL cholesterol levels, or other risk factors. Logistic regression analysis revealed significant additive and dominant effects on the frequency of patients with multi-vessel disease compared with single-vessel disease (T/T vs. T/G vs. G/G, odds ratio:2.1, $p=0.027$; T/T vs. T/G and G/G, odds ratio:3.5, $p=0.005$).

Conclusion: This is the first report to show that a novel ABCG1 – 257T>G promoter polymorphism influences CAD severity in Japanese men.

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Key words; Cholesterol transporter, Promoter, Polymorphism, apolipoprotein A-I, High-density lipoprotein

Introduction

Various clinical and epidemiological studies have demonstrated that high-density lipoprotein (HDL) cholesterol and the risk of cardiovascular events exhibit negative correlation, and that HDL exerts many potential anti-atherogenic effects¹⁾. For example, HDL particles transport cholesterol from peripheral cells to the liver and steroidogenic organs, where cholesterol is used for the synthesis of bile acids, lipoproteins, vita-

min D, and steroid hormones¹⁾. Although the details and the pathogenesis of low-HDL cholesterolemia and increased risk of atherosclerosis are not well understood, a higher risk of coronary artery disease (CAD) in type 2 diabetic patients might be attributed to a reduced reverse cholesterol transport (RCT) system²⁾. Adenosine triphosphate (ATP)-binding cassette transporter (ABC) A1 has been identified as a pivotal gene in the regulation of both plasma HDL cholesterol levels and cellular cholesterol homeostasis, which is a key molecule in the first step of RCT. ABCA1 gene deficiency causes decreases in HDL cholesterol and the capacity of skin fibroblasts and monocyte-derived macrophages to release cholesterol in the extracellular presence of apolipoprotein (apo)A-I or apoE^{1,3)}.

ABCG1 is another member of the ABC trans-

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porter superfamily, which has been mapped to chromosome 21q22.3⁴⁻⁹). In contrast to ABCA1, ABCG1 is a half-transporter containing only one ATP-binding cassette and transmembrane domain^{7, 8}). It is therefore believed that ABCG1 requires a dimeric partner to become active. Wang *et al.* recently reported that ABCG1 and ABCG4 contribute to HDL₂- and HDL₃-dependent cellular cholesterol efflux¹⁰, and that they appear to have an important function related to HDL lipidation¹⁰⁻¹²).

Administration of a high-fat high-cholesterol diet to ABCG1-deficient mice results in massive accumulation of lipids in tissue macrophages, whereas overexpression of human ABCG1 protects murine tissues from dietary fat-induced lipid accumulation¹³. Furthermore, Mauldin *et al.* have shown that reduced ABCG1 function facilitates foam cell formation in type 2 diabetic mice¹⁴. Transplantation of ABCG1-deficient (ABCG1^{-/-}) bone marrow into low-density lipoprotein (LDL) receptor-deficient mice produces contrasting effects on atherosclerotic formation¹⁵⁻¹⁷. In contrast, a decrease in lesion formation and size have been observed in the absence of macrophage ABCG1 in mice^{16, 17}. Recently, it has been demonstrated that total body expression of ABCG1 protects against early atherosclerotic lesion development¹⁸; however, the physiological roles of ABCG1 and its contribution to the progression of atherosclerosis in humans remain unclear. The ABCG1 gene possesses a promoter upstream of exon 1 and another intron promoter, which encodes several transcripts¹⁹⁻²¹. Our previous study demonstrated that ligands for liver-X-receptor (LXR) dramatically increase the activity of the ABCG1 promoter located upstream of exon 1²²; therefore, a ABCG1 nucleotide polymorphism in this LXR-responsive promoter is examined.

Here, we describe the first evidence of the effect of ABCG1 gene transcription in -257T>G polymorphism by *in vitro* reporter assay, and that of ABCG1 on CAD severity in Japanese men.

Subjects and Methods

Subjects

One hundred nine Japanese male patients with CAD selected from Fukuoka University Hospital were genotyped for ABCG1 -257T>G polymorphism (gene accession No.: AP001746.1; dbSNP ID (ss#): 5601744). All subjects had >75% stenosis in at least 1 of the 16 segments of the coronary arteries, determined by coronary angiography. Subject characteristics are described in **Table 1**. This study was approved by the local ethics committee of Fukuoka University

(No. 064) and Fukuoka University Hospital (No. 9-03 (07-48)), and written consent was obtained from all subjects.

Evaluation of Coronary Angiography

Coronary atherosclerosis severity was defined by the number of diseased coronary arteries, and angiographic characteristics of coronary atherosclerotic lesions were defined by AHA classification.

Materials

T0901317 and 9-cis-retinoic acid were purchased from Sigma (St Louis, MO). Fatty acid- and endotoxin-free bovine serum albumin was purchased from Calbiochem (San Diego, CA).

DNA Sequencing

Genomic DNA was extracted from buffy coat in EDTA samples using a QIAamp Blood Mini Kit (Qiagen, Hilden, Germany). The sequence upstream of exon 1 on the ABCG1 gene was amplified from genomic DNA using the following specific primers: sense primer, 5'-ATG AAT GAA AGA AGC CAG ACA CAA A-3' and antisense primer, 5'-CAC AAA CAT AGG TAG TCC AGC TGC-3'. DNA sequencing was performed as described previously²³.

Reporter Plasmid Constructs

Human ABCG1 promoter constructs-Firefly luciferase reporter constructs were generated by PCR amplification of a 1141-bp fragment of human ABCG1 promoter (-1104/+37) and then subcloned with pGL3-Basic vectors (Promega, Madison, WI), as described previously²².

Site-directed mutagenesis-Mutant plasmids were obtained for human ABCG1 promoter using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and each of the following double-stranded oligonucleotides. ABCG1 promoter -257T>G mutation: ABCG1 -257T>G sense, 5'-CGC CCA GTG ACT TGG GAG GGA ACA GAA CTG-3' and ABCG1 -257T>G antisense, 5'-CAG TTC TGT TCC CTC CCA AGT CAC TGG GCG-3'. The setting for mutagenesis was selected according to the manufacturer's protocol as described previously²². The identity of all DNA constructs was confirmed by DNA sequencing. All plasmids were purified using a Hi-speed Plasmid Midi Kit (Qiagen).

Transient Transfection and Reporter Assay

HEK293T cells (RIKEN, Japan) were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (GIBCO-BRL). Cells were

Table 1. Clinical characteristics of patients in different ABCG1 genotype groups

<i>n</i> = 109	T/T (<i>n</i> = 32)	T/G (<i>n</i> = 57)	G/G (<i>n</i> = 20)
Age, y	62.5 ± 2.2	65.8 ± 1.3	64.6 ± 1.9
HT (%)	84.4	77.2	75.0
HL (%)	43.8	52.6	60.0
DM (%)	50.0	40.4	55.0
Smoking (%)	43.8	54.4	60.0
OMI (%)	43.8	52.6	35.0
Low HDL-C (<35mg/dL) (%)	15.6	26.3	35.0*
Number of cardiovascular risks	3.1 ± 0.2	3.0 ± 0.2	3.7 ± 0.3
Number of metabolic factors	2.1 ± 0.2	2.0 ± 0.1	2.3 ± 0.3
BMI (kg/m ²)	23.8 ± 0.7	23.0 ± 0.3	23.2 ± 0.7
Statin (%)	20.0	29.6	31.6
Fibrate (%)	3.3	3.8	5.3
Ca-blocker (%)	63.3	77.4	73.7
ARB (%)	24.1	18.9	31.6
Serum lipid profiles			
TC (mg/dL)	172 ± 33	181 ± 36	179 ± 43
TG (mg/dL)	132 ± 71	124 ± 60	154 ± 80
LDL-C (mg/dL)	103 ± 31	111 ± 32	108 ± 35
HDL-C (mg/dL)	44 ± 11	46 ± 15	41 ± 11
non-HDL (mg/dL)	128 ± 33	136 ± 32	138 ± 44
HDL-C/LDL-C ratio	0.47 ± 0.30	0.47 ± 0.40	0.42 ± 0.40
HDL-C/TC ratio	0.26 ± 0.10	0.26 ± 0.10	0.24 ± 0.20

HT, hypertension; HL, hyperlipidemia; DM, diabetes mellitus; OMI, old myocardial infarction; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; BMI, body mass index; CCB, calcium channel blocker; ARB, angiotensin II type I receptor blockers; Cardiovascular risks are hypertension, diabetes mellitus, smoking, low HDL cholesterolemia (<35mg/dL), high LDL cholesterolemia (>140mg/dL) and high triglyceridemia (>150mg/dL). Metabolic factor is a criterion of the clinical diagnosis of metabolic syndrome (waist circumference substituted for BMI >25). Data are the mean ± S.D.; *, *p* = 0.1 vs. T/T.

transiently transfected with 1 μ g firefly luciferase reporter plasmids and co-transfected with 5 ng pGL4.74 vector (Promega) for control Renilla luciferase by lipofection with 2 μ L Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) using 5×10^5 cells per well according to the manufacturer's instructions. After 6 h lipofection, the cells were incubated with or without T0901317 and 9-cis-retinoic acid for 20 h, as indicated. Luciferase activities were measured as described previously^{22, 24}.

Mutation-Selective (MS) PCR for ABCG1 Promoter - 257T > G Mutation

MS-PCR is performed in a one-step PCR reaction, and therefore, a second restriction-digestion step is not required. The ratio of short to long allele-specific primer was maintained as 2.1:1 on a molar basis to produce bands of similar intensity in the heterozygote when visualized on agarose gel. The MS-PCR reaction mixture contained 2.5 μ L DNA, 2 μ L of each dNTP, 0.2 μ M common upper primer (CCA

CTA TGT TCA CGA ATG TAC), 0.4 μ M short primer (CTA AAG GGC AGT TCT GTT CCG TCA), 0.08 μ M long primer (CAG GAA GTG AGC AGG GTT AGA AAA GGG CAG TTC TGT TCC CAC C), and 0.125 μ L HotstarTaq DNA polymerase (Qiagen) and 2.5 μ L buffer in 25 μ L total volume of the reaction as recommended by the supplier. After denaturing DNA for 15 min at 95°C, the reaction mixture was subjected to 30 cycles of denaturing for 1 min at 94°C, 1 min of annealing at 60°C, 1.5 min extension at 72°C, and a final extension for 10 min at 72°C. The amplified PCR products were separated by 3% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light (**Fig. 1**).

Statistical Analyses

All statistical analyses were performed using SAS software (Statistical Analysis System; SAS Institute Inc. Cary, NC) at Fukuoka University. Differences in categorical variables among subjects with single- and multi-vessel diseases were analyzed by the chi-square

Fig. 1. MS-PCR for genotyping of ABCG1 -257T>G.

For PCR conditions, see "Subjects and Methods." PCR products were analyzed by separating different-sized allelic amplification products using 3.0% gel electrophoresis. Long 161-bp and short 142-bp products indicate G and T allele-specific products, respectively. Lane 1, T/T genotype; lane 2, T/G genotype; lane 3, G/G genotype.

(χ^2) method. The association between the frequency of multi-vessel disease in CAD and the dominant, recessive, and additive effects of the ABCG1 polymorphism G allele were determined by logistic regression analysis. ANOVA and χ^2 analysis were performed to test for differences in age, smoking, body mass index, plasma levels of lipids, hypertension, diabetes mellitus, and a history of myocardial infarction among the genotype groups. *t*-test and ANOVA were used to assess differences in ABCG1 allelic variants and luciferase activity in cells transfected with different constructs.

Results

DNA Sequencing Analysis on ABCG1 Promoter

Twenty healthy volunteers were screened for the DNA sequence on the ABCG1 promoter region upstream of exon 1, which is reported to be a functional promoter with a LXR-responsive element²². Twelve subjects had the ABCG1 promoter -257T>G mutation.

Transcription Activity on ABCG1 Promoter -257T>G Mutation

To investigate whether -257T>G polymorphism influenced promoter activity, transient transfection and luciferase reporter gene assays were performed in cultured cells. In these experiments, the amount of luciferase produced by the construct containing the G allelic sequence was 25.7% lower than that produced by the construct containing the T allelic sequence, significantly ($p < 0.01$) (**Fig. 2A**). ABCG1 promoter

Fig. 2. In vitro ABCG1 promoter activity assay.

Luciferase activities in HEK293T transfected with the aforementioned plasmid constructs after standardizing against activities of Renilla luciferase produced by a cotransfected plasmid. A, ABCG1 transcription activity on the construct containing the T or G allelic sequence. B, ABCG1 transcription activity stimulated with 5 μ mol/L T0901317 (T0) and 9-cis-retinoic acid (9cisRA) on the construct containing the T or G allelic sequence. Data shown are the mean $\pm$ S.D. values of three experiments in quadruplicate.

activity, induced by LXR and retinoid-X-receptor (RXR) agonists, increased 4.6-fold, and the amount of luciferase produced by the construct containing the G allelic sequence was 30.9% lower than that produced by the construct containing the T allelic sequence, significantly ($p < 0.001$) (**Fig. 2B**).

Genotypic Effects of ABCG1 -257T>G Polymorphism on Atherosclerosis Severity

One hundred nine Japanese male patients with CAD underwent genotyping for ABCG1 -257T>G polymorphism by MS-PCR (**Fig. 1**). Frequencies of the T/T, T/G, and G/G genotypes in this study were 29.4% ($n=32$), 52.3% ($n=57$), and 18.3% ($n=20$), respectively. Age, percentage of smokers, prevalence of hypertension, hyperlipidemia, and diabetes mellitus did not significantly differ among genotype groups. There was a trend toward a greater number of cases of low-HDL cholesterolemia among subjects with the G/G genotype, but this was not statistically significant. In addition, there was no significant difference in serum levels of total, LDL, and HDL cholesterol, and triglyceride among genotype groups (**Table 1**).

Logistic Regression Analysis of ABCG1 -257T>G Polymorphism in Single- and Multi-Vessel CAD

Logistic regression analysis revealed an interaction between genotype and CAD severity. Genotype frequencies in the overall sample were grouped according to whether patients had multi- or single-vessel

Table 2. Characteristics of patients overall and with single- and multiple-vessel diseases

	Overall (n = 109)	Single vessel (n = 33)	Multi-vessel (n = 76)
Age (y)	65.0 ± 0.9	63.1 ± 1.9	65.8 ± 1.0
BMI (kg/m ²)	23.2 ± 0.3	23.3 ± 0.6	23.1 ± 0.3
Hypertension (n, %)	85 (78.0%)	27 (81.8%)	58 (76.3%)
Diabetes (n, %)	50 (45.9%)	17 (51.5%)	33 (43.4%)
Hyperlipidemia (n, %)	54 (50.0%)	14 (42.2%)	40 (53.3%)
Lipid-lowering drugs (n, %)	31 (28.4%)	7 (21.2%)	24 (31.6%)
Total cholesterol (mg/dL)	178 ± 3	168 ± 5	183 ± 4*
Triglyceride (mg/dL)	132 ± 6	139 ± 11	128 ± 7
HDL cholesterol (mg/dL)	44 ± 1	44 ± 2	44 ± 2
LDL cholesterol (mg/dL)	108 ± 3	97 ± 5	113 ± 4
Genotype frequencies of the ABCG1 - 257T > G polymorphism			
T/T (n, %)	32 (29.4%)	16 (48.5%)	16 (21.1%)
T/G (n, %)	57 (52.3%)	12 (36.4%)	45 (59.2%)
G/G (n, %)	20 (18.4%)	5 (15.2%)	15 (19.7%)*

HDL, high-density lipoprotein; LDL, low-density lipoprotein; *, $p < 0.05$, by chi-square analysis

Table 3. Odds ratio for multiple-vessel disease according to ABCG1 - 257T > G genotype

Variable	Effect of the G allele	Odds ratio (95% CI)*	p value
T/T vs. T/G vs. G/G	Additive effect	2.1 (1.1–4.0)	0.027
T/T vs. T/G and G/G	Dominant effect	3.5 (1.5–8.6)	0.005
T/T and T/G vs. G/G	Recessive effect	1.4 (0.5–4.2)	0.571

Logistic regression analysis of the additive, recessive, and dominant effects of the ABCG1 G allele on multi-vessel disease. CI, confidence interval; *, denotes confidence interval

CAD. There was a significantly higher level of serum total cholesterol, but not LDL cholesterol, in the group with multi-vessel disease (**Table 2**). Interestingly, the adjusted relative risk associated with the G allele (assuming an additive effect) in the matched-pair analysis was 2.1 (95% confidence interval, 1.1 to 4.0; $p = 0.027$) for multi-vessel CAD compared to single-vessel CAD and 3.5 (95% confidence interval, 1.5 to 8.6; $p = 0.005$) for G/G and T/G genotypes compared to T/T (assuming a dominant effect of the G allele). Additional analyses assuming a recessive effect of the G allele were not significant (**Table 3**).

Discussion

This is the first report to show a novel ABCG1 promoter SNP and the influence of the ABCG1 gene variant on atherosclerotic disease in humans. The present results indicate that, in Japanese men, the presence of the G allele of - 257T > G polymorphism on the ABCG1 gene confers an appreciable increase in the risk of multi-vessel CAD. ABCG1 is upregulated in macrophages of patients with Tangier's disease,

which is caused by a defect in the ABCA1 gene, suggesting cooperative ABCA1 and ABCG1 regulation and a role of the two subfamilies in lipid homeostasis²⁵. Although the mechanism remains elusive, the ABCG1 transporter may potentially assume an important function in HDL lipidation^{10, 11}. In the present result, there was no significant difference in the serum HDL-C level among the genotype of ABCG1 - 257T > G. This result suggests that the turnover of HDL metabolism may accelerate as a consequence of ABCG1 influencing the change in lipid composition in HDL particle, although overall cholesterol concentration in HDL particles is not affected.

In an animal model system, Kennedy *et al.* showed that administration of a high-fat, high-cholesterol diet to ABCG1-deficient mice resulted in massive accumulation of lipids in tissue macrophages, whereas overexpression of human ABCG1 protected the murine tissues from dietary fat-induced lipid accumulation¹³. Transplantation of ABCG1-deficient (ABCG1^{-/-}) bone marrow into LDL receptor-deficient mice was recently reported to have contrasting effects on atherosclerotic formation^{15–17}. Moderate

protection against atherosclerotic lesion formation was shown in macrophage ABCG1 (–/–) on LDL receptor-deficient mice¹⁶. In contrast, Baldan *et al.* observed an increase in lesion formation in mice, and Ranalletta *et al.* found a 32% increase in lesion area in the presence of macrophage ABCG1 in mice¹⁷. Recently, Out *et al.* also demonstrated that total body ABCG1 expression protects against early atherosclerotic lesion development¹⁸.

Several ABC lipid transporters, including ABCA1 and ABCG1, have been shown to be strongly regulated by cholesterol metabolites such as oxysterols^{19, 22, 24}, activating nuclear receptors, LXR α and LXR β ²⁶. LXR α is mainly expressed in the liver, spleen, kidney, adipose tissue, small intestine, and macrophages²⁷, whereas LXR β is expressed ubiquitously²⁸. LXRs form obligate heterodimers with RXR. The LXR/RXR heterodimer binds to lipogenic target gene promoters such as 7 α -hydroxylase (CYP7A), apoE, cholesterol ester transfer protein, SREBP1c, ABCA1, and ABCG1, all of which contain a direct repeat 4 element in their promoter, i.e., two direct hexameric repeats separated by four nucleotides^{27, 29}. The ABCG1 gene has at least two promoters, one upstream of exon 1 and the other upstream of exon 5, and encodes for several transcripts^{19, 20}. The ligands for LXR and RXR markedly increased the activity of the ABCG1 promoter located upstream of exon 1, but did not alter the activity of that located within intron 4, suggesting that the promoter upstream of exon 1 is responsible for LXR/RXR activation as well as the ABCA1 promoter²². In the present results, transcription activity produced by the construct containing the G allelic sequence was significantly lower than that produced by the construct containing the T allelic sequence. Furthermore, the ABCG1 promoter showed increased activity via stimulation of LXR and RXR, and a similar genotype-dependent effect on ABCG1 gene transcription in this condition was identified. These results suggest that ABCG1 –257T>G promoter polymorphism may be an isolated regulating factor for ABCG1 gene transcription activity, independent of LXR and RXR.

The present results are consistent with the notion that ABCG1 gene variations may contribute to inter-individual variability in atherosclerosis susceptibility and severity. ABCG1 expression levels in atherosclerotic tissues might be lower in G allele carriers, and a possible mechanism for the increased incidence of atherosclerosis in such individuals.

In our preliminary analysis of 250 healthy subjects and non CAD patients showed that the frequencies of T/T, T/G, and G/G genotypes were 34.0% ($n=85$), 48.8% ($n=122$), and 17.2% ($n=43$), respec-

tively. This result showed less frequent G/G genotypes than in the present result; however, there are potential limitations to the present study. First, we did not select angiographically defined normal subjects as controls, because a selection bias is known to exist. Angiographically defined normal subjects generally have more risk factors for CAD than patients with clinical symptoms who have not been selected for angiography, because a person with both chest pain and a known risk factor is more likely to be referred for angiography than a person with just clinical symptom. Our findings indicate that ABCG1 is associated with CAD progression; however, it is not clear whether ABCG1 contributes to the occurrence of CAD in non-atherosclerotic subjects. Other limitations of this study are that only Asian men were used and the sample size was relatively small. Further investigations using large clinical trials are required to confirm our preliminary observations concerning the contribution of ABCG1 to CAD or atherosclerosis.

In summary, the results of this clinical study indicate that, in Japanese men, the presence of the G allele of –257T>G polymorphism on the ABCG1 gene confers an appreciable increase in the risk of multi-vessel CAD. Although the mechanism remains elusive, the ABCG1 transporter may potentially assume an important function in HDL lipidation^{10, 11}. It is conceivable that inactivation of the ABCG1 transporter may facilitate the progression of atherosclerosis in patients with CAD.

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