

# Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart  
Association®



*Learn and Live* SM

## **Genotypic Effect of the -565C>T Polymorphism in the ABCA1 Gene Promoter on ABCA1 Expression and Severity of Atherosclerosis**

Theodosios Kyriakou, Conrad Hodgkinson, David E. Pontefract, Srikanth Iyengar, W. Martin Howell, Yuk-ki Wong, Per Eriksson and Shu Ye  
*Arterioscler. Thromb. Vasc. Biol.* 2005;25;418-423; originally published online Nov 4, 2004;

DOI: 10.1161/01.ATV.0000149379.72018.20

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association,  
7272 Greenville Avenue, Dallas, TX 75214

Copyright © 2005 American Heart Association. All rights reserved. Print ISSN: 1079-5642. Online  
ISSN: 1524-4636

The online version of this article, along with updated information and services, is  
located on the World Wide Web at:

<http://atvb.ahajournals.org/cgi/content/full/25/2/418>

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular  
Biology is online at

<http://atvb.ahajournals.org/subscriptions/>

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters  
Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax:  
410-528-8550. E-mail:

[journalpermissions@lww.com](mailto:journalpermissions@lww.com)

Reprints: Information about reprints can be found online at

<http://www.lww.com/reprints>

# Genotypic Effect of the $-565C>T$ Polymorphism in the ABCA1 Gene Promoter on ABCA1 Expression and Severity of Atherosclerosis

Theodosios Kyriakou, Conrad Hodgkinson, David E. Pontefract, Srikanth Iyengar, W. Martin Howell, Yuk-ki Wong, Per Eriksson, Shu Ye

**Objective**—Loss-of-function mutations of the ATP-binding cassette transporter A1 (ABCA1) gene cause Tangier disease, a rare genetic disorder with accumulation of lipid-laden macrophages and increased risk of atherosclerosis. Common variants of this gene may be a genetic factor for atherosclerosis in the general population. This study was performed to test the reported association between the  $-565C>T$  polymorphism and atherosclerosis severity and to investigate whether this variant per se had an effect on promoter activity of the ABCA1 gene.

**Methods and Results**—A cohort of patients with coronary atherosclerosis were genotyped for the  $-565C>T$  polymorphism. Logistic regression analyses showed that homozygotes of the  $-565T$  allele had greatest mean number of diseased coronary arteries, particular in nonsmokers. Real-time reverse-transcriptase polymerase chain reaction showed that in atherosclerotic plaques removed from patients undergoing endarterectomy, ABCA1 expression levels were lowest in those who had the T/T genotype and highest in those of the C/C genotype. Transfection and reporter assays demonstrated that in cultured macrophages, the  $-565T$  allelic promoter had a lower activity in driving gene expression than the  $-565C$  allelic promoter. Electrophoretic mobility shift assays displayed differential binding of nuclear proteins to the 2 alleles.

**Conclusions**—These results indicate that the  $-565C>T$  polymorphism has an allele-specific effect on ABCA1 gene expression and provide further evidence of a genotypic effect on coronary atherosclerosis severity. (*Arterioscler Thromb Vasc Biol.* 2005;25:418-423.)

**Key Words:** ABCA1 ■ genetics ■ promoter ■ polymorphism ■ atherosclerosis

ATP-binding cassette transporter A1 (ABCA1) plays a pivotal role in efflux of intracellular cholesterol and phospholipids.<sup>1,2</sup> Loss-of-function mutations in the ABCA1 gene cause Tangier disease, a rare genetic disorder with accumulation of lipid-laden macrophages in tissues, absence of plasma high-density lipoprotein (HDL), and an increased risk of coronary artery disease (CAD) in some families.<sup>3-5</sup> A hypothesis has been put forward that ABCA1 gene variants that have high frequencies but modest effects may contribute to interindividual differences in CAD susceptibility and severity in the general population.

A polymorphism arising from a C-to-T substitution at position  $-565$  (designated as  $-477$  previously) in the ABCA1 gene promoter has been shown to be associated with severity of coronary atherosclerosis.<sup>6,7</sup> To verify this finding, we examined this polymorphism in a cohort of patients with angiographically documented CAD from Southern England. We also investigated whether ABCA1 was expressed at

different levels in individuals with different  $-565C>T$  genotype, and whether the polymorphism had an effect on promoter activity of the ABCA1 gene.

## Subjects and Methods

### Subjects

A cohort of 1170 white patients with CAD recruited from the Southampton General Hospital were genotyped for the  $-565C>T$  polymorphism. All subjects had  $>50\%$  stenosis in at least 1 of 16 segments of the coronary arteries, determined by coronary angiography. The characteristics of the subjects have been described previously.<sup>8</sup> Data for fasting levels of triglycerides, total cholesterol, HDL cholesterol, and low-density lipoprotein cholesterol were available for 1006, 1089, 630, and 319 subjects, respectively. The study was approved by the local ethical committee and all subjects gave written consent.

### Determination of Genotypes

Genotypes for the  $-565C>T$  polymorphism was determined by the tetra-primer ARMS PCR method<sup>9</sup> with the following primers:

Original received September 22, 2003; final version accepted October 7, 2004.

From the Human Genetics Division (T.K., C.H., D.E.P., W.M.H., S.Y.), School of Medicine, University of Southampton, Southampton, UK; the Cardiothoracic Unit (D.E.P., S.I., Y.W.), Southampton General Hospital, Southampton, UK; and the Atherosclerosis Research Unit (P.E.), King Gustaf V Research Institute, Karolinska Hospital, Stockholm, Sweden.

T.K. and C.H. contributed equally to this work.

Correspondence to Dr Shu Ye, Human Genetics Division, Duthie Building (mp 808), Southampton General Hospital, Southampton SO16 6YD, United Kingdom. E-mail Shu.Ye@soton.ac.uk

© 2005 American Heart Association, Inc.

*Arterioscler Thromb Vasc Biol.* is available at <http://www.atvbaha.org>

DOI: 10.1161/01.ATV.0000149379.72018.20

AAGCAGCCATTACCCAGAGGACTGGCC (forward inner primer), GCCTAGGCTGGGGTGAGGGGAAGTCA (reverse inner primer), GATGTTCTCTCGGGTCTCTGAGGGACC (forward outer primer), and AGCCAAGGGCACCAGTGAATTGCTTC (reverse outer primer).

### Real-Time Reverse-Transcriptase Polymerase Chain Reaction

RNA was extracted from a section of atherosclerotic plaques removed from patients undergoing carotid endarterectomy and converted to cDNA using an oligo-dT<sub>15</sub> primer. An adjacent section was stained with hemoxylin and eosin, and the percentages of fibrous tissue area and soft-lipid area were determined histologically with the use of a grid. Macrophages in an adjacent section of the atherosclerotic plaques were immunohistochemically stained with an antibody for CD68. Real-time polymerase chain reaction of the ABCA1 gene was performed in duplicates, using the following primers: GGACATGCGCAAAGTTCTGA (forward primer, located in exon 5) and CAGGAAATCTTGAAGCTTCAAG (reverse primer, located in exon 6). Polymerase chain reaction specificity was confirmed by dissociation curve analysis and gel electrophoresis. The  $2^{-\Delta\Delta Ct}$  method described by Livak and Schmittgen<sup>10</sup> was used to analyze the results. In brief, the Ct (threshold cycle) value of the ABCA1 gene was subtracted by the Ct value of a reference housekeeping gene (36B4, acidic ribosomal phosphoprotein P0)<sup>11–13</sup> to standardize for the amounts of RNA template and efficiencies of reverse transcription. The resulting  $\Delta Ct$  values were then converted to a linear form using  $2^{-\Delta Ct}$  and compared between genotype groups.

### Transient Transfection and Reporter Assays

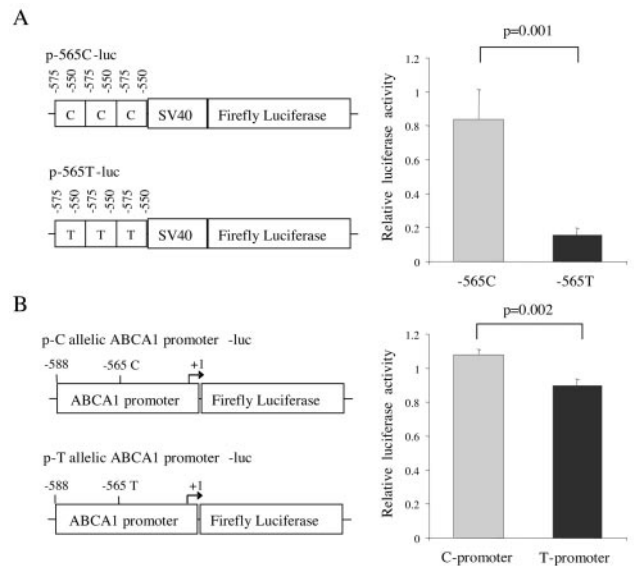
Promoter activity was analyzed using methods described previously.<sup>14</sup> Two sets of plasmid constructs were generated. In one set of these constructs, oligonucleotides corresponding to the sequence from nucleotide –575 to –550 (relative to the transcription start site) in the ABCA1 gene promoter, with either a C or T at the –565 polymorphic site,<sup>7</sup> were inserted into the pGL3-promoter vector (Promega, UK) (Figure 1A). In the other set of constructs, ABCA1 gene promoter sequences (position –588bp to +21bp), with either a C or T at the –565 polymorphic site, were inserted into the pGL3 basic vector (Promega) (Figure 1B). These constructs, together with the pRL-TK plasmid (Promega), were used to transfect cultured macrophages (RAW264.7 cells from ATCC). At 48 hours after transfection, the cells were lysed and subjected to luciferase activity assay using a dual-luciferase reporter assay system (Promega). The result is expressed as the ratio of firefly luciferase activity over *Renilla* luciferase activity.

### Electrophoretic Mobility Shift Assay

Double-stranded oligonucleotide probes (C: AGAGGACTGTC-CGCCTTCCCCTACC; and T: AGAGGACTGTCCTTC-CCCTACC) corresponding to the sequence from nucleotide –575 to –550 in the ABCA1 gene promoter,<sup>7</sup> with either a C or T at the –565 polymorphic site were labeled with [ $\gamma$ -<sup>32</sup>P] ATP. The probes were incubated with nuclear protein extracts from RAW264.7 cells or THP-1 cells, in the presence or absence of competitors, ie, unlabeled probe C (referred to as competitor C), unlabeled probe T (referred to as competitor T), or a nonspecific sequence (referred to as nonspecific competitor). Protein–DNA complexes were resolved by polyacrylamide gel electrophoresis and detected by autoradiography.

### Statistical Analyses

The HWE program (<ftp://linkage.rockefeller.edu/software/utilities>) was used to examine whether the observed genotype distribution deviated from Hardy–Weinberg equilibrium. ANOVA and  $\chi^2$  analysis were performed to test differences between genotype groups in age, gender ratio, smoking habit, body mass index, plasma levels of total cholesterol, HDL cholesterol and triglyceride, hypertension, diabetes mellitus, and family CAD history. Ordinal logistic regression analyses were performed to examine differences in number of



**Figure 1.** Promoter activity assay. A, Left panel shows schematic representation of the plasmid constructs containing 3 concatenated copies of a 26-bp promoter sequence corresponding to the sequence from nucleotide –575 to –550 of the C or T allele, upstream of a luciferase reporter gene. Right panel shows luciferase activities in macrophages transfected with the aforementioned plasmid constructs after standardizing against activities of *Renilla* luciferase produced by a cotransfected plasmid. Data shown are mean ( $\pm$ SEM) values of 3 experiments in quadruplicates. B, Left panel shows schematic representation of the plasmid constructs containing the ABCA1 gene promoter with either a C or T at the –565 polymorphic site, upstream of a luciferase reporter gene. Right panel shows luciferase activities in macrophages transfected with the ABCA1 promoter–luciferase gene plasmid constructs after standardizing against activities of *Renilla* luciferase produced by a cotransfected plasmid. Data shown are mean ( $\pm$ SEM) values of 3 experiments in quadruplicates.

diseased coronary arteries between genotype groups. The *t* test and ANOVA were used to assess differences in ABCA1 transcript abundance in atherosclerotic plaques from patients with different genotypes and differences in luciferase activity in cells transfected with different constructs.

## Results

### Genotypic Effects of the –565C>T Polymorphism on Atherosclerosis Severity

A total of 1159 of the CAD patients were successfully genotyped for the –565C>T polymorphism. The frequencies of the C/C, C/T, and T/T genotypes in this cohort were 0.30 ( $n=351$ ), 0.49 ( $n=565$ ), and 0.21 ( $n=243$ ), respectively. This genotype distribution was consistent with Hardy–Weinberg equilibrium.

There was no significant difference in plasma levels of total cholesterol, HDL cholesterol, low-density lipoprotein cholesterol, and triglycerides among the genotype groups (Table 1). Age, gender ratio, percentage of smokers, prevalence of hypertension, and diabetes mellitus did not significantly differ among the genotype groups (Table 1).

In the sample as a whole, there was a trend toward greater number of diseased coronary arteries in T/T homozygotes, but the differences were not statistically significant (Table 2). Logistic regression analysis revealed an interaction between

**TABLE 1. Demographic, Biochemical, and Clinical Characteristics of Subjects in Different ABCA1 Genotype Groups**

	C/C	C/T	T/T	P
Age, y	62.59 (10.09)	63.35 (9.83)	64.28 (10.00)	0.115
Male, %	76.42	76.84	75.41	0.907
Current and ex-smokers, %	75.78	75.22	70.78	0.329
Body mass index, kg/m <sup>2</sup>	27.37 (4.28)	27.62 (4.29)	27.38 (4.16)	0.620
Total cholesterol, mmol/L	5.15 (1.07)	5.16 (1.02)	4.98 (0.97)	0.067
HDL-C, mmol/L	1.29 (0.64)	1.28 (0.32)	1.26 (0.35)	0.709
LDL-C, mmol/L	2.78 (0.67)	2.77 (0.78)	2.89 (0.87)	0.576
Triglycerides, mmol/L	1.84 (1.09)	1.88 (1.12)	1.87 (1.58)	0.886
Hypertension, %	40.63	47.89	43.44	0.088
Type 2 diabetes mellitus, %	9.30	10.16	12.66	0.420
Family CAD history, %	48.30	48.77	47.95	0.975

Mean (SD) shown for continuous variables.

HDL-C indicates high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; CAD, coronary artery disease.

genotype and smoking in determining the number of diseased vessels ( $P=0.009$ ). Therefore, further analyses of ABCA1 genotypic effects were performed in smokers and nonsmokers separately. In nonsmokers, the number of diseased vessels were greatest in homozygotes for the T allele, intermediate in heterozygotes, and smallest in homozygotes for the C allele ( $P=0.001$  for T/T versus C/C, and  $P=0.03$  for T/C versus C/C; Table 2). These differences remained significant after adjusting for age, gender, body mass index, cholesterol levels, HDL levels, and diabetes ( $P=0.002$  for T/T versus C/C, and  $P=0.02$  for T/C versus C/C; Table 2). In smokers, these measurements did not significantly differ among the genotype groups (Table 2). There was also an interaction between gender and genotype ( $P=0.02$ ). The association between the T allele and greater number of disease vessels was most pronounced in female nonsmokers, less pronounced in male nonsmokers, and not significant in female and male smokers (Table 2).

#### Less ABCA1 Expression in $-565T$ Allele Carriers

To investigate whether ABCA1 expression in atherosclerotic plaques differed between patients of different genotypes for the  $-565C>T$  polymorphism, ABCA1 mRNA levels in atherosclerotic plaques were quantified using the real-time reverse-transcriptase polymerase chain reaction method. The assays showed that the amounts of ABCA1 transcript were lowest in T/T homozygotes and highest in C/C homozygotes, and the differences remained after adjusting for age, gender, smoking, macrophage contents in atheroma, percentage of fibrous tissue in atheroma, and percentage of soft lipid area in atheroma ( $P=0.041$ ; Table 3).

#### Allele-Specific Effect of the $-565C>T$ Polymorphism on Promoter Activity

To investigate whether the  $-565C>T$  polymorphism had an effect on promoter activity, transient transfection and luciferase reporter gene assays were performed in cultured macrophages. In these experiments, the amount of luciferase produced by the construct containing the T allelic sequence

was lower than that produced by the construct containing the C allelic sequence (Figure 1).

#### Allele-Specific Effect of the $-565C>T$ Polymorphism on Binding of Nuclear Proteins to the ABCA1 Gene Promoter

To investigate whether the  $-565C>T$  polymorphism was located at a transcription factor binding site and, if so, whether the binding of the transcription factor(s) differed for the C and T alleles, electrophoretic mobility shift assays were performed in which radiolabeled probes corresponding to the C or T allele were incubated with nuclear protein extracts from monocytes/macrophages, ie, RAW264.7 and THP-1 cells, respectively. In the assays with RAW264.7 cell nuclear protein extracts (Figure 2A), 3 major DNA-protein complexes were readily detected using the probe corresponding to the C allele (lane 1), and the intensities of these bands were markedly reduced in the presence of unlabeled C allele probe (lanes 2 and 3), but not affected by unlabeled T allele probe (lanes 4 and 5) or a nonspecific competitor (lane 6). The intensities of these bands were substantially weaker in the assays using the probe corresponding to the T allele (lanes 7 to 12; Figure 2A). In the assays with THP-1 cell nuclear protein extracts (Figure 2B), a major DNA-protein complex was detected using the C allele probe, and its band intensity was markedly reduced in the presence of unlabeled C allele probe. The intensity of the corresponding band was substantially lower in the assays using the T allele probe (Figure 2B).

#### Discussion

The results of this study are consistent with the notion that ABCA1 gene variations may contribute to interindividual variability in atherosclerosis susceptibility and severity. The study showed that individuals carrying the T allele were more likely to have more severe atherosclerosis, supporting the findings from a study by Lutucuta et al.<sup>6</sup> In addition, we found that ABCA1 expression levels in ex vivo atherosclerotic tissues were lower in T allele carriers, a possible mechanism for greater atherosclerosis in such individuals. In

TABLE 2. Severity of Coronary Atherosclerosis in Different Genotype Groups

Gender	Genotype	Single Vessel Disease	Double Vessel Disease	Triple Vessel Disease	Mean (SD) Number of Diseased Vessels	Odds Ratio (95% CI)	Odds Ratio (95% CI) Adjusted for Covariates*
Sample as a whole							
Male and female	T/T	92 (19.4%)	83 (21.1%)	69 (23.2%)	1.90 (0.81)	1.17 (0.86–1.57), NS	1.09 (0.80–1.48), NS
	C/T	238 (50.2%)	190 (48.2%)	142 (47.7%)	1.83 (0.80)	0.98 (0.77–1.26), NS	0.94 (0.73–1.20), NS
	C/C	144 (30.4%)	121 (30.7%)	87 (29.1%)	1.84 (0.80)	Reference	Reference
	Total	474 (100%)	394 (100%)	298 (100%)			
Nonsmokers							
Male and female	T/T	18 (15.4%)	28 (29.5%)	25 (29.8%)	2.10 (0.78)	2.64 (1.46–4.79), <i>P</i> =0.001	2.63 (1.43–4.83), <i>P</i> =0.002
	C/T	57 (48.7%)	37 (38.9%)	46 (54.8%)	1.92 (0.86)	1.77 (1.07–2.94), <i>P</i> =0.03	1.82 (1.08–3.07), <i>P</i> =0.02
	C/C	42 (35.9%)	30 (31.6%)	13 (15.5%)	1.66 (0.73)	Reference	Reference
	Total	117 (100%)	95 (100%)	84 (100%)			
Male	T/T	10 (16.9%)	17 (30.4%)	16 (29.1%)	2.14 (0.77)	2.16 (1.00–4.69), <i>P</i> =0.05	2.50 (1.11–5.59), <i>P</i> =0.03
	C/T	30 (50.8%)	21 (37.5%)	30 (54.5%)	2.00 (0.87)	1.60 (0.82–3.13), NS	1.89 (0.94–3.79), NS
	C/C	19 (32.2%)	18 (32.1%)	9 (16.4%)	1.78 (0.76)	Reference	Reference
	Total	59 (100%)	56 (100%)	55 (100%)			
Female	T/T	8 (13.8%)	11 (28.2%)	9 (31.0%)	2.04 (0.79)	3.38 (1.33–8.60), <i>P</i> =0.01	3.12 (1.19–8.22), <i>P</i> =0.02
	C/T	27 (46.6%)	16 (41.0%)	16 (55.2%)	1.81 (0.84)	2.00 (0.91–4.39), NS	2.24 (0.99–5.10), <i>P</i> =0.05
	C/C	23 (39.7%)	12 (30.8%)	4 (13.8%)	1.51 (0.68)	Reference	Reference
	Total	58 (100%)	39% (100%)	29 (100%)			
Smokers							
Male and female	T/T	74 (20.8%)	55 (18.6%)	43 (20.3%)	1.82 (0.79)	0.84 (0.59–1.19), NS	0.78 (0.54–1.11), NS
	C/T	179 (50.4)	151 (51.0%)	95 (44.8%)	1.80 (0.78)	0.81 (0.61–1.08), NS	0.77 (0.57–1.02), NS
	C/C	102 (28.7%)	90 (30.4%)	74 (34.9%)	1.89 (0.81)	Reference	Reference
	Total	355 (100%)	296 (100%)	212 (100%)			
Male	C/C	62 (21.8%)	41 (17.1%)	38 (19.8%)	1.83 (0.83)	0.73 (0.50–1.09), NS	0.67 (0.46–1.02), NS
	C/T	143 (50.2%)	127 (52.9%)	83 (43.2%)	1.83 (0.78)	0.75 (0.55–1.02), NS	0.71 (0.52–1.01), NS
	T/T	80 (28.1%)	72 (30.0%)	71 (37.0%)	1.96 (0.82)	Reference	Reference
	Total	285 (100%)	240 (100%)	192 (100%)			
Female	C/C	12 (17.1%)	14 (25.0%)	5 (25.0%)	1.77 (0.72)	1.72 (0.72–4.15), NS	1.27 (0.49–3.29), NS
	C/T	36 (51.4%)	24 (42.9%)	12 (60.0%)	1.67 (0.75)	1.24 (0.60–2.55), NS	1.27 (0.58–2.79), NS
	T/T	22 (31.4%)	18 (32.1%)	3 (15.0%)	1.56 (0.63)	Reference	Reference
	Total	70 (100%)	56 (100%)	20 (100%)			

\*Adjusted for age, gender, body mass index, smoking, cholesterol levels, HDL levels, and diabetes. NS indicates not significant.

concordance with this finding, the in vitro assays in macrophages showed that the T allelic promoter had a lower activity in driving gene expression than the C allelic promoter, and that there were differential binding of nuclear proteins with the 2 allelic promoters, which could be an explanation for the difference in promoter activity between the 2 alleles. These data suggest that the  $-565C>T$  polymorphism is not merely a genetic marker for other polymorphisms at this genomic locus through linkage disequilibrium but rather has a direct effect on ABCA1 expression.

A number of polymorphisms in the ABCA1 gene have been identified. In a recent study in which 13 polymorphisms in the promoter and 10 in the coding region of the gene were analyzed in relation to plasma apoAI levels and risk of myocardial infarction, Tregouet et al found that among the promoter polymorphisms studied, only the  $-565C>T$  was associated with apoAI levels (no associa-

tion of myocardial infarction with any of the promoter polymorphisms was found, and there was no significant linkage disequilibrium between the promoter polymorphisms and coding region polymorphisms), which highlights the importance of the  $-565C>T$  polymorphism.<sup>15</sup> Previously Lutucuta et al<sup>6</sup> showed that patients carrying the  $-565T$  allele had more severe coronary atherosclerosis. In agreement with their finding, we observed in the present study a trend toward greater atherosclerosis in  $-565T$  allele carriers in the sample as a whole. In addition, we detected interactions of genotype with smoking and gender, with a significant association of the T allele with greater atherosclerosis severity in nonsmokers, particularly in females. An interaction between another ABCA1 gene polymorphism (ie, R219K) and smoking has been reported previously, although the underlying mechanisms remain unknown.<sup>16</sup>

**TABLE 3. Results of Real-Time Reverse-Transcriptase Polymerase Chain Reaction**

Genotype	Mean (SEM)
C/C	23.16 (2.09), n=10
T/C	24.10 (1.92), n=14
T/T	28.41 (1.59), n=4
	*P=0.041

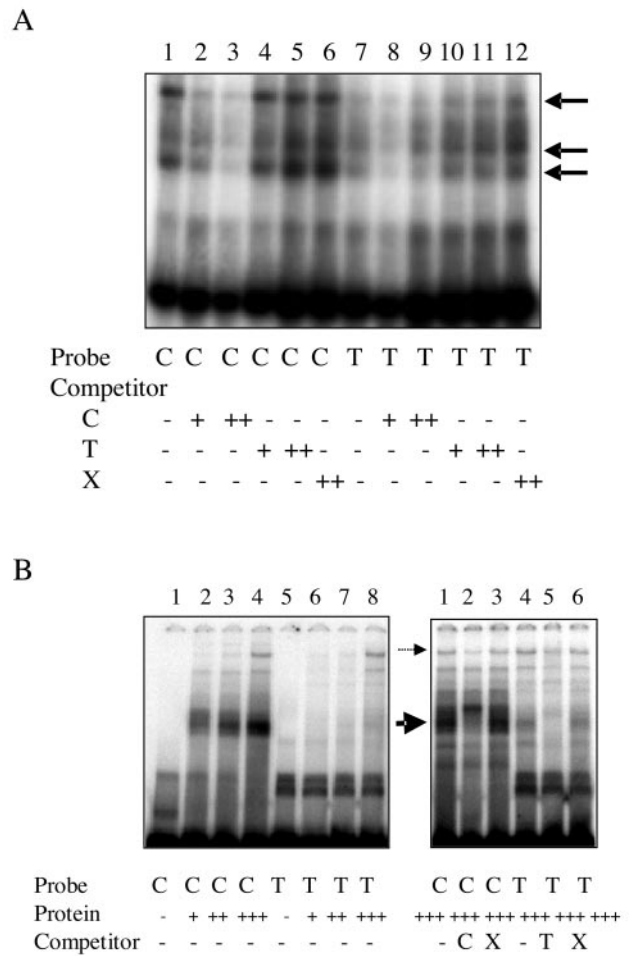
Shown are mean ΔCt values [the Ct (threshold cycle) value of the ABCA1 gene subtracted by the Ct value of the reference housekeeping gene] in different genotype groups; the larger the ΔCt value, the lower the mRNA level.

\*Adjusted for age, gender, smoking, macrophage contents in atheroma, percentage of fibrous tissue in atheroma, and percentage of soft lipid area in atheroma.

Several other polymorphisms in the ABCA1 gene have been associated with various cardiovascular traits. For example, the R219K polymorphism has been shown to be associated with risk of myocardial infarction and/or severity of atherosclerosis,<sup>15-19</sup> the V825I, M883I, and R1587K polymorphisms with various cardiovascular traits,<sup>17,20,21</sup> the -191G>C, -17C>G, and 69C>T polymorphisms with risk of coronary events in patients with coronary atherosclerosis,<sup>22</sup> and the 319insG polymorphism with severity of atherosclerosis.<sup>22</sup> Taken together, these data suggest that the development and outcome of atherosclerosis might be influenced by a qualitative change of the ABCA1 protein caused by coding region variants and a quantitative change in ABCA1 expression caused by regulatory region variants.

There is, however, some differences in the findings of different studies. For example, in a study of 465 Japanese patients with myocardial infarction or angina pectoris, Takagi et al<sup>23</sup> did not find an association between severity of atherosclerosis and the -565C>T polymorphism, nor did they find an association between severity of atherosclerosis and the ABCA1 gene R219K polymorphism, which has been shown to be associated with atherosclerosis severity in several other studies.<sup>15-19</sup> Thus, it appears that the genotypic effects of ABCA1 may be influenced by other factors such as genetic backgrounds and environmental factors.

There is emerging evidence suggesting that ABCA1 gene variants can exert phenotypic effects on atherosclerosis independent of changes in plasma lipid levels.<sup>6,16-18,22</sup> The results of the present study are in agreement with this notion. There was no significant difference in plasma levels of total cholesterol, HDL cholesterol, low-density lipoprotein cholesterol, and triglycerides among the -565C>T genotype groups in the sample examined in this study. Lutucuta et al<sup>6</sup> also found no significant difference in lipid levels between the -565C>T genotype groups, whereas Zwartz et al found no association between lipid levels and the -191G>C, -17G>C, C69T, and 319ins polymorphisms, which were associated with coronary events or atherosclerosis severity. Interestingly, a study of ABCA1 transgenic mice<sup>24</sup> and a study of mice that were selectively deficient in leukocyte ABCA1<sup>25</sup> showed that ABCA1 had a significant effect on the development of atherosclerosis in the absence of a significant influence on plasma HDL cholesterol level. However, the increase in ABCA1 level in the transgenic mice resulted in a



**Figure 2.** Electrophoretic mobility shift assay. A, Representative results of electrophoretic mobility shift assays with nuclear extracts from RAW264.7 cells. Nuclear protein extracts derived from RAW264.7 monocytes/macrophages were incubated with radiolabeled probes corresponding to the C allele (lanes 1 to 6) or the T allele (lanes 7 to 12) in the absence or presence of competitors. Lanes 1 and 7, no competitor; lanes 2 and 8, competitor C in 10-fold molar excess (+); lanes 3 and 9, competitor C in 20-fold molar excess (++); lanes 4 and 10, competitor T in 10-fold molar excess (+); lanes 5 and 11, competitor T in 20-fold molar excess (++); lanes 6 and 12, a nonspecific competitor (X) in 20-fold more excess (+++). Arrows indicate DNA-protein complexes. B, Representative results of electrophoretic mobility shift assays with nuclear extracts from THP-1 cells. Nuclear protein extracts derived from THP-1 monocytes/macrophages were incubated with radiolabeled probes corresponding to the C allele (lanes 1 to 4 in the panel on the left, and lanes 1 to 3 in the panel on the right) or the T allele (lanes 5 to 8 in the panel on the left, and lanes 4 to 6 in the panel on the right) in the absence or presence of competitors. C indicates competitor C; T, competitor T; X, nonspecific competitor; arrows, DNA-protein complexes; -, no extract; +, 0.01 mg/mL extract; ++, 0.03 mg/mL extract; +++, 0.1 mg/mL extract.

significant increase in efflux of cholesterol from macrophages,<sup>24</sup> suggesting that changes in ABCA1 activity and reverse cholesterol transport may alter the net flux of cholesterol from the vessel wall toward the liver, without necessarily altering plasma lipid levels.

In summary, the results of this study showed that the -565C>T polymorphism of the ABCA1 gene has an allelic-specific effect on promoter activity and ABCA1 mRNA

expression in atherosclerotic plaques, and provide further evidence of a genotypic effect of this polymorphism on atherosclerosis severity, consistent with the notion that common polymorphisms in the ABCA1 gene may contribute to interindividual variability in susceptibility to and severity of atherosclerosis.

### Acknowledgments

This work was supported by the British Heart Foundation (PG98183 and PG/02/053).

### References

- Attie AD, Kastelein JP, Hayden MR. Pivotal role of ABCA1 in reverse cholesterol transport influencing HDL levels and susceptibility to atherosclerosis. *J Lipid Res.* 2001;42:1717–1726.
- Tall AR, Costet P, Wang N. Regulation and mechanisms of macrophage cholesterol efflux. *J Clin Invest.* 2002;110:899–904.
- Brooks-Wilson A, Marcil M, Clee SM, Zhang LH, Roomp K, van Dam M, Yu L, Brewer C, Collins JA, Molhuizen HO, Loubser O, Ouelette BF, Fichter K, Ashbourne-Excoffon KJ, Sensen CW, Scherer S, Mott S, Denis M, Martindale D, Frohlich J, Morgan K, Koop B, Pimstone S, Kastelein JJ, Hayden MR. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat Genet.* 1999;22:336–345.
- Bodzioch M, Orso E, Klucken J, Langmann T, Bottcher A, Diederich W, Drobnik W, Barlage S, Buchler C, Porsch-Ozcurumez M, Kaminski WE, Hahmann HW, Oette K, Rothe G, Aslanidis C, Lackner KJ, Schmitz G. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat Genet.* 1999;22:347–351.
- Rust S, Rosier M, Funke H, Real J, Amoura Z, Piette JC, Deleuze JF, Brewer HB, Duverger N, Deneffe P, Assmann G. Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat Genet.* 1999;22:352–355.
- Lutucuta S, Ballantyne CM, Elghannam H, Gotto AM, Jr., Marian AJ. Novel polymorphisms in promoter region of atp binding cassette transporter gene and plasma lipids, severity, progression, and regression of coronary atherosclerosis and response to therapy. *Circ Res.* 2001;88:969–973.
- Santamarina-Fojo S, Peterson K, Knapper C, Qiu Y, Freeman L, Cheng JF, Osorio J, Remaley A, Yang XP, Haudenschild C, Prades C, Chimini G, Blackmon E, Francois T, Duverger N, Rubin EM, Rosier M, Deneffe P, Fredrickson DS, Brewer HB, Jr. Complete genomic sequence of the human ABCA1 gene: analysis of the human and mouse ATP-binding cassette A promoter. *Proc Natl Acad Sci U S A.* 2000;97:7987–7992.
- Ye S, Dunleavy L, Bannister W, Day LB, Tapper W, Collins AR, Day IN, Simpson I. Independent effects of the -219 G>T and varepsilon 2/ varepsilon 3/ varepsilon 4 polymorphisms in the apolipoprotein E gene on coronary artery disease: The Southampton Atherosclerosis Study. *Eur J Hum Genet.* 2003;11:437–443.
- Ye S, Dhillon S, Ke X, Collins AR, Day IN. An efficient procedure for genotyping single nucleotide polymorphisms. *Nucleic Acids Res.* 2001;29:E88.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25:402–408.
- Joseph SB, Castrillo A, Laffitte BA, Mangelsdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. *Nat Med.* 2003;9:213–219.
- Joseph SB, Laffitte BA, Patel PH, Watson MA, Matsukuma KE, Walczak R, Collins JL, Osborne TF, Tontonoz P. Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. *J Biol Chem.* 2002;277:11019–11025.
- Morgan AR, Rerkasem K, Gallagher PJ, Zhang B, Morris GE, Calder PC, Grimble RF, Eriksson P, McPheat WL, Shearman CP, Ye S. Differences in matrix metalloproteinase-1 and matrix metalloproteinase-12 transcript levels among carotid atherosclerotic plaques with different histopathological characteristics. *Stroke.* 2004;35:1310–1315.
- Zhang B, Ye S, Herrmann SM, Eriksson P, de Maat M, Arveiler D, Luc G, Cambien F, Hamsten A, Watkins H, Henney AM. Functional polymorphism in the regulatory region of gelatinase B gene in relation to severity of coronary atherosclerosis. *Circulation.* 1999;99:1788–1794.
- Tregouet DA, Ricard S, Nicaud V, Arnould I, Soubigou S, Rosier M, Duverger N, Poirier O, Mace S, Kee F, Morrison C, Deneffe P, Tiret L, Evans A, Deleuze JF, Cambien F. In-depth haplotype analysis of ABCA1 gene polymorphisms in relation to plasma ApoA1 levels and myocardial infarction. *Arterioscler Thromb Vasc Biol.* 2004;24:775–781.
- Cenarro A, Artieda M, Castillo S, Mozas P, Reyes G, Tejedor D, Alonso R, Mata P, Pocovi M, Civeira F. A common variant in the ABCA1 gene is associated with a lower risk for premature coronary heart disease in familial hypercholesterolaemia. *J Med Genet.* 2003;40:163–168.
- Clee SM, Zwinderman AH, Engert JC, Zwarts KY, Molhuizen HO, Roomp K, Jukema JW, van Wijland M, van Dam M, Hudson TJ, Brooks-Wilson A, Genest J, Jr, Kastelein JJ, Hayden MR. Common genetic variation in ABCA1 is associated with altered lipoprotein levels and a modified risk for coronary artery disease. *Circulation.* 2001;103:1198–1205.
- Brousseau ME, Bodzioch M, Schaefer EJ, Goldkamp AL, Kielar D, Probst M, Ordovas JM, Aslanidis C, Lackner KJ, Bloomfield RH, Collins D, Robins SJ, Wilson PW, Schmitz G. Common variants in the gene encoding ATP-binding cassette transporter 1 in men with low HDL cholesterol levels and coronary heart disease. *Atherosclerosis.* 2001;154:607–611.
- Evans D, Beil FU. The association of the R219K polymorphism in the ATP-binding cassette transporter 1 (ABCA1) gene with coronary heart disease and hyperlipidaemia. *J Mol Med.* 2003;81:264–270.
- Tan JH, Low PS, Tan YS, Tong MC, Saha N, Yang H, Heng CK. ABCA1 gene polymorphisms and their associations with coronary artery disease and plasma lipids in males from three ethnic populations in Singapore. *Hum Genet.* 2003;113:106–117.
- Harada T, Imai Y, Nojiri T, Morita H, Hayashi D, Maemura K, Fukino K, Kawanami D, Nishimura G, Tsuchida K, Monzen K, Yamazaki T, Mitsuyama S, Shintani T, Watanabe N, Seto K, Sugiyama T, Nakamura F, Ohno M, Hirata Y, Yamazaki T, Nagai R. A common Ile 823 Met variant of ATP-binding cassette transporter A1 gene (ABCA1) alters high density lipoprotein cholesterol level in Japanese population. *Atherosclerosis.* 2003;169:105–112.
- Zwarts KY, Clee SM, Zwinderman AH, Engert JC, Singaraja R, Loubser O, James E, Roomp K, Hudson TJ, Jukema JW, Kastelein JJ, Hayden MR. ABCA1 regulatory variants influence coronary artery disease independent of effects on plasma lipid levels. *Clin Genet.* 2002;61:115–125.
- Takagi S, Iwai N, Miyazaki S, Nonogi H, Goto Y. Relationship between ABCA1 genetic variation and HDL cholesterol level in subjects with ischemic heart diseases in Japanese. *Thromb Haemost.* 2002;88:369–370.
- Singaraja RR, Fievet C, Castro G, James ER, Hennuyer N, Clee SM, Bissada N, Choy JC, Fruchart JC, McManus BM, Staels B, Hayden MR. Increased ABCA1 activity protects against atherosclerosis. *J Clin Invest.* 2002;110:35–42.
- Van Eck M, Bos IS, Kaminski WE, Orso E, Rothe G, Twisk J, Bottcher A, Van Amersfoort ES, Christiansen-Weber TA, Fung-Leung WP, Van Berkel TJ, Schmitz G. Leukocyte ABCA1 controls susceptibility to atherosclerosis and macrophage recruitment into tissues. *Proc Natl Acad Sci U S A.* 2002;99:6298–6303.