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Identification and characterization of novel loss of function mutations in ATP-binding cassette transporter A1 in patients with low plasma high-density lipoprotein cholesterol

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ABSTRACT

Objectives: The current literature provides little information on the frequency of mutations in the ATP-binding cassette transporter A1 (ABCA1) in patients with low high-density lipoprotein cholesterol (HDL) levels that are referred to the clinic. In 78 patients with low plasma levels of HDL cholesterol that were referred to our clinic, we routinely screened for *ABCA1* gene mutations and studied the functionality of newly identified *ABCA1* missense mutations.
Methods: The coding regions and exon–intron boundaries of the *ABCA1* gene were sequenced in 78 subjects with HDL cholesterol levels below the 10th percentile for age and gender. Novel mutations were studied by assessing cholesterol efflux capacity (using apolipoprotein A–I as acceptor) after transient expression of ABCA1 variants in BHK cells.
Results: Sixteen out of 78 patients (21%) were found to carry 19 different *ABCA1* gene variants (1 frameshift, 2 splice-site, 4 nonsense and 12 missense variation) of which 14 variations were novel. Of three patients with homozygous mutations and three patients having compound heterozygous mutations only one patient presented with the clinical characteristics of Tangier Disease (TD) in the presence of nearly complete HDL deficiency. Seven out of eight newly identified *ABCA1* missense mutations were found to exhibit a statistically significant loss of cholesterol efflux capacity.
Conclusion: This study shows that one out of five patients who are referred to our hospital because of low HDL cholesterol levels have a functional *ABCA1* gene mutation. It is furthermore demonstrated that *in vitro* studies are needed to assess functionality of ABCA1 missense mutations.

1. Introduction

Twin studies have indicated that the variation in plasma high-density lipoprotein (HDL) cholesterol levels is largely determined by genetic factors [1]. Many genes have been implicated in HDL metabolism [2] and this number is still expanding [3,4].

One of the major HDL candidate genes is the ATP-binding cassette transporter A1 (*ABCA1*) which is a cell membrane double transporter protein that plays an important role in cholesterol homeostasis. It is generally accepted that ABCA1 controls the rate-limiting step in the transport of cellular free cholesterol and phospholipids to apolipoprotein (apo) A–I which leads to the formation of pre-β high-density lipoprotein (pre-β HDL). Through the action of lecithin:cholesterol acyltransferase (LCAT), this pre-β HDL can mature into larger HDL subspecies [5–7].

Defects in the *ABCA1* gene cause Tangier Disease (TD) [8], an autosomal recessive disorder characterized by HDL deficiency and accumulation of cholesterol in peripheral tissues. TD patients suffer from many symptoms including peripheral neuropathy, hepatosplenomegaly, and corneal opacification. While carriers of mutations in the *ABCA1* gene are reported to exhibit an increased risk of atherosclerosis [9], it has also been reported that not all TD patients suffer from overt atherosclerosis [10]. Recent epidemiological data suggest that loss of ABCA1 function is not necessarily

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associated with increased risk of ischemic heart disease or cerebrovascular disease [11,12]. Heterozygous carriers for detrimental *ABCA1* mutations do not present with specific clinical symptoms but present with markedly lower HDL cholesterol levels compared to age- and gender-matched controls [13].

ABCA1 is mainly expressed in the small intestine, liver, brain and cells of reticuloendothelial system. The 220 kDA protein is synthesized in the endoplasmic reticulum and transported to the plasma membrane via vesicles, but it is also found in intracellular compartments such as late endosomes/lysosomes, the trans-Golgi network and endoplasmic reticulum [6,14].

More than 50% of the over 90 identified *ABCA1* mutations in the current literature [15] are missense mutations. Most of these mutations appear to be localized in extracellular loops, nuclear binding domains and carboxy terminal region [12,16,17]. Functionally defective ABCA1 variants fail to mediate lipid efflux to apo A–I and as a consequence the non-lipidated apo A–I fails to undergo maturation into larger HDL subspecies and will undergo rapid renal clearance [18].

Previous studies have demonstrated that missense mutations in *ABCA1*, identified in patients with Tangier Disease or individuals with Familial hypoalphalipoproteinemia, can cause different degrees of impairment in lipid transfer activity [13].

Thus far, only few investigators have routinely sequenced the *ABCA1* gene. There is only one report on patients with isolated low HDL cholesterol that are referred to the clinic [19]. Two other groups have reported ABCA1 gene variation at the lower end of the HDL cholesterol distribution curve of prospective epidemiological studies [11,17]. In the current study, we sequenced the *ABCA1* gene in 78 patients that were referred to our clinic who presented with HDL cholesterol levels below the 10th percentile for age and gender. We identified an unexpected high number of ABCA gene variants ($n = 19$), of which 14 had not been described earlier. *In vitro* as well as confocal imaging experiments were carried out to evaluate whether the newly identified mutations were functional and could therefore explain the low HDL cholesterol phenotype of the respective patients.

2. Materials and methods

2.1. Study population

The current study is part of a research effort aiming at the characterization of mutations in established and newly proposed HDL genes, and the identification of novel genes that regulate HDL cholesterol levels. In a first step, we have selected 78 individuals with extremely low HDL cholesterol (<10th percentile for age and gender). In a second step, we have sequenced the coding regions of established HDL genes, i.e. ATP-binding cassette transporter AI (ABCA1), apolipoprotein A–I (apo A–I), and lecithin:cholesterol acyltransferase (LCAT). Patients were either seen in our outpatient clinic or they were referred to our hospital. With the exception of five patients from south-east Asia, one patient from Belgium and one patient from Spain, all other patients were of Dutch ancestry. Of note, patients #2 and #14 (see Table 1) were referred with a suspicion of Tangier Disease. In this screening effort, we identified one mutation in APOAI in two subjects, 13 mutations in LCAT in 20 subjects and 19 mutations in ABCA1 in 16 individuals.

2.2. Biochemical measurements

Blood was obtained after an overnight fast in EDTA-coated tubes and directly placed on ice. Plasma was isolated by centrifugation at 4 °C, 3000 × *g* for 15 min and stored at −80 °C for further analyses. Plasma cholesterol, LDL cholesterol, HDL cholesterol and triglyceride levels were analyzed using a commercially available enzymatic method (Randox, Westburg, USA) on the Cobas Mira autoanalyzer (Roche, Basel, Switzerland).

2.3. Mutation screening in ABCA1

Genomic DNA was extracted from 10 ml whole blood on an AutopureLS apparatus according to manufacturer's protocol (Gentra Systems, Minneapolis, USA). Primers were designed to amplify coding sequence and exon–intron boundaries of the *ABCA1* gene using web-based Primer3 software [20]. PCR amplification was carried out with 50 ng of genomic DNA in a 25 μl reaction volume containing 1× Taq DNA polymerase buffer (Qiagen, Hilden, Germany), 50 μmol/l of each dNTP, 0.4 μmol/l of each primer, and 1 U Taq DNA polymerase. A Touchdown PCR program (96 °C for 5 min, then 20 cycles of 30 s at 96 °C, 30 s at 65 °C to 55 °C with 0.5 °C decrement/cycle and 30 s at 72 °C, followed by 30 cycles of 30 s at 96 °C, 30 s at 55 °C and 30 s at 72 °C) on a T3 biocycler PCR apparatus (Biometra, Germany) was used for DNA amplification. The sequence reactions were performed using fluorescently labeled dideoxy chain terminations with a BigDye terminator ABI prism kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol and analyzed on an Applied Biosystems automated DNA sequencer (model 370). Sequences were analyzed with the Sequencher Package (Gene Codes Co., Ann Arbor, MI, USA).

2.4. Generation of ABCA1 gene expression vectors

Wild-type ABCA1-GFP pcDNA3.1 vector was provided by Prof. S. Calandra (University of Modena, Italy). This vector carries the human cDNA of *ABCA1* fused in frame with a Green Fluorescence Protein (GFP) cDNA. Eight novel missense variations [c.299C > G (p.S100C), c.1724A > G (p.D575G), c.1779C > G (p.F593L), c.3167T > C (p.L1056P), c.3757G > A (p.E1253K), c.4535C > T (p.T1512M), c.5573T > C (p.V1858A), c.5821T > C (p.C1941R)] were introduced into this chimeric construct by site-directed mutagenesis using Stratagene QuikChange XL site-directed mutagenesis kit according to manufacturer's instructions (La Jolla, CA, USA).

2.5. Functional assessment of ABCA1 gene mutations

Baby hamster kidney (BHK) cells were obtained from ATCC (Manassas, VA). Cells were cultured in DMEM F-12 GlutaMax (GIBCO) containing 10% fetal bovine serum (FBS) and Penicillin (100 U/ml)–Streptomycin (100 μg/ml) at 37 °C in a humidified 5% CO_2 incubator. Transient transfections were carried out when the cells were at 90% confluency using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Two days after transfection, transfection efficiency was evaluated by FACS analysis. The cells were harvested, centrifuged, extensively washed with phosphate buffered saline, resuspended in the same buffer and analyzed in a FacsCalibur cell sorter (BD Biosciences, Bedford, MA) using CellQuestPro software. Non-transfected cells were used as negative control. To assess cholesterol efflux potential, the transfected cells were incubated with 2 μCi/ml [^{3}H]cholesterol for 24 h. Cholesterol efflux was measured after 4 h incubation with or without apo A–I (20 μg/ml; Calbiochem). Radioactivity in the medium and cells was determined by scintillation counting and the fractional cholesterol efflux was calculated as the percentage of cpmmedium/(cpmmedium + cpmcell). For each construct, efflux to apo A–I was measured in triplicate in three independent experiments.