Genetic Variation in ABCG1 and Risk of Myocardial Infarction and Ischemic Heart Disease

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Objective—ATP binding cassette transporter G1 (ABCG1) facilitates cholesterol efflux from macrophages to mature high-density lipoprotein particles. Whether genetic variation in ABCG1 affects risk of atherosclerosis in humans remains to be determined.

Methods and Results—We resequenced the core promoter and coding regions of ABCG1 in 380 individuals from the general population. Next, we genotyped 10 237 individuals from the Copenhagen City Heart Study for the identified variants and determined the effect on lipid and lipoprotein levels and on risk of myocardial infarction (MI) and ischemic heart disease (IHD). g.-376C>T, g.-311T>A, and Ser630Leu predicted risk of MI in the Copenhagen City Heart Study, with hazard ratios of 2.2 (95% confidence interval: 1.2–4.3), 1.7 (1.0–2.9), and 7.5 (1.9–30), respectively. These results were confirmed for g.-376C>T in a case-control study comprising 4983 independently ascertained IHD cases and 7489 controls. Expression levels of ABCG1 mRNA were decreased by approximately 40% in g.-376C>T heterozygotes versus noncarriers (probability values: 0.005–0.009). Finally, in vitro specificity protein 1 (Sp1) bound specifically to a putative Sp1 binding site at position −382 to −373 in the ABCG1 promoter, and the presence of the −376 T allele reduced binding and transactivation of the promoter by Sp1.

Conclusion—This is the first report of a functional variant in ABCG1 that associates with increased risk of MI and IHD in the general population. (Arterioscler Thromb Vasc Biol. 2012;32:506-515.)

Key Words: ABC transporter, atherosclerosis, ischemic heart disease, ABCG1, myocardial infarction

Macrophages are key elements in the development of atherosclerosis. Because these cells are unable to limit their lipid uptake, they rely on efficient cholesterol efflux transporters to prevent lipid accumulation. ATP binding cassette transporter G1 (ABCG1) is a transmembrane cholesterol transporter that effluxes cellular cholesterol from macrophages by delivering cholesterol to mature high-density lipoprotein (HDL) particles. As discussed by Westerterp et al, it is controversial whether whole-body Abcg1 knockout mice have increased atherosclerosis. In Abcg1−/−Apolipoprotein E-deficient mice fed a wild-type diet, atherosclerosis was decreased, whereas in Abcg1−/− mice fed an atherogenic diet, atherosclerosis was increased. Whether genetic variation in ABCG1 affects risk of disease in humans remains to be determined.

Because ABCG1 has been reported to be a key transporter of cholesterol from lipid-poor to mature HDL particles based on in vitro studies, we reasoned that genetic variation in ABCG1 might affect levels of HDL cholesterol in plasma. Therefore, to increase the likelihood of identifying genetic variants with effect on HDL cholesterol, we resequenced the core promoter, coding regions, and consensus splice sites of ABCG1 in individuals in the general population with the lowest 2% (n = 190) and highest 2% (n = 190) HDL cholesterol levels for age and sex. Next, we genotyped 10 237 individuals from the general population, the Copenhagen City Heart Study (CCHS), for the variants identified, and determined the association with plasma levels of HDL cholesterol and other lipids and lipoproteins, and the ability of these variants to predict risk of myocardial infarction (MI) and ischemic heart disease (IHD) with 34 years of follow-up. Finally, genetic variants associated with risk of MI or IHD in the CCHS were validated in a case-control study comprising 4983 independently ascertained IHD cases, of which 2921 had an MI, and 7489 healthy controls from the CCHS.

One variant, −376C>T in genomic DNA (g.−376C>T), located in a putative specificity protein 1 (Sp1) binding site, consistently associated with an increased risk of MI.
and IHD in both studies. We therefore measured expression levels of \textit{ABCG1} mRNA in \(g.-376C>T\) heterozygotes versus noncarriers and complemented the human studies with extensive functional studies of the \textit{ABCG1} promoter in vitro and in vivo.

**Methods**

**Human Studies**

**Subjects**

Studies were approved by institutional review boards and Danish ethical committees and were conducted according to the Declaration of Helsinki. Written informed consent was obtained from participants. All participants were white and of Danish descent.

**The CCHS**

The CCHS is a prospective cardiovascular study of the Danish general population initiated in 1976 to 1978 with follow-up examinations in 1981 to 1983, 1991 to 1994, and 2001 to 2003. Individuals were randomly selected based on the national Danish Civil Registration System to reflect the adult Danish general population, aged 20 to 80 years or above. We genotyped 10,237 individuals for the genetic variants identified by resequencing the core promoter of \textit{ABCG1} (nucleotides \(-1632\) to \(+123\) relative to \(+1\) in ATG, NT\_030188; Supplemental Figure I, available online at http://atvb.ahajournals.org/), the coding regions corresponding to the main transcript,\textsuperscript{12,13} and all consensus splice sites in individuals from the CCHS with the 2% lowest (\(n=190\)) and 2% highest (\(n=190\)) HDL cholesterol levels for age and gender (Supplemental Table I). Follow-up started in 1976 and ended at the date of death, occurrence of event, emigration, or on August 2010 (last update of the complete national Danish registries), whichever came first. Follow-up time was 34 years (243,000 person-years) and was 100% complete, ie, none were lost to follow-up.

Information on diagnoses of IHD (World Health Organization, International Classification of Diseases, 8th edition: codes 410–414; 10th edition: codes 120–125) was collected and verified by reviewing all hospital admissions and diagnoses entered in the national Danish Patient Registry, all causes of death entered in the Danish Causes of Death Registry, and medical records from hospitals and general practitioners. IHD was fatal or nonfatal MI or characteristic symptoms of stable angina pectoris, including revascularization procedures.\textsuperscript{14} A diagnosis of MI followed the changing definitions over time. After year 2000, the diagnosis was based on either of the following: (1) typical rise and fall of biochemical markers of myocardial necrosis (troponin or creatine kinase muscle brain function [CK-MB]) with at least one of the following: ischemic symptoms, development of pathological Q waves on the ECG, ECG changes indicative of ischemia, or coronary artery intervention; or (2) pathological findings of an acute, healed, or healing MI,\textsuperscript{15} with later changes as indicated.\textsuperscript{16} A total of 2076 participants had IHD, of whom 985 had MI.

**The Copenhagen Ischemic Heart Disease Study**

To validate whether \textit{ABCG1} variants associated with risk of MI and IHD in an independent study, a case-control study was conducted, the Copenhagen Ischemic Heart Disease Study (CIHDS). The cases were 4983 consecutive patients from the greater Copenhagen area referred for coronary angiography to Copenhagen University Hospital, during the period 1991 through 2010. These patients had documented IHD based on characteristic symptoms of angina pectoris,\textsuperscript{17} plus at least one of the following: stenosis/atherosclerosis on coronary angiography, a previous MI, or a positive bicycle exercise test. The diagnosis of MI was established with the same criteria as described above. Of the 4983 cases with IHD, 2921 had MI. Cases with IHD were compared with 7489 controls without ischemic cardiovascular events (IHD or ischemic cerebrovascular disease) from the CCHS.

**mRNA Expression Analyses of \textit{ABCG1} g.-376C>T**

Total RNA was isolated from the buffy coat of 34 g.-376C>T heterozygotes and 68 noncarriers, matched 1:2 by age and sex, followed by first-strand cDNA synthesis and quantitative real-time polymerase chain reaction analysis using the relative standard curve method and \textit{GAPDH} as the endogenous control. For details, please see the Supplemental Methods.

**Biochemical Analyses, Covariates, Sequencing, Genotyping, and Statistical Analyses**

Please see the Supplemental Methods.

**In Vitro and In Vivo Cell Studies of \textit{ABCG1} g.-376C>T**

Binding of Sp1 to the \textit{ABCG1} promoter and Sp1-mediated transactivation were explored with DNA affinity precipitation assays, competition assays with biotinylated DNA probes, chromatin immunoprecipitation assays in HEK293T cells, and transactivation assays in \textit{Drosophila} S2 cells as detailed in the Supplemental Methods.

**Results**

We resequenced the core promoter (nucleotide \(-1632\) to nucleotide \(+123\); Supplemental Figure I), the coding regions corresponding to the main transcript,\textsuperscript{12,13} and all consensus splice sites of \textit{ABCG1} in 380 individuals with extreme levels of HDL cholesterol. Next, we genotyped 10,237 individuals from the general population, the CCHS, for all promoter variants (except insertions/deletions), nonsynonymous, synonymous, and splice-site variants identified (for a complete list of all 19 variants genotyped, see Supplemental Table I). Three variants, g.-311T>A, g.-376C>T, and Ser630Leu, which predicted risk of MI and IHD in the CCHS, were validated in a second study, including 4983 independently ascertained IHD cases. One variant, g.-376C>T (minor allele frequency=0.2%, no TT homozygotes identified), located in a putative Sp1 binding site, consistently associated with an increased risk of MI and IHD in both studies. We therefore measured expression levels of \textit{ABCG1} mRNA in g.-376C>T heterozygotes versus noncarriers and complemented the human studies with extensive functional studies of the \textit{ABCG1} promoter in vitro and in vivo.

Characteristics of participants in the CCHS and the CIHDS by disease status are shown in the Table.

**Genetic Variation in \textit{ABCG1} and HDL Cholesterol**

Genetic variants identified by resequencing 190 individuals with the 2% lowest and 190 individuals with the 2% highest HDL cholesterol levels for age and sex in the CCHS are shown in Supplemental Table I, as are the allele frequencies in the extreme HDL cholesterol groups, and in the entire CCHS. Pairwise linkage disequilibria for variants with allele frequencies \(\geq 0.002\) are shown in Supplemental Figure II. Two of 16 variants (3 singletons excluded), g.-1082C>T and G327>T, were associated with modest reductions in HDL...
Risk of MI and IHD

Multifactorially adjusted hazard ratios for MI were 2.2 (95% confidence interval: 1.2–4.3), 1.7 (1.0–2.9), and 7.5 (95% confidence interval: 4.1–5.9) for g.−376C>T heterozygotes versus noncarriers, multifactorially adjusted odds ratios were 1.9 (0.8–4.2) for MI and 2.4 (1.2–4.6) for IHD (Figure 3, left panels), confirming the results from the CCHS. To maximize power, we combined cases from CCHS and CIHDS and compared them with 7489 controls without ischemic cardiovascular disease from the CCHS. For g.−376C>T heterozygotes versus noncarriers, multifactorially adjusted odds ratios were 1.9 (0.8–4.2) for MI and 2.4 (1.2–4.6) for IHD (Figure 3, left panels), confirming the results from the CCHS. To maximize power, we combined cases from CCHS and CIHDS and compared them with 7489 controls without ischemic cardiovascular disease from the CCHS. Results for g.−376C>T were similar (Figure 3, right panel). The validation results for IHD for g.-311T>A and Ser630Leu did not reach statistical significance in the CIHDS alone or in the combined study of CCHS and CIHDS and compared them with 7489 controls without ischemic cardiovascular disease from the CCHS. For g.−376C>T heterozygotes versus noncarriers, multifactorially adjusted odds ratios were 1.9 (0.8–4.2) for MI and 2.4 (1.2–4.6) for IHD (Figure 3, left panels), confirming the results from the CCHS. To maximize power, we combined cases from CCHS and CIHDS and compared them with 7489 controls without ischemic cardiovascular disease from the CCHS. Results for g.−376C>T were similar (Figure 3, right panel). The validation results for IHD for g.-311T>A and Ser630Leu did not reach statistical significance in the CIHDS alone or in the combined study of CCHS and CIHDS (Figure 3, left and right panels), whereas risk of MI for the Ser630Leu variant reached statistical significance in the combined study (P=0.04).

mRNA Expression Levels in g.−376C>T Heterozygotes Versus Noncarriers

The mRNA expression of ABCG1 was decreased by 36% in 34 g.−376C>T heterozygotes versus 68 noncarriers, matched 1:2 by age and gender (Figure 4, P=0.005). These results were reproduced in an independent experiment (P=0.009; data not shown), using theuffy coat from the same people but isolated at different times. The cell composition of the Buffy coat did not differ between g.−376C>T heterozygotes and noncarriers for neutrophils, eosinophils, monocytes, basophils, lymphocytes, or other leukocytes (probability values: 0.24–0.97).
Figure 1. High-density lipoprotein (HDL) cholesterol levels and hazard ratios for myocardial infarction and ischemic heart disease as a function of ATP binding cassette transporter G1 (ABCG1) genotype in the Copenhagen City Heart Study. Probability values were found by test for trend or Mann-Whitney U test. CI indicates confidence interval.
Binding of Sp1 to the ABCG1 Promoter, and Sp1-Mediated Transactivation

Primers and probes used in DNA affinity precipitation, chromatin immunoprecipitation, and transactivation assays are shown in Supplemental Tables II and III. Positions of putative Sp1 sites and other putative nuclear factor binding sites in the ABCG1 promoter are shown in Supplemental Figure I.

The ABCG1 core promoter contains 3 putative Sp1 binding sites at positions −566, −382 (GCCCAAGCCCC; position −376 underlined), and −184, respectively12 (Supplemental Figure I). To determine whether the g.−376C>T variant was biologically functional, we performed in vitro and in vivo cell studies. First, we used DNA affinity precipitation assays to establish that the g.−376C>T variant was located in a functional Sp1 site. A DNA probe corresponding to the g.−391/−361 ABCG1 promoter bearing the C allele (−376C) bound Sp1 with high affinity (Figure 5A, lane 3). In contrast, Sp1 could not bind to a similar DNA probe bearing the T allele at position −376 (−376T, Figure 5A, lane 4). As a positive control, we used a DNA probe corresponding to the g.−118/+205 ABCA1 promoter shown previously to harbor a functional Sp1 binding site (Figure 5A, lane 5).17 As a negative control, we used an unrelated probe corresponding to the region around the TATA box of the human ABCA1 promoter (Figure 5A, lane 6). Thus, these experiments showed that Sp1 bound specifically to the putative Sp1 site described by Langmann12 at position −382 (GCCCAGCCCC; position −376 underlined; Supplemental Figure I) in vitro, and that the presence of the g.−376T allele markedly reduced binding. Competition assays further substantiated that Sp1 bound to the g.−391/−361 region of the ABCG1 promoter. As shown in Figure 5B (lanes 3–6), binding of Sp1 to the ABCG1 probe was competed out by increasing amounts of an oligonucleotide corresponding to the g.−63/+45 region of the human p21 promoter (p21 [Sp1wt]) bearing a well-characterized Sp1 target site,18 whereas a mutated human p21 promoter (p21 [Sp1mut]) was unable to displace Sp1 from the ABCG1 probe (Figure 5B, lanes 7–9).

The binding of endogenous Sp1 to the human ABCG1 promoter in vivo was established by chromatin immunoprecipitation assays in HEK293T cells. As shown in Figure 5C, Sp1 was found to be constitutively associated with chromatin fragments corresponding to the proximal ABCG1 promoter (−487/−144) which includes 2 Sp1 sites at position −382 and −184, respectively (3.2-fold enriched binding compared with control), whereas binding of Sp1 to these sites was reduced by 44% in the presence of mithramycin A, a well-established Sp1 inhibitor19 (a representative original image is shown in Supplemental Figure III). Finally, transactivation assays in Drosophila S2 cells that lack endogenous Sp1 or related activities,20 showed that the human −1080/+14 ABCG1 core promoter bearing the g.−376C allele (plus 2 additional putative Sp1 sites at positions −566 and −184; shown in Supplemental Figure I and IV), was strongly transactivated by Sp1 (103.5-fold) (Figure 5D, left panel). Mutating the Sp1 site centered at nucleotide −376C to −376T decreased the Sp1-mediated transactivation of the ABCG1 promoter by 64% (Figure 5D, right panel). The promoter retained partial activity, probably because of binding of Sp1 to the 2 other sites, which were still intact. In contrast, Sp2 and Sp3 did not stimulate the ABCG1 promoter to significant

Figure 2. Cumulative incidence of myocardial infarction, ischemic heart disease, and proportion surviving as a function of age and by ATP binding cassette transporter G1 (ABCG1) g.−376C>T genotype in the Copenhagen City Heart Study. Red indicates ABCG1 g.−376C>T heterozygotes (CT), and green indicates noncarriers (CC). Probability values were determined by log-rank test.

Binding of Sp1 to the ABCG1 Promoter, and Sp1-Mediated Transactivation

Primers and probes used in DNA affinity precipitation, chromatin immunoprecipitation, and transactivation assays
levels in either of the 2 experiments (Figure 5D). Thus, these experiments showed that Sp1 also bound to the human *ABCG1* promoter in vivo (Figure 5C) and was strongly transactivated specifically by Sp1 in cells that lack endogenous Sp1 (Figure 5D, left panel) and that this transactivation was severely reduced by mutating the Sp1 site at $\gg H11002 \ 376C$ to $\gg H11002 \ 376T$ (Figure 5D, right panel).

**Discussion**

The principal findings of this study are as follows: (1) *ABCG1*, $\gg H11002 \ 376C$\textendash$T$, located in a putative Sp1 binding site, predicted an increased risk of MI and IHD and reduced longevity in the general population without affecting levels of HDL cholesterol or other lipids or lipoproteins. (2) The increased risk of MI and IHD was validated in a large case-control study. (3) Expression levels of *ABCG1* mRNA were reduced in $\gg H11002 \ 376C$\textendash$T$ heterozygotes compared with noncarriers. (4) Extensive functional studies in vitro and in vivo showed that $\gg H11002 \ 376C$\textendash$T$ was indeed located in a functional Sp1 binding site and that the presence of the $\gg H11002 \ 376T$ allele substantially reduced binding and transactivation of the promoter by Sp1. This is the first report of a functional genetic variant in *ABCG1*, which predicts an increased risk of MI and IHD in humans.

Based on data from in vitro studies, ABCG1 has been suggested to have a critical role in mediating cholesterol efflux to mature HDL particles, thus preventing cellular lipid accumulation,21–23 and has consequently been suggested to protect against atherosclerosis. Recently, 3 groups reported transplantation studies of *Abcg1*-deficient (*Abcg1\textendash/\textendash*) bone marrow into *Ldl receptor*-deficient mice with apparently contrasting effects on atherosclerotic lesion formation.24–26 These discrepancies were suggested to be due to differences in dietary composition.27 Studies of whole-body deficiency of *Abcg1* have also lead to conflicting results. In *Abcg1\textendash/\textendash*/*Apolipoprotein E*-deficient mice fed a wild-type diet, atherosclerosis was decreased,7 whereas in *Abcg1\textendash/\textendash* mice on a C57BL/6 background without *Ldl receptor* or *Apolipoprotein E* deficiency, atherosclerosis was increased.8 In the latter model, although the cholesterol levels were lower, the diet included 0.5% cholate, which results in increased inflammatory responses in certain tissues and very small atherosclerotic lesions in normal mice. Thus, this area of investigation is at present highly controversial.

A recent case-only study of 109 Japanese men with coronary artery disease reported increased severity of coronary artery disease associated with a common polymorphism, rs5601744 T\textendash>G, in the *ABCG1* promoter.28 This polymorphism is located 19 720 base pairs upstream of the ATG start site of the main transcript. In the CCHS, the frequency of the minor allele (G allele) was 0.21 and did not tag the
However, as mentioned above, the evidence to support that ABCG1 is a key transporter of cholesterol from lipid-poor to mature HDL is based mainly on in vitro studies of macrophages. In addition to macrophages, ABCG1 is expressed in other cell types, such as endothelial cells and pancreatic β cells, and deficiency of ABCG1 in these cells may therefore also contribute to atherogenesis.6,31,32 Consequently, the reduced levels of ABCG1 mRNA in g.−376C>T heterozygotes observed in the present study, may lead to reduced endothelial nitric oxide synthase activity and increased atherogenesis, as shown in Abcg1−/−/Ldlr−/− mice compared with Ldlr−/− mice, when transplanted with wild-type bone marrow to specifically investigate the role of vascular ABCG1.6 Reduced endothelial nitric oxide synthase activity may in turn lead to increased monocyte-endothelial cell interactions and vascular inflammation, as shown in aortic endothelial cells isolated from ABCG1 knockout mice.32 Finally, impaired insulin secretion has been observed in pancreatic β cells from Abcg1 knockout mice both in vivo and in vitro, without an effect on cellular cholesterol content or efflux.31 All these effects may potentially contribute to the lipid and lipoprotein independent effects of g.−376C>T on risk of MI and IHD observed in the present study and may suggest that ABCG1 is not a key transporter of cholesterol from lipid-poor to mature HDL in vivo.

Neither metaanalyses of all genome-wide association studies of blood lipid traits33 nor metaanalyses of susceptibility to coronary artery disease34 have identified ABCG1 as a candidate gene. However, to be captured in these studies, the variant either must be on the array or must be tagged by common single-nucleotide polymorphisms on the array. The latter is unlikely for rare variants.35 Neither the rare g.−376C>T (minor allele frequency: 0.2%), which associated with MI and IHD, nor the relatively rare variants, g.-1082C>T and G237 (minor allele frequency: 0.6% and 4.0%), which associated with MI and IHD, nor the relatively rare variants, g.-1082C>T and G237 (minor allele frequency: 0.6% and 4.0%), which associated with MI and IHD, nor the relatively rare variants, g.-1082C>T and G237 (minor allele frequency: 0.6% and 4.0%), which associated with MI and IHD, nor the relatively rare variant in the present study, were present on or tagged by other single-nucleotide polymorphisms on these commercial arrays.33,34 This may explain why ABCG1 was not identified as a candidate gene for coronary heart disease or HDL cholesterol levels in these genome-wide association studies.

In a recent study, Edmondson et al used a custom-made array designed to include 66 HDL cholesterol gene loci selected on the basis of HDL biology, candidate genes, and results from previous genome-wide association studies.36 In this study, rs914189 in ABCG1 (intervening sequence 11–293G>G; minor allele frequency 0.21) was associated with HDL cholesterol levels; however, risk of coronary heart disease was not determined. The 2 rare variants associated with HDL cholesterol in our study were not present on this array.

In conclusion, we have identified a functional genetic variant in the ABCG1 promoter that associates with an increased risk of MI and IHD in the general population. This is the first report to demonstrate a role for genetic variation in ABCG1 in risk of ischemic vascular disease in the general population.
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Disclosures
None.

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Figure 5. Binding of specificity protein 1 (Sp1) to the ATP binding cassette transporter G1 (*ABCG1*) promoter, and Sp1-mediated transactivation. A and B, DNA affinity precipitation assays with biotinylated DNA probes. A, Extracts of HEK293T cells overexpressing Sp1 were incubated with biotinylated DNA probes corresponding to the −391/−361 region (1 Sp1 site at position −382/−373) of the human *ABCG1* promoter bearing the −376C allele (lane 3) or the −376T allele (lane 4), the −118/+205 region of the human *ABCA1* promoter (−90/−85 binds Sp1,17 lane 5), or the −31/−9 region (TATA) of the *ABCA1* promoter (which does not bind Sp1,17 lane 6). B, Competition assays with biotinylated DNA probes corresponding to the −391/−361 region of the human *ABCG1* promoter bearing the −376 C allele (lanes 3–9) in the presence of an increasing molar excess (0.5, 1.0, and 1.5 μmol/L) of nonbiotinylated double-stranded probes corresponding to the wild-type (wt) (lanes 4–6), or mutated (lanes 7–9) −63/−45 region of the human p21 promoter.18 The Sp1 promoter binding assays in A and B were performed twice using different extracts and the results were identical. WB, Western blot. C, Chromatin immunoprecipitation assays in HEK293T cells treated with 0.2 μmol/L mithramycin A for 24 hours or untreated cells using an antibody for Sp1. Polymerase chain reaction primers corresponding to the −487/−144 region of the *ABCG1* promoter (including 2 Sp1 sites at positions −382/−373 and −184/−176). Mithramycin A is a well-established inhibitor of Sp1 binding to DNA. The chromatin immunoprecipitation assays were performed 2 times, and the polymerase chain reactions of each experiment were replicated twice. *P<0.05. D, Transactivation assays in *Drosophila* S2 cells using the −1080/+14 *ABCG1* promoter, including putative Sp1 sites at positions −566/−558, −382/−373, and −184/−176 (left panel, −376C allele; right panel, −376T allele) (1 μg each) along with *Drosophila* expression vectors for Sp1, Sp2, or Sp3 (5 ng each). The hsp-lacZ plasmid (0.5 μg) was used for normalization. Relative luciferase activity is shown. The values for the transactivation assays are the average of 4 transfections.


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Supplement Material

Supplemental Methods

In vitro and in vivo cell studies of ABCG1 g.-376C>T

Materials

Dulbecco’s modified Eagle’s medium (DMEM) and penicillin/streptomycin for cell culture were purchased from Invitrogen/Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from BioChrom Labs (Terre Haute, IN). Charcoal-stripped serum was prepared after treatment of FBS with charcoal and dextran. Restriction enzymes and T4 DNA ligase were purchased from Minotech (Heraklion, Greece) or New England Biolabs (Beverly, MA). GoTaq DNA polymerase, dNTPs, the luciferase assay system, and the Wizard SV gel and PCR cleanup system were purchased from Promega (Madison, WI). Mithramycin A was purchased from Sigma-Aldrich (St. Louis, MI). Trizol reagent for RNA extraction and SuperScript Rnase H reverse transcriptase were purchased from Invitrogen. The Super Signal West Pico chemiluminescent substrate was purchased from Pierce (Rockford, IL). Anti-Sp1 (H225 and 1C6) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse peroxidase-conjugated secondary antibody was purchased from Chemicon International Inc. (Temecula, CA). Streptavidin-coupled superparamagnetic beads were purchased from Invitrogen.

Plasmids

The human ABCG1 promoter -1,080/+14 was amplified from genomic DNA by PCR and cloned into the pGL3 basic vector (Promega) at the KpnI–XhoI sites. The mutation C>T at position -376 was generated by overlapping PCR and confirmed by sequencing. The sequences of the primers used for the cloning of the ABCG1 promoter and for mutagenesis are given in Supplemental Table II. The expression vector pCDNA3-Bio-Sp1, used for the DNA affinity precipitation experiments has been described previously1. The Drosophila pPac expression vectors for human Sp1, Sp2 and Sp3 have been described previously2.
Cell Cultures and Treatments

Human embryonic kidney cells (HEK293T) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, l-glutamine, and penicillin/streptomycin at 37°C in a 5% CO₂ atmosphere. Drosophila Schneider S2 cells were cultured in Schneider’s insect medium supplemented with 10% insect fetal bovine serum and penicillin/streptomycin at 25°C. For the treatment of cells with mithramycin, 10% FBS was replaced by 5% charcoal-stripped serum (CSS), and mithramycin was added for 24 h at a final concentration of 0.2 µM.

DNA affinity precipitation

For DNA affinity precipitation (DNAP) assays, whole cell extracts from HEK293T cells that had been transfected with PCDNA3-Bio-Sp1 were used. HEK (human embryonic kidney fibroblasts) were used, because they are an excellent system for protein overexpression, they are easily manipulated, and because they express endogenous Sp1 transcription factors and the ABCG1 transporter. Dynabeads were washed once with 1×B&W buffer [5 mM Tris/HCl (pH 7.5), 0.5 mM EDTA and 1 mM NaCl], mixed with 0.5 µM of biotinylated oligonucleotide and incubated at room temperature (25°C) for 15 min. The oligonucleotide-coupled beads were washed twice with 1×B&W buffer and once with 1×BBRC buffer (10% glycerol, 10 mM Tris/HCl, pH 7.5, 50 mM KCl, 4 mM MgCl₂ and 0.2 mM EDTA). The protein–DNA binding reactions were allowed to proceed for 5 h on a rotary shaker at 4°C. Each reaction mixture included 150 µg of whole cell extracts, 8 µg of competitor poly (dI/dC) and the biotinylated oligonucleotide-coupled Dynabeads. Non-coupled Dynabeads were utilized to confirm the specificity of the DNA-protein interaction. Double stranded biotinylated oligonucleotides corresponding to the −391/−361 region of the human ABCG1 promoter bearing the -376C or -376T alleles were coupled to Dynabeads and used as probes. As positive and negative controls, respectively, we used probes corresponding to the human ABCA1 promoter region -118/+205 (which includes a well-characterized Sp1 site at position -90/-85³), and the ABCA1 promoter region -31/-9 (which includes the ABCA1 TATA-box) (Supplemental Table III). Oligonucleotides used for cloning the ABCG1 promoter, and for mutagenesis
are given in Supplemental Table II. DNAP competition assays were performed using increasing molar excess of non-biotinylated oligonucleotides corresponding to the wild type \(-63/-45\) region of the human p21\(_{CDKN1A}\) promoter bearing two well characterized Sp1 binding sites [p21 (Sp1wt)] or the same region bearing mutations in the two Sp1 sites [p21 (Sp1 mut)]\(^4\). Sp1 bound to the oligonucleotides was detected by SDS/PAGE and immunoblotting using anti-Sp1 (1C6) antibody.

**Chromatin Immunoprecipitation Assays**

Chromatin immunoprecipitation (ChIP) assay was performed as described previously\(^3\).

Immunoprecipitated chromatin was analyzed by PCR using primers corresponding to the proximal \(-487/-144\) region of the human \(ABCG1\) gene promoter containing to putative Sp1 sites at postions \(-382/-373\) and \(-184/-176\), respectively (Supplemental Table II for primers; Supplemental Figure 1 for location of putative Sp1 sites). In control reactions, the chromatin immunoprecipitations were performed in the absence of the anti-Sp1 antibody (ab). The products of the PCR amplifications were analyzed by agarose gel electrophoresis. Quantification was performed using the Tinascan v.2 software and is presented as the ratio of specific (+ anti-Sp1 ab) vs non-specific (- anti-Sp1 ab) binding of Sp1 to chromatin (see Supplemental Figure III for a representative image).

**Transient transfections and transactivation assays**

For the DNA affinity precipitation assays, \(9\times10^5\) HEK293T cells in 10cm plates were transiently transfected with 20\(\mu\)g of an expression vector for Sp1 using the \(Ca_3(PO_4)_2\) coprecipitation method. For the transactivation assays, \(5\times10^5\) Drosophila S2 cells/well were plated in 12-well plates and transient transfections were performed using the \(Ca_3(PO_4)_2\) coprecipitation method. In each well a total amount of 3\(\mu\)g DNA was used including 1\(\mu\)g of reporter plasmid [\((-1080/+14)ABCG1(-376C)-luc\) or \((-1080/+14)ABCG1(-376T)-luc\) – mutation in bold and underlined] containing three putative Sp1 sites at positions \(-566/-558\), \(-382/-373\), and \(-184/-176\), respectively (Supplemental Figure I and Supplemental Figure IV), 5ng of the pPac-Sp1, -Sp2 or -Sp3 expression vectors, 0.5\(\mu\)g of the hs-LacZ vector expressing \(\beta\)-galactosidase for normalization purposes and salmon sperm DNA as fill up DNA. Luciferase assays
from Promega Corp. were used and performed according to the manufacturer’s instructions. Normalization for transfection efficiency was performed by β-galactosidase assays.

4 **Human studies**

5 **Biochemical analyses**

6 Colorimetric and turbidimetric assays were used to measure plasma levels of total cholesterol, triglycerides, HDL cholesterol after precipitation of apolipoprotein B containing lipoproteins, apolipoproteins B and –AI (all Boehringer Mannheim GmbH, Mannheim, Germany). Low density lipoprotein cholesterol was calculated using the Friedewald equation, or measured directly if triglycerides were ≥4mmol/L.

10 **Covariates**

11 The risk factors, diabetes mellitus, smoking and hypertension were dichotomized and defined as diabetics (self-reported disease, use of anti-diabetic medication and/or non-fasting plasma glucose >11.0 mmol/L), smokers (current smoker), and antihypertensive therapy (use of antihypertensive drugs). Body mass index was weight (kg) divided by height squared (m²). Alcohol consumption was grams of alcohol consumed per day. Physical activity was the fraction of individuals with less than four hours per week of light physical activity during leisure time.

18 **Sequencing**

19 Genomic DNA was isolated from frozen whole blood (QiaAmp4 DNA Blood Mini Kit; QIAGEN GmbH, Hilden, Germany). Nineteen PCR fragments were amplified covering the core promoter (nt -1632 to nt +123; Supplemental Figure I), the coding regions corresponding to the main transcript (but using the ATG start site defined in reference 6; the remainder of the promoter and exon 1 sequence are identical in the two papers), and all consensus splice sites of ABCG1 in 380 individuals from the CCHS
with extreme HDL cholesterol levels. All PCR fragments were subsequently sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems Inc., Foster City, CA, USA).

**Genotyping**

An ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA) was used for genotyping by TaqMan-based assays. All promoter (except ins/dels), nonsynonymous, synonymous, and splice-site variants identified by resequencing (n=19; Supplemental Table I) were genotyped in all >10,000 participants in the CCHS. Three genetic variants, g.-311T>A, g.-376C>T, and S630L, which predicted risk of MI in this study (Figure 1), were further genotyped in the CIHDS. TaqMan® probes and PCR primers are available from the authors. Genotypes were verified by sequencing 50 of each genotype for common variants, and all heterozygotes and homozygotes for less common variants.

**mRNA expression analyses of ABCG1 g.-376C>T**

Total RNA was isolated from buffy coat from 34 g.-376C>T heterozygotes and 68 noncarriers, matched 1:2 by age and sex, using TRIzol (Invitrogen, Taastrup, Denmark), and treated with DNase (QIAGEN GmbH, Hilden, Germany) before purification on spin columns (QIAGEN). First-strand cDNA was synthesized from 500 ng total RNA using 15U AMV reverse transcriptase, 40U RNAsin, 0.25 µg random hexamer primers (all Promega, Madison, WI, USA), and 2.5M dNTPs (KJ. Ross Petersen aps, Klampenborg, Denmark). Sense and antisense primers (Tag Copenhagen, Copenhagen, Denmark) are given in Supplemental Table II.

Quantitative real-time PCR analyses were performed on an ABI PRISM® 7900HT Sequence Detection System using the fast SYBR-green kit (Applied Biosystems Inc.). Each 20 µL of PCR contained 10 µL fast SYBR-green mix, 0.5 µM of each primer, 2 µL cDNA synthesized from 500 ng total RNA and PCR grade H$_2$O. For quantification, the relative standard curve method was used to determine the concentrations of ABCG1 and GAPDH (endogenous control) mRNA$^8$. Standard curves of $C_T$ values plotted against the logarithm of the input total RNA were prepared by assaying serial dilutions.
(from 1:1 to 1:8000) of noncarrier RNA from 40 ng/µl to 0.005 ng/µl, with \textit{ABCG1} and \textit{GAPDH} assays.

The relative amounts of \textit{ABCG1} and \textit{GAPDH} mRNA in noncarriers and g.-376C>T heterozygotes were quantitated by linear extrapolation of the \(C_T\) values using the equation to the line obtained from their respective standard curves. A relative \textit{ABCG1} expression value was obtained by dividing the \textit{ABCG1} value by the \textit{GAPDH} value. The mean \(C_T\) values for \textit{ABCG1} and \textit{GAPDH} in noncarriers were 30.2 and 25.7, respectively, and 29.5 and 25.7 in g.-376C>T heterozygotes. Real-time quantitative PCR was performed in duplicate in two independent experiments, using the buffy coat from the same persons, but isolated at different times. PCR fragments were run on a 2% E-Gel\textsuperscript{®}48 (Invitrogen, Taastrup, Denmark) to ensure that no DNA was amplified during real-time PCR. Primers for \textit{ABCG1} and \textit{GAPDH} are listed in Supplemental Table II.

**Statistical analyses**

We used the statistical software package Stata (version 10.1; STATA Corp., College Station, Texas, USA) and Origin v.6 software (OriginLab®, Mass, USA). Two-sided probability values <0.05 were considered significant. In human studies, Pearson \(\chi^2\) test and Mann-Whitney U test were used for two-group comparisons of categorical and continuous variables, respectively. For trend test, groups of individuals were classified by \textit{ABCG1} genotypes and ranked 0, 1, and 2 with 0 (noncarriers) as the reference group. In functional studies \textit{in vitro}, Student’s t-test was used for two group comparisons, and one-way analysis of variance (ANOVA) was used for multiple comparisons.

In the prospective study, the CCHS, Kaplan-Meier plots and log-rank tests evaluated the cumulative incidence of MI, IHD, and longevity as a function of genotype. Cox proportional hazards regression models estimated hazard ratios for MI and IHD as a function of genotype. For all survival statistics, age was the time scale using left truncation (or delayed entry)\textsuperscript{9}. This implies that age is automatically adjusted for, and therefore not included as a covariate in the model.

In the case-control study, the CIHDS, and in the combined study of CCHS and CIHDS, logistic regression analysis adjusted for sex and age, in 10-year age groups, or multifactorially adjusted
for age, sex, diabetes mellitus, smoking and antihypertensive therapy, was used to estimate odds ratios for MI and IHD as a function of genotype.

For mRNA expression analysis g.-376C>T heterozygotes were compared with noncarriers, matched 1:2 for age and sex, using linear regression.
Supplemental Table I. Genetic variation in regulatory and coding regions of the *ABCG1* gene in individuals with the lowest 2% and highest 2% HDL-cholesterol levels among 10,237 participants in the Copenhagen City Heart Study (CCHS).

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<tr>
<th>Gene region</th>
<th>Nucleotide substitution</th>
<th>allele frequency in %</th>
<th>P-value</th>
<th>Allele frequency in general population in %</th>
<th>Amino acid residue</th>
<th>rs number / reference</th>
</tr>
</thead>
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<td>1 (0.3)</td>
<td>0.32</td>
<td>0.2</td>
<td>-</td>
<td>New</td>
</tr>
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<td>Promoter</td>
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<td>0.65</td>
<td>0.6</td>
<td>-</td>
<td>New</td>
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<td>0.32</td>
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<td>-</td>
<td>New</td>
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<td>101 (27)</td>
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<td>-</td>
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<td>26.0</td>
<td>-</td>
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<td>0.2</td>
<td>-</td>
<td>New</td>
</tr>
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<td>Promoter</td>
<td>g.-530A&gt;G</td>
<td>23 (6)</td>
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<td>2 (0.5)</td>
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<td>0.4</td>
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<td>g.-269G&gt;A</td>
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<td>1 (0.3)</td>
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<td>17 (5)</td>
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<td>1 (0.3)</td>
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<td>Intron 4</td>
<td>IVS4 –6 C&gt;T</td>
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<tr>
<td>Exon 7</td>
<td>724 G&gt;A</td>
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<tr>
<td>Exon 9</td>
<td>981 C&gt;T</td>
<td>14 (4)</td>
<td>7 (2)</td>
<td>0.18</td>
<td>4.0</td>
<td>G327</td>
</tr>
<tr>
<td>Exon 9</td>
<td>1046 A&gt;G</td>
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<td>Exon 15</td>
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<td>1 (0.3)</td>
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<td>0.02</td>
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</tr>
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</table>

1 *Nucleotide 1 denotes A in the startcodon ATG in exon 1, corresponding to base position 53,133 in ABCG1 consensus sequence AP001746. +The Copenhagen City Heart Study. HDL-C= high density lipoprotein cholesterol
Supplemental Table II. Oligonucleotides used in *ABCG1* promoter cloning, mutagenesis, chromatin immunoprecipitation assays and real time PCR for *ABCG1*.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence*</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCG1-1080-F</td>
<td>5’-CCCCTACAGGTGCTGGAAATG-3’</td>
<td>Cloning of the -1,080/+14 ABCG1 promoter region</td>
</tr>
<tr>
<td>ABCG1+14-R</td>
<td>5’-CCGCTGAGGAAACGCGGAGTCTC-3’</td>
<td>Cloning of the -1,080/+14 ABCG1 promoter region</td>
</tr>
<tr>
<td>ABCG1-391F</td>
<td>5’-CCGCTGAGGAAACGCGGAGTCTC-3’</td>
<td>Mutagenesis of -376C&gt;T</td>
</tr>
<tr>
<td>ABCG1-361R</td>
<td>5’-CCGCTGAGGAAACGCGGAGTCTC-3’</td>
<td>Mutagenesis of -376C&gt;T</td>
</tr>
<tr>
<td>ABCG1-487F</td>
<td>5’-CCGCTGAGGAAACGCGGAGTCTC-3’</td>
<td>Chromatin Immunoprecipitation Assay</td>
</tr>
<tr>
<td>ABCG1-144R</td>
<td>5’-CCGCTGAGGAAACGCGGAGTCTC-3’</td>
<td>Chromatin Immunoprecipitation Assay</td>
</tr>
<tr>
<td>Rt-ABCG1 – F</td>
<td>5’-ATGGGAGTCTTTCTTGGAAACAC-3’</td>
<td>Real time PCR analysis of <em>ABCG1</em></td>
</tr>
<tr>
<td>Rt-ABCG1 – R</td>
<td>5’-GGACTGTGCCACCAGGAGGAGTCT-3’</td>
<td>Real time PCR analysis of <em>ABCG1</em></td>
</tr>
<tr>
<td>GAPDH – F</td>
<td>5’-TGCGGAGGAGTCTGGCGGA-3’</td>
<td>Real time PCR analysis of <em>GAPDH</em></td>
</tr>
<tr>
<td>GAPDH – R</td>
<td>5’-GAAGGGTGCATGGGCAA-3’</td>
<td>Real time PCR analysis of <em>GAPDH</em></td>
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*KpnI (GGTACC) and XhoI (CTCGAG) sites are underlined.*
### Supplemental Table III. Oligonucleotides used in DNA affinity precipitation assays.

<table>
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<th>Name</th>
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<tr>
<td>ABCG1-391wt-F-Bio</td>
<td>5'-Bio-CCCGTGCTGGCCACAGCCCGAGTTCCGG-3'</td>
<td>Biotinylated primer corresponding to the -391/-361 region of the ABCG1 promoter bearing the -376C allele</td>
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<tr>
<td>ABCG1-361wt-R</td>
<td>5'-CCCGAACTCGCCGGGCTGGGAGCCACGAGGAG-3'</td>
<td>Non-biotinylated primer corresponding to the -391/-361 region of the ABCG1 promoter bearing the -376C allele</td>
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<td>ABCG1-391mut-F-Bio</td>
<td>5'-Bio-CCCGTGCTGGCCACAGCCCGAGTTCCGG-3'</td>
<td>Biotinylated primer corresponding to the -391/-361 region of the ABCG1 promoter bearing the -376T allele</td>
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<tr>
<td>ABCG1-361mut-R</td>
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<td>Non-biotinylated primer corresponding to the -391/-361 region of the ABCG1 promoter bearing the -376T allele</td>
</tr>
<tr>
<td>ABCA1-118-F-Bio</td>
<td>5'-TGAACATACATAACAGACAGCGCAGGAGGA-3'</td>
<td>Biotinylated primer corresponding to the -118/-94 region of the ABCA1 promoter</td>
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<td>ABCA1+205R</td>
<td>5'-CCGCTCGAGGTCTTTCTTTCTACCCCTTGGCA-3'</td>
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<td>ABCA1-31-F-Bio</td>
<td>5'-Bio-CCGAATCTATAAACAGGGAACGTAG-3'</td>
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<td>Non-biotinylated primer corresponding to the -31/-9 region of the ABCA1 promoter (TATA)</td>
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Supplemental Figures and Figure Legends

Supplemental Figure I

---

**Supplemental Figure I**

---

E-BOX  
AP-2  
NFY  
NFKB  
SRE  
SP-1  
SP1
**Supplemental Figure II**

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<th>g.-1082C&gt;T</th>
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<th>g.-686G&gt;A</th>
<th>g.-530A&gt;G</th>
<th>g.-376C&gt;T</th>
<th>g.-367G&gt;A</th>
<th>g.-217C&gt;A</th>
<th>IVS4-6C&gt;T</th>
<th>G327 C&gt;T</th>
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**D' level**
- **= 1.0**
- **≥0.90**
- **<0.90**

**r² level**
- **>0.90**
- **>0.80**
- **<0.80**

3
Supplemental Figure III

<table>
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<th>input</th>
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<tr>
<td></td>
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</table>

![Image of gel electrophoresis pattern with bands indicating the effects of Mithramycin in different conditions (input, no ab, +a-Sp1).]
Supplemental Figure IV

Putative Sp1 binding sites

-566 → -558
-382 → -373
-184 → -176

(-1080/+14) ABCG1 -LUC

(-1080/+14) ABCG1 mut376 -LUC

-376 C
+14

-376 T
Supplemental Figure I. Nucleotide sequence of the core promoter and the first exon-intron boundary of the \textit{ABCG1} gene adapted from Langmann et al. Putative transcription factor binding sites are shown above the underlined core sequences. Note that there are three putative Sp1 binding sites at positions -566/-558, -382/-373, and -184/-176, respectively. The C at position -376 in the second Sp1 site (-382/-373; nt -1632 to +123 relative to +1 in ATG, NT_030188) which has been substituted by a T in heterozygotes in this study, is marked in bold and underlined. Promoter sequence and intron 1 is depicted in lower case letters, exon 1 in capitals.

Supplemental Figure II. Pairwise linkage disequilibria (LD) between eleven variants (minor allele frequency $\geq 0.002$) identified after screening the \textit{ABCG1} gene in individuals with the lowest 2% and highest 2% HDL cholesterol levels in the Copenhagen City Heart Study (CCHS) and subsequent genotyped in the CCHS. $D'$ values above and $r^2$ values below the gray diagonal. Pairwise LD was estimated using the software Haploview 4.0 (www.broad.mit.edu/mpg/haploview/download.php).

Supplemental Figure III. Representative image of the chromatin immunoprecipitation analysis. Symbols – and + represent chromatin immunoprecipitation assays performed in the absence or in the presence of 0.2 mM mithramycin, respectively. Input: non-immunoprecipitated chromatin (positive control); no ab: immunoprecipitation in the absence of the a-Sp1 antibody (negative control); +a-Sp1: immunoprecipitation in the presence of the a-Sp1 antibody.

Supplemental Figure IV. Illustration of -376C and -376T reporter genes. The (-1080/+14)\textit{ABCG1-luc} represents the wildtype -376C reporter gene, whereas the (-1080/+14)\textit{ABCG1mut376-luc} represents the mutated -376T reporter gene. In addition to the putative Sp1 binding site at position -382/-373 (GCCCAG\underline{CCC}; position -376 underlined), two putative Sp1 sites are present at positions -566/-558 and -184/-176, respectively.
Supplemental References


