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

Silvia Lutucuta, Christie M. Ballantyne, Hesham Elghannam, Antonio M. Gotto, Jr, A.J. Marian

Abstract—Identification of mutations in the ATP binding cassette transporter (ABCA1) gene in patients with Tangier disease, who exhibit reduced HDL cholesterol (HDL-C) and apolipoprotein A1 (apoA1) levels and premature coronary atherosclerosis, has led to the hypothesis that common polymorphisms in the ABCA1 gene could determine HDL-C and apoA1 levels and the risk of coronary atherosclerosis in the general population. We sequenced a 660-bp 5′ fragment of the ABCA1 gene in 24 subjects and identified 3 novel polymorphisms: −477C/T, −419A/C, and −320G/C. We developed assays, genotyped 372 participants in the prospective Lipoprotein Coronary Atherosclerosis Study (LCAS), and determined the association of the variants with fasting plasma lipids and indices of quantitative coronary angiograms obtained at baseline and 2.5 years after randomization to fluvastatin or placebo. Distribution of −477C/T and −320G/C genotypes were 127 CC, 171 CT, and 74 TT and 130 GG, 168 GC, and 75 CC, respectively, and were in complete linkage disequilibrium (P<0.0001). Data for −477C/T are presented. The −419A/C variant was uncommon (present in 1 of 63 subjects). Heterozygous subjects had a modest reduction in HDL-C (P=0.09) and apoA1 (P=0.05) levels and a lesser response of apoA1 to treatment with fluvastatin (P=0.04). The mean number of coronary lesions causing 30% to 75% diameter stenosis was greater in subjects with the TT genotype (3.1±2.1) or CT genotype (2.9±1.9) than in subjects with the CC genotype (2.2±1.8) (P=0.002). Similarly, compared with subjects with the CC genotype, greater numbers of subjects with the TT or CT genotype had ≥1 coronary lesion (P=0.001). No association between the genotypes and progression of coronary atherosclerosis or clinical events was detected. We conclude that ABCA1 genotypes are potential risk factors for coronary atherosclerosis in the general population. (Circ Res. 2001;88:969-973.)

Key Words: atherosclerosis ■ genetics ■ HDL cholesterol ■ ATP binding cassette transporter ■ apolipoprotein A1

Plasma levels of HDL cholesterol (HDL-C) and apolipoprotein A1 (apoA1) are under tight control of genetic factors, which are largely unknown. Recent identification of mutations in the ATP binding cassette transporter (ABCA1) gene in patients with Tangier disease,1–3 who also have very low plasma levels of HDL-C and apoA1 and an increased risk of premature coronary atherosclerosis, suggests a major role for the ABCA1 protein in regulating plasma HDL-C and apoA1 levels. This notion is further supported by the observation of an age-dependent reduction in HDL-C levels and an increased frequency of coronary artery disease in members of families with Tangier or familial hypoalphalipoproteinemia (HA) who were heterozygous for mutations in the ABCA1 gene.4 These discoveries in conjunction with the well-established role of HDL-C in protection against coronary atherosclerosis5,6 have led to the hypothesis that common polymorphisms in the ABCA1 gene could affect plasma levels of HDL-C and apoA1 and thus serve as genetic risk factors for coronary atherosclerosis in the general population.4,7 We sequenced the promoter region of the ABCA1 gene, detected novel polymorphisms, and analyzed their association with plasma levels of lipids, the severity and progression of coronary atherosclerosis, and the response to therapy in a prospective study of a well-characterized cohort.8

Materials and Methods

Study Population
All subjects provided informed consent, and the institutional review board approved the study. The design9 and primary result8 of the Lipoprotein and Coronary Atherosclerosis Study (LCAS) have been published. In brief, 429 subjects who were aged 35 to 75 years and had ≥1 coronary lesion causing 30% to 75% diameter stenosis and LDL cholesterol (LDL-C) of 115 to 190 mg/dL despite diet were randomized to fluvastatin (40 mg daily) or placebo. Total choles-
terol, LDL-C, HDL-C, triglyceride, lipoprotein(a), and apolipoprotein levels were measured in all subjects, and quantitative coronary angiography was performed in 340 subjects at baseline and 2.5 years after randomization. Baseline and final plasma level of lipids and indices of quantitative coronary angiograms were determined and used to assess the association of the genotypes with plasma lipids, with the severity, progression, and regression of coronary atherosclerosis, and with the response to treatment (pharmacogenetics). Clinical events monitored were definite or probable myocardial infarction, unstable angina requiring hospitalization, percutaneous coronary interventions, coronary artery bypass grafting, and death of any cause.

**Identification of Novel Polymorphisms in the Promoter Region**

A 660-bp 5′ fragment of the ABCA1 gene was amplified by polymerase chain reaction (PCR) (forward primer 5′-AGCAGTAAAGATGTTCCTCTGGG-3′, reverse 5′-CCGAGGCCAGGAGGTATTACTATCG-3′) in 12 subjects with HDL <35 mg/dL and 12 subjects with HDL >75 mg/dL. Subjects with the correct Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Genetic Analyzer 310 (PE Biosystem). Sequences were analyzed and compared with the published sequence of the ABCA1 promoter sequence to detect polymorphisms.

**Genotyping**

We identified 3 novel polymorphisms. −477C/T, −419A/C, and −320G/C. We designed PCR–restriction fragment length polymorphism assays, and an investigator who had no knowledge of the angiographic and clinical data performed the genotyping in 372 subjects in the LCAS population. The −477C/T polymorphism creates a novel site for the AciI restriction enzyme (C/CGC) in the presence of the C allele. A set of primers was designed to amplify a 164-bp product was 133 bp long. Subjects with GG (n = 166), and CC (n = 75) genotypes were identified by gel electrophoretic patterns (GG, 102 and 31 bp; GC, 133, 102, and 31 bp; and CC, 133 bp). The frequency of the C allele was 0.42. The −477C/T and −320G/C variants were in complete linkage disequilibrium, and the −320G allele co-segregated with the −477C allele (127 GG/CC, 163 GC/CT, 74 CC/TT, 5 GG/CT, 3 CC/CT, and 0 GG/TT, GC/CC, GC/CT, and CC/CC; χ² = 699, P < 0.0001). The results of association studies with phenotypes were similar for the 2 polymorphisms, and data for the −477C/T polymorphism is presented.

The size of the PCR amplicon encompassing the −419A/C polymorphic site was 164 bp. Only 1 of 63 subjects carried the A allele, identified by the presence of 140- and 24-bp digestion products (allele frequency of 0.01).

**−477C/T Variants and Demographic and Clinical Phenotypes**

Demographic and clinical characteristics, such as age, sex, ethnic background, height, weight, body mass index, systolic and diastolic blood pressure, waist/hip ratio, history of smoking, and history of myocardial infarction, were not significantly different among the genotypes (data not shown). Diabetes mellitus was present in 7.1%, 2.3%, and 1.4% of the subjects with CC, CT, and TT genotypes, respectively (P = 0.074).

**−477C/T Variants and Plasma Lipids at Baseline and Response to Fluvastatin**

Plasma lipids at baseline and response to fluvastatin in −477C/T variants are shown in Table 1. There was a trend toward lower mean plasma levels of HDL-C (P = 0.094) and apoA1 (P = 0.054) in heterozygous subjects. There were no significant interactions between the genotypes and in the response of plasma lipids to treatment with fluvastatin, with the exception of apoA1, which showed a significant genotype-treatment interaction (P = 0.0385), as shown in Table 2.

**−477C/T Variants and Severity of Coronary Atherosclerosis**

There were strong associations between the genotypes and 2 indices of severity of coronary atherosclerosis, as shown in Table 1. The mean number of qualifying lesions (30% to 75% diameter stenosis) was greater in subjects with the TT genotype (3.1 ± 2.1) or CT genotype (2.9 ± 1.9) than in those with the CC genotype (2.2 ± 1.8) (P = 0.002). Similarly,
Lipids (N=547)

$\text{the CT genotype (90%) had greater numbers of subjects with the TT genotype (88%) or}$

$\text{the CT genotype (90%) had greater numbers of subjects with the TT genotype (88%) or}$

$\text{greater numbers of subjects with the TT genotype (88%) or the CT genotype (90%) had ≥1 qualifying lesion on quantitative coronary angiograms than did those with the CC}$

$\text{CC genotype (75%) (P=0.002). When study subjects were stratified according to the mean plasma levels of HDL-C}$

$\text{the -477C/T genotypes were strongly associated with the number of qualifying coronary lesions, in}$

$\text{in a gene-dose-dependent manner, only in subjects with the lower HDL-C levels (2.0 [CC] versus 2.8 [CT] versus 3.4 [TT], F=7.2, P=0.001). Analysis in subjects with the plasma levels of HDL-C of <35 mg/dL also showed}$

$\text{a strong association between the genotypes and the number of}$

$\text{TABLE 1. ABCA1-477C/T Genotypes and Baseline Plasma Lipids and Severity of}$

$\text{Coronary Atherosclerosis}$

<table>
<thead>
<tr>
<th>Lipids</th>
<th>CC (N=127)</th>
<th>CT (N=171)</th>
<th>TT (N=74)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC, mg/dL</td>
<td>219.7±23.8</td>
<td>219.5±24.1</td>
<td>224.6±25.9</td>
</tr>
<tr>
<td>HDL-C, mg/dL</td>
<td>44.2±11.4</td>
<td>42.7±10.6</td>
<td>46.0±12.4*</td>
</tr>
<tr>
<td>LDL-C, mg/dL</td>
<td>144.0±19.4</td>
<td>144.5±20.4</td>
<td>147.1±20.3</td>
</tr>
<tr>
<td>TG, mg/dL</td>
<td>156.2±55.8</td>
<td>163.1±58.4</td>
<td>159.6±55.7</td>
</tr>
<tr>
<td>ApoA1, mg/dL</td>
<td>135.8±27.1</td>
<td>128.7±25.9</td>
<td>136.2±27.9†</td>
</tr>
<tr>
<td>ApoB, mg/dL</td>
<td>134.5±22.4</td>
<td>136.0±20.1</td>
<td>135.3±20.8</td>
</tr>
<tr>
<td>Lp(a), mg/dL</td>
<td>35.9±35.2</td>
<td>37.6±34.2</td>
<td>34.3±29.8</td>
</tr>
</tbody>
</table>

$\text{Angiographic indices}$

| No. of qualifying lesions | 2.2±1.8 | 2.9±1.9 | 3.1±2.1‡ |
| No. of total occlusions | 0.33±0.58 | 0.35±0.64 | 0.35±0.85 |
| Subjects with ≥1 qualifying lesion, n (%) | 95 (75) | 155 (90) | 65 (80)§ |
| Subjects with ≥1 total occlusion, n (%) | 35 (28) | 46 (27) | 16 (22) |
| Mean MLD, mm | 1.68±0.39 | 1.66±0.42 | 1.69±0.39 |

$\text{Values are mean±SD or as indicated. TC indicates total cholesterol; TG, triglyceride; apoB,}$

$\text{apoB, apolipoprotein B; and Lp(a), lipoprotein(a).}$

$\text{*P=0.002, †P=0.038, ‡P=0.002, and §P=0.001.}$

$\text{TABLE 2. ABCA1-477C/T Genotypes and Progression and Regression of Coronary Atherosclerosis, Response to Therapy, and}$

$\text{Clinical Events}$

<table>
<thead>
<tr>
<th>Lipids (N=372)</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects, n</td>
<td>65</td>
<td>80</td>
<td>35</td>
<td>61</td>
<td>88</td>
<td>39</td>
</tr>
<tr>
<td>ΔTC, %</td>
<td>0.03±12.3</td>
<td>−0.05±12.5</td>
<td>−4.4±14.7</td>
<td>−16.9±12.1</td>
<td>−14.5±14.5</td>
<td>−15.4±11.0</td>
</tr>
<tr>
<td>ΔHDL-C, %</td>
<td>4.9±16.9</td>
<td>3.0±14.7</td>
<td>2.9±14.2</td>
<td>7.8±15.9</td>
<td>10.8±15.4</td>
<td>8.1±16.2</td>
</tr>
<tr>
<td>ΔLDL-C, %</td>
<td>−2.3±16.4</td>
<td>−4.0±17.9</td>
<td>−8.1±18.8</td>
<td>−27.6±15.0</td>
<td>−23.5±20.6</td>
<td>−26.1±13.1</td>
</tr>
<tr>
<td>ΔTG, %</td>
<td>8.1±38.9</td>
<td>15.6±43.8</td>
<td>0.1±32.2</td>
<td>4.2±46.8</td>
<td>−3.8±31.8</td>
<td>5.6±48.1</td>
</tr>
<tr>
<td>ΔApoA1, %</td>
<td>1.8±14.8</td>
<td>1.0±13.8</td>
<td>0.7±18.3</td>
<td>2.6±18.1</td>
<td>12.0±21.5</td>
<td>1.8±17.2*</td>
</tr>
<tr>
<td>ΔApoB, %</td>
<td>7.6±19.0</td>
<td>3.5±17.6</td>
<td>4.7±26.9</td>
<td>−20.0±21.9</td>
<td>−14.4±15.9</td>
<td>−15.7±13.3</td>
</tr>
<tr>
<td>ΔLp(a), %</td>
<td>−5.1±28.7</td>
<td>−1.0±33.6</td>
<td>−10.0±35.8</td>
<td>−8.4±33.8</td>
<td>0.9±36.9</td>
<td>−2.2±38.4</td>
</tr>
</tbody>
</table>

$\text{Angiographic indices (N=316)}$

| No. of qualifying lesions | 2.2±1.8 | 2.9±1.9 | 3.1±2.1‡ |
| No. of total occlusions | 0.33±0.58 | 0.35±0.64 | 0.35±0.85 |
| Subjects with ≥1 qualifying lesion, n (%) | 95 (75) | 155 (90) | 65 (80)§ |
| Subjects with ≥1 total occlusion, n (%) | 35 (28) | 46 (27) | 16 (22) |
| Mean MLD, mm | 1.68±0.39 | 1.66±0.42 | 1.69±0.39 |

$\text{Values are mean±SD or as indicated. MLD indicates minimal lumen diameter.}$

$\text{*P=0.038.}$
coronary lesions (2.1±1.5 [CC] versus 2.8±2.0 [CT] versus 4.4±1.2 [TT], \( P=0.003 \)). Analysis of the data after exclusion of 14 subjects with diabetes mellitus did not change the observed association between the genotypes and the number of qualifying coronary lesions (2.2±1.9 [CC] versus 2.8±1.9 [CT] versus 3.1±2.1 [TT]; \( F=6.9, P=0.001 \)). The number of total occlusions, the number of subjects with \( \geq 1 \) total occlusion, and the mean MLD did not differ significantly among the genotypes (Table 1).

### -477C/T Variants and Progression/Regression of Coronary Atherosclerosis

The mean number of new coronary lesions or total occlusions, the number of subjects who had progression of coronary atherosclerosis, and the changes in mean MLD during the course of 2.5 years of follow-up were not significantly different among the genotypes (Table 2). There were no significant interactions between the genotypes and response to treatment with fluvastatin with regard to angiographic indices of the progression/regression of atherosclerosis. No significant association between the -477C/T genotypes and the progression or regression of coronary atherosclerosis and response to therapy with fluvastatin was detected in subjects stratified according to the mean plasma levels of HDL-C and in subjects with plasma levels of HDL-C \(<35\) mg/dL.

### Clinical Events

Morbid or fatal events occurred in 54 patients (14%). Distribution of clinical events among the genotypes in the placebo and fluvastatin groups was not significantly different (Table 2).

### Discussion

We identified 3 novel polymorphisms in the promoter region of the \( ABCA1 \) gene and determined the association of the common polymorphisms with plasma levels of lipids, with angiographic indices of the severity, progression, and regression of coronary atherosclerosis, with clinical events, and with the response to treatment (pharmacogenetics) in a prospective study of a well-characterized population. We show that the -477C/T variants were strongly associated with the severity of coronary atherosclerosis (as determined by quantitative coronary angiography) and modestly associated with plasma levels of HDL-C and apoA1 and with the response of apoA1 to treatment with fluvastatin. No other association between the genotypes and biochemical, angiographic, and clinical phenotypes or genotype-treatment interaction was detected.

The hypothesis that common variants of the \( ABCA1 \) gene could modulate plasma levels of HDL-C and apoA1 stems largely from the discovery of mutations in \( ABCA1 \) in patients with Tangier disease and HA, who exhibit significantly reduced plasma levels of HDL-C and apoA1 and a high incidence of premature atherosclerosis.\(^1\)\(^-\)\(^3\)\(^,\)\(^11\) Members of families with Tangier disease or HA who are heterozygous for mutations in the \( ABCA1 \) gene exhibit a wide variation in HDL-C levels, an age-dependent decline in HDL-C levels, and an increased risk of coronary atherosclerosis.\(^1\)\(^-\)\(^3\) In the LCAS population, which is a representative of a general population with mildly to moderately elevated LDL-C levels,\(^8\) we observed only a modest association between HDL-C or apoA1 levels and heterozygosity for polymorphisms in \( ABCA1 \), which remained unchanged when analyzed in subjects aged \( \geq 50 \) years (\( n=313 \)). Thus, unlike familial disorders, such as Tangier disease or HA, in which common variants in \( ABCA1 \) reduce HDL-C levels significantly,\(^4\) our data suggest that common variants in the promoter region of the \( ABCA1 \) gene exert, at most, a modest effect on plasma levels of HDL-C and apoA1. Low HDL-C levels in the general population, unlike familial disorders, could result from a variety of disorders, of which only a portion operate through the reverse cholesterol transport, a function that has been attributed to apoA1 protein.\(^7\) The lack of a major effect on HDL-C levels in this general population is consistent with \( ABCA1 \) acting as a flippase at the plasma membrane that stimulates cholesterol and phospholipid efflux to apoA1 and HDL-C; the efflux to HDL-C is relatively minor.\(^7\) Multiple complex pathways are involved in the determination of plasma HDL-C levels, which include not only HDL-C synthesis and catabolism but also the metabolism of triglyceride-rich lipoproteins. Although a complete absence of \( ABCA1 \) leads to marked reductions in HDL-C levels, subjects heterozygous for mutations in the \( ABCA1 \) gene in families with Tangier disease and HA exhibit wide variations in HDL-C levels and apoA1,\(^4\) illustrating the limitations in assessing the function of both total reverse cholesterol transport and the initial step mediated by \( ABCA1 \) by examining only plasma HDL-C levels. In families with Tangier disease and HA, reduction in the relative efflux was greater in heterozygous subjects who had low plasma levels of HDL-C.\(^4\) Although the primary determinant of low HDL-C levels in LCAS was clearly not related to the 477T variant, we postulate that in patients with low levels of HDL-C, the 477T variant in the \( ABCA1 \) promoter leads to a reduction in the expression of \( ABCA1 \) and reverse cholesterol transport, resulting in increased cholesterol accumulation in the vessel wall and increased atherosclerotic burden as assessed by an increased number of lesions at baseline angiography. Angiographic progression may be driven more by plaque destabilization and thrombosis instead of a gradual accumulation of cholesterol in macrophages and thus showed no relation to the 477T variant. In accordance with the results of studies in families with Tangier disease and HA, our results demonstrated a strong association between the severity of coronary atherosclerosis and the \( ABCA1 \) genotypes in the LCAS population. The latter finding suggests dissociation between the proatherosclerotic effect of mutations and their impact on plasma levels of HDL-C and apoA1 in the general population, which has also been reported in subjects with mutations in cholesteryl ester transfer protein, resulting in high HDL-C levels but an increased risk of coronary atherosclerosis.\(^12\) In the absence of a significant association between the -477C/T genotypes and the plasma levels of HDL-C and apoA1, the progression or regression of coronary atherosclerosis, and the response to therapy, the observed association of the T allele with the severity of coronary atherosclerosis requires confirmation in additional data sets. The disparity
between the effect of \textit{ABCA1} variants on HDL-C levels and the severity of atherosclerosis in the present study also raises the possibility of statistical errors. Regarding \( \beta \) error, the sample size of the present study provided \( >90\% \) power to detect a 15\% difference in mean plasma levels of HDL-C (6.6 mg/dL) and apoA1 (20 mg/dL) among the genotypes. The possibility of an \( \alpha \) error, leading to a spurious association between the \textit{ABCA1} variants and the severity of the coronary atherosclerosis, is unlikely given the strength of the association \( (P=0.001) \). We made no adjustment for multiple testing, and if it is assumed that the hypotheses being tested are not fully independent of one another, a significant probability value \( (P<0.05) \) should be regarded as a potential association. We also note that the choice of end points, the duration of the study, and the inclusion criteria were determined before genetic analysis. In addition, the duration of LCAS (2.5 years) was relatively short, and the number of new clinical events was relatively low (54 events), which may not be sufficient to detect possible genotype-treatment interactions or association of the genotypes with clinical events or the progression/regression of coronary atherosclerosis. Overall, there were no genetic or biological gradients or a trend toward an association with multiple dependent phenotypes to suggest the possibility of type II \( (\beta) \) error. Furthermore, 108 subjects in LCAS were also treated with cholestyramine. Distributions of the genotypes among those who were or were not treated with adjunctive cholestyramine were not significantly different, and there were also no significant cholestyramine therapy-genotype interactions regarding plasma lipids or angiographic phenotypes (data not shown). The main results of the LCAS showed that treatment with fluvastatin reduced mean LDL-C by 24\% and slowed the progression of coronary lesions significantly.\(^8\)

In summary, we identified 2 common and 1 uncommon polymorphism in the promoter region of the \textit{ABCA1} gene and showed a strong association between the \textendash{}477C/T genotypes and the severity of coronary atherosclerosis, defined by quantitative coronary angiography in the LCAS population. There was a modest association between the genotypes and plasma levels of HDL-C and apoA1 and the response of apoA1 to treatment with fluvastatin. We conclude that \textit{ABCA1} \textendash{}477C/T genotypes are potential risk factors for coronary atherosclerosis in the LCAS population, representative of the general population with mildly to moderately elevated LDL-C.\(^8\)

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### References

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