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Biochimica et Biophysica Acta 1689 (2004) 47–57

BIOCHIMICA ET BIOPHYSICA ACTA

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Two novel missense mutations in ABCA1 result in altered trafficking and cause severe autosomal recessive HDL deficiency

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Received 4 February 2003; received in revised form 6 January 2004; accepted 22 January 2004

Available online 20 February 2004

Abstract

Extremely low concentrations of high density lipoprotein (HDL)-cholesterol and apolipoprotein (apo) AI are features of Tangier disease caused by autosomal recessive mutations in ATP-binding cassette transporter A1 (*ABCA1*). Less deleterious, but dominantly inherited mutations cause HDL deficiency. We investigated causes of severe HDL deficiency in a 42-year-old female with progressive coronary disease.

ApoAI-mediated efflux of cholesterol from the proband's fibroblasts was less than 10% of normal and nucleotide sequencing revealed inheritance of two novel mutations in *ABCAI*, V1704D and L1379F. ABCA1 mRNA was approximately 3-fold higher in the proband's cells than in control cells; preincubation with cholesterol increased it 5-fold in control and 8-fold in the proband's cells, but similar amounts of ABCA1 protein were present in control and mutant cells. When transiently transfected into HEK293 cells, confocal microscopy revealed that both mutant proteins were retained in the endoplasmic reticulum, while wild-type ABCA1 was located at the plasma membrane.

Severe HDL deficiency in the proband was caused by two novel autosomal recessive mutations in *ABCA1*, one (V1704D) predicted to lie in a transmembrane segment and the other (L1379F) in a large extracellular loop. Both mutations prevent normal trafficking of ABCA1, thereby explaining their inability to mediate apoA1-dependent lipid efflux.

Keywords: ABCA1; Protein trafficking; HDL deficiency; Apolipoprotein AI; Cholesterol efflux

1. Introduction

Tangier disease [1–4] is a rare autosomal recessive disorder characterised by extremely low levels of high density lipoprotein (HDL)-cholesterol and of apolipoprotein (apo) AI in plasma, and by the accumulation of cholesterol in macrophage-rich tissues, notably the tonsils, which acquire a characteristic orange-yellow colour. Hepatosplenomegaly and peripheral neuropathy are also commonly associated with this disorder [5]. Tangier disease is caused by mutations in the ATP-binding cassette transporter A1 (*ABCA1*) gene that impair apoAI-mediated cholesterol efflux from cells [6–12], and these patients invariably have defects in both alleles of *ABCA1*. Some heterozygous relatives of Tangier patients display an intermediate phenotype of low to normal HDL-cholesterol and a 50% reduction in apoAI-mediated efflux of cholesterol [13]. In the absence of functional ABCA1 protein, newly secreted apoAI is rapidly degraded and cholesterol efflux deficiency ensues [14]. The disorder is often, but not always, associated with an increased risk of coronary artery disease [5].

Familial HDL deficiency is characterised by less severe HDL deficiency than that seen in Tangier disease, but with a dominant mode of inheritance. Nevertheless, it too results from mutations in *ABCA1* [12]. As yet, no clear distinction has emerged between the mutations in *ABCA1* that result in autosomal recessive Tangier disease and those that cause dominant HDL deficiency, or between those that do or do

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doi:10.1016/j.bbadis.2004.01.007

not result in all the clinical manifestations of accumulation of cholesterol in the reticuloendothelial system. In this study, we describe a patient who has inherited two defective alleles of *ABCA1* from apparently unaffected parents, each encoding a previously undescribed single amino acid substitution (L1379F and V1704D). The patient has almost complete HDL deficiency and severe premature coronary artery disease, but does not have yellow tonsils or other overt signs of cholesterol deposition in macrophages.

2. Materials and methods

2.1. Patient and family studies

The proband was a 42-year-old pre-menopausal woman of English origin who was assessed in the lipid clinic at Chelsea and Westminster Hospital, London after diagnosis of coronary artery disease treated by coronary artery bypass graft. Family members were evaluated separately, with informed consent.

All blood samples for lipid and lipoprotein analysis were taken after an overnight fast of at least 10 h. Low density lipoprotein cholesterol (LDL)-cholesterol concentrations were calculated using the Friedwald formula [15]. ApoAI and apolipoprotein B were measured by nephelometry. A skin biopsy was taken aseptically under local anaesthesia with informed consent from the proband. Dermal fibroblasts were grown from 0.25 cm^2 explants of dermis cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mmol/l glutamine, 100 units penicillin/ml and 100 μg streptomycin/ml.

2.2. Measurement of LCAT activity

Plasma LCAT activity was measured using the egg lecithin:human apoAI: [^{3}H]-cholesterol proteoliposome substrate method as described previously [16]. This assay largely reflects LCAT concentration and was carried out by incubating plasma (7.5 μl) with substrate (242.5 μl) at 37 °C for 1 h. Lipids were extracted by addition of chloroform–methanol and separated by thin-layer chromatography; radioactivity in the free cholesterol and cholesteryl ester bands was measured and the values used to calculate the percentage of cholesterol esterified per hour.

2.3. Agarose gel electrophoresis and immunoblotting

Lipoproteins in plasma (2 μl) were separated by electrophoresis on pre-cast agarose gels (Hydragel, Sebia) and either stained with Sudan black or transferred onto Hybond-ECL nitrocellulose membranes (Amersham Pharmacia) by pressure blotting. For immunostaining, monoclonal antibodies against human apoAI (Calbiochem) or LCAT (S. Schepelmann and J.S. Owen, unpublished data) were diluted 1:1000 in 3% (w/v) bovine serum albumin in phosphate-buffered saline (PBS). A goat anti-mouse Ig-HRP (Amersham Pharmacia) was used as a secondary antibody (1:5000 in 10% (w/v) milk powder/PBS-Tween), followed by detection by enhanced chemiluminescence (Amersham Pharmacia).

2.4. Measurement of cholesterol efflux

Control and proband skin fibroblasts were grown to 50–70% confluence in 12-well tissue culture plates. Cells were labeled by incubation with 1 μCi/ml [^{3}H]-cholesterol in DMEM containing 5% fetal bovine serum for 72 h. The monolayers were washed and incubated with BSA (1 mg/ml) in serum-free DMEM for 48 h to allow the labeled cholesterol to equilibrate with intracellular cholesterol pools. Cholesterol efflux was induced by the addition of 5 μg/ml human apoAI [17] in serum-free DMEM to quadruplicate wells; control wells received DMEM alone. Media were removed after 6 h, the cells washed once with PBS and then dissolved in 0.1 M NaOH. Radioactivity in media and cells were measured and the cholesterol efflux rate calculated as the % of dpm in medium/(dpm in medium + dpm in cells). The mean of values for patient's cells (apoAI-mediated efflux minus mean of unstimulated cells) was compared to the value for control cells (apoAI-mediated efflux minus mean of unstimulated cells) by an unpaired Student's *t*-test.

2.5. Nucleotide sequence analysis

DNA was extracted from venous whole blood [18]. Genotyping was as previously described [19] with markers from the ABI Prism linkage mapping set (PE Applied Biosystems). The coding regions and proximal promoter (starting from 395 bp upstream from the ATG of *ABCA1*) were amplified by PCR with primers located within 50–70 bp of each splice junction (primer details available on request: c.albrecht@csc.mrc.ac.uk). PCR reactions were carried out in a volume of 25 μl containing 100 ng of DNA, 250 nM of each primer, 200 μM of each dNTP, 1 × Thermopol buffer (New England Biolabs, Hitchen, Herts, UK) and 1 U of *Vent* polymerase (New England Biolabs). Amplification conditions were as follows: initial denaturation for 1 min at 97 °C, 35 cycles of 1 min at 96 °C, 1 min at 52–59 °C (depending on the primer pair) and 1 min at 72 °C, with a final extension at 72 °C for 10 min. Amplification products were analysed on 2% low melting agarose gels, purified from the gel with a gel extraction kit (Qiagen) and sequenced with both PCR primers using an ABI 3700 Prism automated sequencer. Sequences were analysed with Sequence Navigator software, and compared with the published sequence of ABCA1 cDNA (GenBank accession no: AF285167). ABCA1 mRNA was sequenced after amplification by RT-PCR from total RNA, isolated from cultured skin fibroblasts using RNA-Bee™ RNA isolation reagent according to the manufacturer's instructions (Biogenesis Ltd.). The RT reaction comprised 0.04 U of oligo-p$(dT)_{15}$ primer, 5 mM $MgCl_2$, 1 mM of each dNTP, 40 U RNAse inhibitor, 1 × reaction buffer (Roche)

and 20 U AMV reverse transcriptase (Roche). RT reaction mix (1–5 μl) was then used as template in a PCR reaction as described above, with primers that produced nine overlapping fragments of the coding regions (details available on request).

2.6. Analysis of polymorphisms in DNA by WAVE™ analysis

PCR fragments of *ABCA1* exons 28, 36 and 6 were amplified as described above with the following primers:

exon 28,	5′-TCTAACACTTGCCGTTTCCTGCTGTC and 5′-CCTCGTAAACATCTTTGGTCTGCTCG
exon 36,	5′-TTGTCTGTGTGTCCATGTCCTCACTG and 5′-GCTGGAACATTTCCTGATGATAGCCAG
exon 6,	5′-GGACCCAGCTTCCAATCTTCATAATCC and 5′-GCCTCACATTCCGAAAGCATTAGTGC

PCR products (8 μl of reaction mix) were denatured by heating to 94 °C, followed by cooling to 25 °C over 25 min to enhance heteroduplex formation, and analysed on a WAVE™ Nucleic Acid Fragment Analysis System (Transgenomic). The optimum temperatures for analysis of each fragment were predicted with Wavemaker software (Transgenomic) and confirmed empirically.

2.7. Quantification of mRNA

Skin fibroblasts were preincubated for 24 h in DMEM medium containing either 10% fetal bovine serum or cholesterol (30 μg/ml) complexed to fatty acid-free bovine serum albumin (2% w/v). Cell monolayers from two 9-cm dishes were washed twice with sterile Puck's saline A (Gibco BRL, Life Technologies), scraped into a sterile 50 ml tube in 5 ml of Puck's saline and pelleted by centrifugation for 10 min at 3000 rpm. Total cellular RNA was extracted as described above. Contaminating DNA was removed by incubation of total RNA with DNAse I (Promega). For cDNA synthesis, RNA (1 μg) was transcribed with a First Strand cDNA synthesis Kit for RT-PCR (Roche), according to the supplier's instructions. For Taqman analysis, the cDNA was diluted 20-fold with nuclease-free water.

Primers and probe for Taqman analysis of ABCA1 mRNA were designed with PrimerExpress software (PE Applied Biosystems) and the reaction optimised according to PE User bulletin number 2. The forward primer was 5′-GGGAGGCTCCCGGAGTT in exon 3, the reverse primer was 5′-GTATAAAAGAAGCCTCCGAGCATC in exon 4, and the FAM-labeled probe, spanning exons 3 and 4, was 5′-AACTTTAACAAATCCATTGTGGCTCGCCTGT. Single tube Taqman analysis was performed on an ABI prism 7700 sequence detection system with 300 nM of forward and reverse primers in the presence of 200 nM 5′ FAM-3 TAMRA-tagged probe. The internal standard was GAPDH mRNA, assayed with commercially supplied reagents (PE Applied Biosystems). Reactions were carried out in triplicate and contained 5 μl of diluted cDNA in a total volume of 25 μl. The amount of ABCA1 mRNA in cells was expressed relative to that of GAPDH, and was calculated as $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{target} - Ct_{GAPDH}$. Fold induction (relative expression in patient/relative expression in control) was expressed as $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct = \Delta Ct_{patient} - \Delta Ct_{control}$.

2.8. Immunoblotting of ABCA1 protein in cell membranes

Skin fibroblasts were preincubated in medium containing either 10% fetal bovine serum or 2% bovine serum albumin plus 30 μg/ml cholesterol for 24 h [11]. Cells in two 9-cm dishes were washed twice with PBS and harvested by scraping from the dish. A total membrane fraction was prepared as described by Rosenberg et al. [20]. Briefly, the cells were lysed by homogenisation in 1 ml ice-cold buffer A (50 mM maltose, 50 mM Tris–HCl pH 7.5, 2 mM EGTA, 2 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine). Cell debris was removed by centrifugation at 500 × *g* for 10 min, the supernatant diluted 3-fold with buffer B (buffer A with 300 mM maltose), and the membrane fraction pelleted by centrifugation at 100,000 × *g* for 60 min. The membrane pellet was resuspended in 100 μl buffer B and protein content measured (Biorad protein assay). Membrane proteins were fractionated on reduced SDS-PAGE (12% acrylamide) and transferred to nitrocellulose membranes. ABCA1 protein was detected with a rabbit antipeptide antiserum, diluted 1:1500 (Abcam Ltd., Cambridge, UK) followed by peroxidase-labeled anti rabbit IgG, diluted 1:3000 (Dako).

2.9. Plasmids/DNA construction

Full-length human ABCA1 cDNA was generated by reverse transcriptase PCR of mRNA obtained from skin fibroblasts of the patient and a healthy individual (see Nucleotide sequence analysis). Wild-type ABCA1 cDNA was cloned into pGEM®-11Zf vector (Promega) and replaced with the corresponding DNA fragment (*Asp*718–*Bam*HI) containing each mutation (4425 or 5401). Enhanced green fluorescent protein (eGFP) was fused in frame to the C-terminus of ABCA1 by overlapping PCR strategy as described previously [21] using the following primers: 5′-CCAGAGGAGATGCTTTCCTTAA/5′-CTTGCTCACCATTACATAGCTTTC for ABCA1 and 5′-GAAAGCTATGTAATGGTGAGCAAG/5′-TCTTTGTCGCGGCCGCTTTACTTGTACAGCTCGTCCATGCC (including a *Not*I restriction site) for eGFP. The PCR reactions were carried out with 100 ng DNA as described above (see Nucleotide sequence analysis) at an annealing temperature of 55 °C. The gel purified PCR products were pooled and 1 μl subjected to another PCR reaction with the outermost primers (annealing temperature 55 °C, extension 1.5 min at 72 °C, min). The PCR product was cloned into pCR® 4Blunt-TOPO vector (Invitrogen) and *Bam*HI–*Not*I ABCA1 cDNA fragments in the pGEM®-11Zf constructs replaced

with the ABCA1-eGFP hybrid. The resulting wild-type and mutant ABCA1-eGFP cDNAs were cloned with *Sal*I–*Not*I into pCI-neo mammalian expression vector (Promega) and all constructs verified by complete sequencing.

2.10. Analysis of expression of ABCA1 eGFP fusion proteins in HEK 293 cells

HEK 293 cells were plated on poly-L-lysine (Sigma) coated glass cover slips in six-well plates 12 h before transfection. Cells were transfected with plasmids containing wild-type or mutated ABCA1-eGFP cDNA according to the calcium phosphate transfection protocol (Clontech) with 2 μg of DNA/well, washed twice with PBS after 6 h, and fixed in 4% formaldehyde/4% sucrose 24 h after transfection.

Fixed cells were permeabilised with 0.1% Triton X-100 for 4 min and stained with DAPI (15 mg/ml; 1:10,000 dilution; Molecular Probes) for nuclear DNA. In order to identify the intracellular localisation of the wild-type and mutant ABCA1 proteins, cells were also stained with the mouse monoclonal antibody against sarco/endoplasmic reticulum calcium ATPase (SERCA 2; clone 11D8; Affinity Bioreagents Inc.) for 2 h at room temperature after blocking with 0.2% fish skin gelatin in PBS. The antibody against SERCA2 was detected by a goat anti mouse IgG secondary antibody conjugated to Alexa 568 (Molecular Probes, 1:400 dilution for 30 min, room temperature).

Cells were imaged by a Leica SP confocal microscope through a 63X 1.32 NA PlanApoChromat oil immersion objective. eGFP was excited by a 488 nm line of an Argon laser and Alexa 568 by a 568 nm line of a Krypton laser. In order to avoid bleedthrough, the fluorophores were excited sequentially. The emitted fluorescence was collected separately through a triple dichroic mirror 488/568/663. The emission filter bands for eGFP and Alexa 568 fluorescence were restricted to 500–552 nm and 594–620 nm, respectively. DAPI staining of nuclear DNA was excited by a 351 nm line of a UV laser and emission fluorescence collected by a 396–508 nm bandpass filter.

Stack of confocal sections separated by 1 μm increments were taken and images analysed by Metamorph software (Universal Imaging Corporation, USA).

3. Results

3.1. Clinical findings

The 42-year-old proband presented with progressive and premature coronary artery disease requiring coronary artery bypass surgery; at 1 year follow-up, angiography confirmed severe progressive coronary atheroma. The proband's fasting lipid profile revealed marked HDL deficiency, with HDL-cholesterol less than 0.1 mmol/l; total cholesterol was 3.8

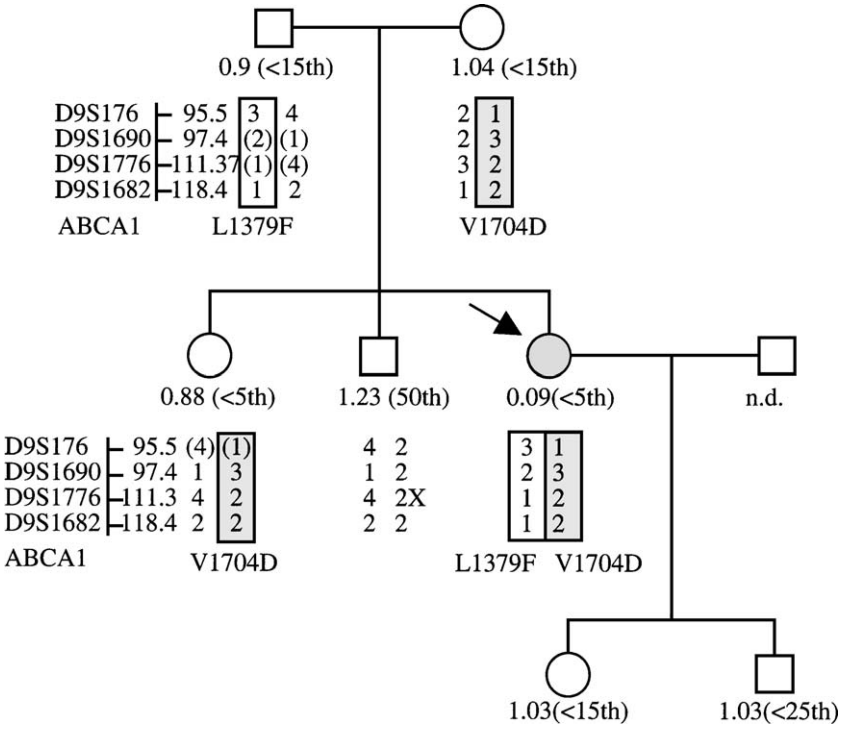

Fig. 1. Pedigree of the HDL-deficient proband. The proband is indicated with an arrow; values below each symbol indicate HDL-cholesterol concentration (mmol/l) and the centile in England according to age and gender [22]. Markers that flank ABCA1 on chromosome 9q31 [26] are shown with their position in bp $\times 10^{-6}$; ABCA1 is at ~ 101.15 (UCSC genome browser, http:// genome.ucsc.edu; April 2003 freeze). Genotypes are shown below each symbol (those in brackets were deduced). A recombination in the maternal allele of the proband's unaffected brother is indicated (×). In the proband, the paternal allele (clear box) carried the L1379F variant of *ABCA1*, and the maternal allele (shaded box) carried the V1704D variant.

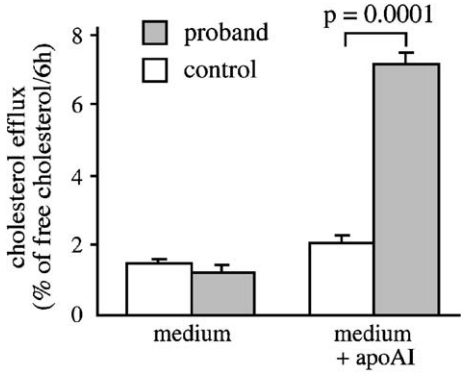

Fig. 2. Efflux of cholesterol from skin fibroblasts in culture. Skin fibroblasts were equilibrated with [^{3}H]-cholesterol and incubated in serum-free medium with or without human apoA1 (5 μg/ml). Cholesterol efflux was measured as the percentage of labeled cholesterol present in the medium after 6 h. The mean of values from three separate determinations is shown. The significance of the difference between proband and control was determined by unpaired Student's *t*-test.

mmol/l, triglycerides 2.52 mmol/l and the calculated LDL-cholesterol was 2.8 mmol/l. Lipoprotein analysis showed apoAI was < 0.3 g/l, apoB 0.83 g/l and Lp(a) 98 mg/l. Apart from HDL deficiency, no other risk factors for premature coronary disease were identified in the proband, except that she had been a modest cigarette smoker (10 per day). Although our study was focused on identifying the proband's clinical and biochemical phenotype, HDL-cholesterol levels were also measured in her immediate family. For both parents, her sister and her daughter aged 17 years these were below the 10th centile for their age and gender [22] (Fig. 1). The HDL-cholesterol of her 15-year-old son was just above the 10th centile for 16–24-year-olds, whereas that of her brother was close to the mean for his age and gender. The proband's father had coronary heart disease, but had only become symptomatic at age 70 years and her mother was well at age 79 years. On physical examination, the proband did not have enlarged yellow tonsils, hepatosplenomegaly or peripheral neuropathy; renal function was normal. Possible underlying causes of apoAI or HDL deficiency were investigated, including LCAT deficiency [23] and mutations in the genes encoding apoAI [23] or ABCA1 [9–12].

3.2. Investigation of inherited defects in LCAT or apoAI

Mutations in the genes for LCAT and apoAI were excluded as the cause of HDL deficiency. LCAT activity in the proband's plasma, as determined in a proteoliposomal assay [16], was at the lower end of the normal range, with an esterification rate of 8.5%/h, 67 nmol/ml/h (reference range of 7.7–12.8%/h; 60–101 nmol/ml/h) [24]; 74% of plasma cholesterol was esterified (reference range 68–75%) [24]. Immunoblotting of plasma proteins fractionated by agarose gel electrophoresis revealed normal amounts of LCAT protein, but undetectable apoAI in the proband's plasma. Analysis of the sequence of the apoAI gene in the proband's DNA [25] revealed no mutations in the coding sequence, intron/exon splice junctions or proximal promoter.

3.3. Investigation of inherited defects in ABCA1 as the cause of HDL deficiency

There was no difference in the basal rate of cholesterol efflux from the proband's skin fibroblasts cultured in serum-free medium compared to that in control fibroblasts (1.5 vs. 1.2% of cellular free cholesterol in 6 h) (Fig. 2). However, in the presence of apoAI, efflux from the proband's cells barely increased over the basal level, whereas efflux from control cells was increased by approx. 6-fold to 7.0% of cellular free cholesterol, a value consistent with other studies [11]. This marked reduction of apoAI-mediated cholesterol efflux from the proband's fibroblast (< 10% of normal) suggests defective ABCA1 activity in her cells. Analysis of markers flanking *ABCA1* on chromosome 9q31 [26] revealed that the proband had inherited two different alleles of *ABCA1*, and that neither of her unaffected siblings had inherited the same two alleles (Fig. 1).

Table 1
Genetic variants in *ABCA1* in the HDL-deficient proband

Exon	Base no.[a]	Codon[b]	Base substitution	Amino acid change	Comments
1	273		hmz C to G	none	common [27]
2	368–370	Leu 26	hmz TTA to CTG	none	CTG in genomic sequence [27]
5	764	Leu 158 (98)	htz A/G	none	common [29]
6	947	Arg 219 (159)	htz G/A	Arg to Lys	common [28]
14	2330	Ile 680 (620)	hmz C	none	common [28]
16	2763	Ile 825 (765)	hmz G	Ile to Val	common [28]
17	2930	Met 883 (823)	hmz A	Met to Ile	common [28]
28	4425	Leu 1379 (1319)	htz C/T	Leu to Phe	novel, rare
30	4571	Thr 1427 (1367)	htz G/A	none	common [28]
34	5050	Arg 1587 (1527)	htz G/A	Arg to Lys	common [28]
36	5401	Val 1704 (1644)	htz T/A	Val to Asp	novel, rare

[a] Based on published sequence for cDNA (GenBank accession AF285167).
[b] Based on ATG start codon = bp 291-3 in cDNA sequence above (previous codon no. in parentheses).

ABCA1 defects could not be excluded as the underlying cause of the apoAI deficiency, and hence the nucleotide sequence of the gene was determined. This revealed that the proband was heterozygous at a number of sites in *ABCA1*, as listed in Table 1. The majority of these were polymorphisms that have been observed in control populations [27–29]. However, two single base pair substitutions were novel, namely $C_{4425}T$ in exon 28 (based on GenBank accession no. AF28517), predicted to substitute L1379 with Phe, and $T_{5401}A$ in exon 36, predicted to substitute V1704 with Asp. Neither of these variants was found in 164 alleles of a control English population [30]. In comparison, the allele frequency of the R219K polymorphism was 0.22 (K allele), similar to that found in other European populations [31]. The proband's father was heterozygous for L1379F, and her mother and sister for the V1704D variant (Fig. 1).

3.4. Expression of normal and mutant ABCA1 in vitro

The L1379F and V1704D mutations were introduced into the full-length cDNA for ABCA1, fused at its carboxy-terminus to eGFP. The C-terminal eGFP tag does not alter either the expression and function of the ABCA1 protein or its localisation [32,33]. Both wild-type (WT) and mutant

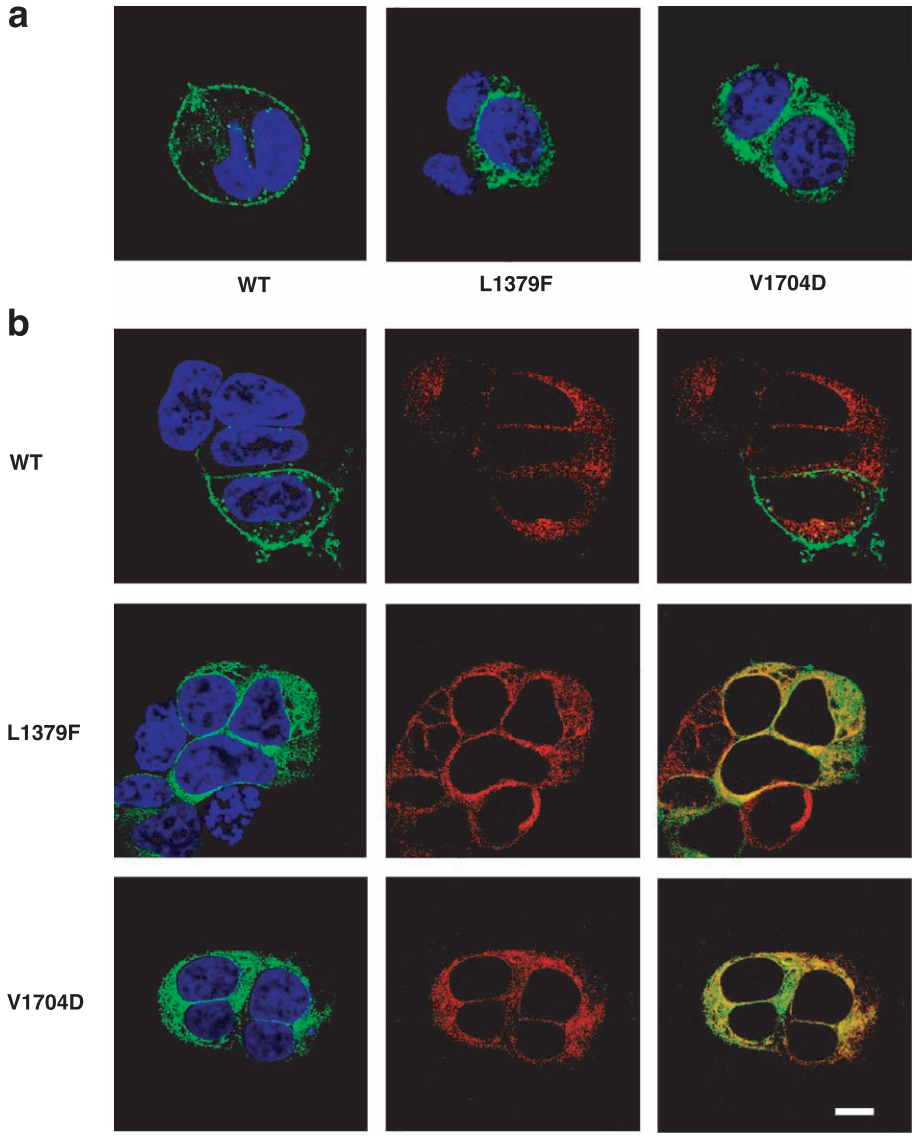

Fig. 3. Expression of ABCA1-eGFP fusion proteins in HEK 293 cells. (a) Confocal sections of transiently transfected HEK 293 cells expressing wild-type ABCA1-eGFP (left panel), ABCA1-eGFP L1379F (middle panel) and ABCA1-eGFP V1704D (right panel); eGFP fluorescence is shown in green and DAPI staining of nuclear DNA in blue. Both mutations caused severe alteration in ABCA1 trafficking to the plasma membrane as compared to the wild-type (WT). (b) HEK 293 cells expressing wild-type ABCA1-eGFP, ABCA1-eGFP L1379F and ABCA1-eGFP V1704D were stained for SERCA 2 as an ER marker. Confocal sections are shown for eGFP (green, left column), SERCA 2 (red, middle column) and the co-localisation of eGFP and SERCA 2 in the overlay (yellow, right column). A large extent of co-localisation was found for both mutants, suggesting retention of L1379F and V1704D in the ER. In contrast, wild-type ABCA1 localised predominantly to the plasma membrane and no co-localisation with the ER marker was apparent. Dimension bar indicates 10 μm.

ABCA1-eGFP proteins were found to be expressed in transiently transfected HEK293 cells. As expected [32,33], normal ABCA1-eGFP was present mainly at the cell surface (Fig. 3a, left panel) with punctate intracellular vesicles visible in some cells and possible accumulation in the Golgi in others (data not shown). In marked contrast to this, trafficking of both mutated proteins to the plasma membrane was severely altered in comparison to the wild-type (Fig. 3a, middle and right panels). Their intracellular localisation pattern suggested that both were present largely in the endoplasmic reticulum (ER). This was confirmed by the observation that both mutant eGFP fusion proteins, but not wild-type ABCA1-eGFP, co-localised extensively with sarco/endoplasmic reticulum calcium ATPase (SERCA 2), a marker of the ER [34] (Fig. 3b).

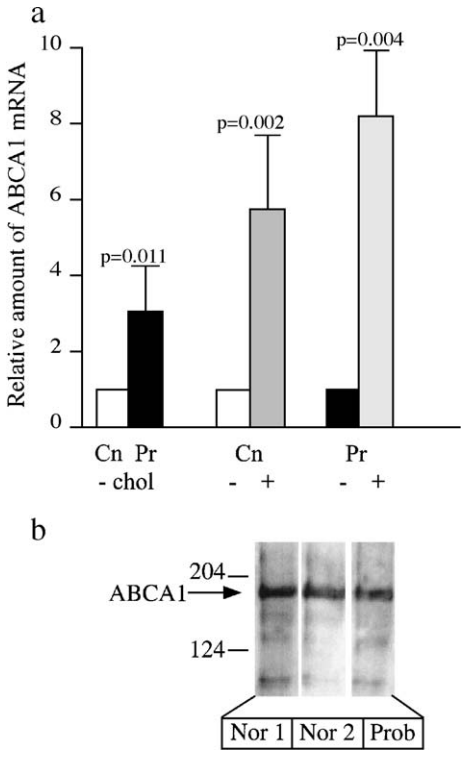

Fig. 4. Quantification of ABCA1 mRNA and protein in cultured skin fibroblasts. (a) Total RNA was isolated from skin fibroblasts incubated for 24 h in medium containing 10% serum or in serum-free medium containing 2% BSA and 30 μg/ml cholesterol. Basal ABCA1 mRNA levels in the proband's cells incubated with serum is expressed relative to the mean of values (normalized to 1.0) from cells from four different normolipaemic controls (left). Also shown is the effect of cholesterol loading on ABCA1 mRNA levels in control cells (mean of four, centre) and the proband's cells (right), relative to the value in cells incubated with serum. Each value represents the mean ± S.D. of two Taqman real-time PCR determinations of at least three different preparations of RNA. The variation between control cell lines was less than 10% of the mean for cells incubated in medium and 15% for cells incubated with cholesterol. Significant differences (*P* values) were determined by unpaired Student's *t*-test. (b) Membrane proteins from cultured fibroblasts (40 μg in approx. 25 μl of extract) were fractionated by reducing SDS-PAGE (12% acrylamide gels) and immunoblotted with rabbit polyclonal anti-ABCA1 peptide. Bound antibody was detected with peroxidase-conjugated anti-rabbit IgG by chemiluminescence (2 min exposure). The positions of marker proteins (kDa) on the blot are indicated.

3.5. ABCA1 expression in cultured skin fibroblasts

Cultured skin fibroblasts from the proband contained 3-fold more ABCA1 mRNA relative to GAPDH mRNA than control cells when assayed by real-time PCR (Fig. 4a). The variation in ABCA1 mRNA content relative to GAPDH mRNA in cultured skin fibroblasts from four different control subjects was less than 10% ($\Delta Ct = 6.84 \pm 0.15$). When the cells were preincubated with cholesterol to induce ABCA1 expression, the mean relative level of ABCA1 mRNA increased by approximately 6-fold in control cells ($\Delta Ct = 4.16 \pm 0.27$, $n = 4$), and more than 8-fold in the proband's cells (Fig. 4a). In contrast, there was no discernible difference between the amount of ABCA1 protein in membranes from the proband's and control cells when estimated by semi-quantitative immunoblotting (Fig. 4b).

4. Discussion

We describe a patient of English origin with severe HDL deficiency and premature coronary disease who is heterozygous for two rare alleles of *ABCA1* that are predicted to cause single amino acid substitutions, L1379F and V1704D. These mutant alleles have not been described previously and, as far as we are aware, this is the first individual of English origin known to have a disorder caused by mutations in *ABCA1*. There is strong evidence that these mutations in *ABCA1* are the underlying cause of the HDL deficiency. Firstly, the clinical and biochemical phenotype of the proband is consistent with defective *ABCA1*, and other possible causes of HDL deficiency were excluded. Secondly, the two *ABCA1* variants were not found in 164 alleles of English origin and, finally, both mutations were found to disrupt trafficking to the plasma membrane of ABCA1 protein when expressed in cultured cells in vitro. Thus, the data are consistent with a diagnosis of HDL deficiency caused by autosomal recessive defects in *ABCA1*.

Both L1379 and V1704 are conserved between human, mouse and chicken ABCA1 and the regions in which they lie are strongly conserved between ABCA1 and ABCA4 (formerly known as ABCR [35], Fig. 5). Simple Modular Architecture Research Tool (SMART; http://www.smart.embl-heidelberg.de) analysis predicts a secondary structure consistent with that known for other ABC transporters, for example P-glycoprotein, encoded by *MDR1* [36], and the bacterial lipid flippase MsbA [37]. ABCA1 is predicted to consist of two tandem repeats, each comprising a transmembrane domain with six membrane-spanning segments and an intracellular nucleotide binding domain. This topology is supported by observations that FLAG or HA peptides inserted in the extracellular loops of ABCA1 do not disrupt its function and are accessible at the cell surface [38,39].

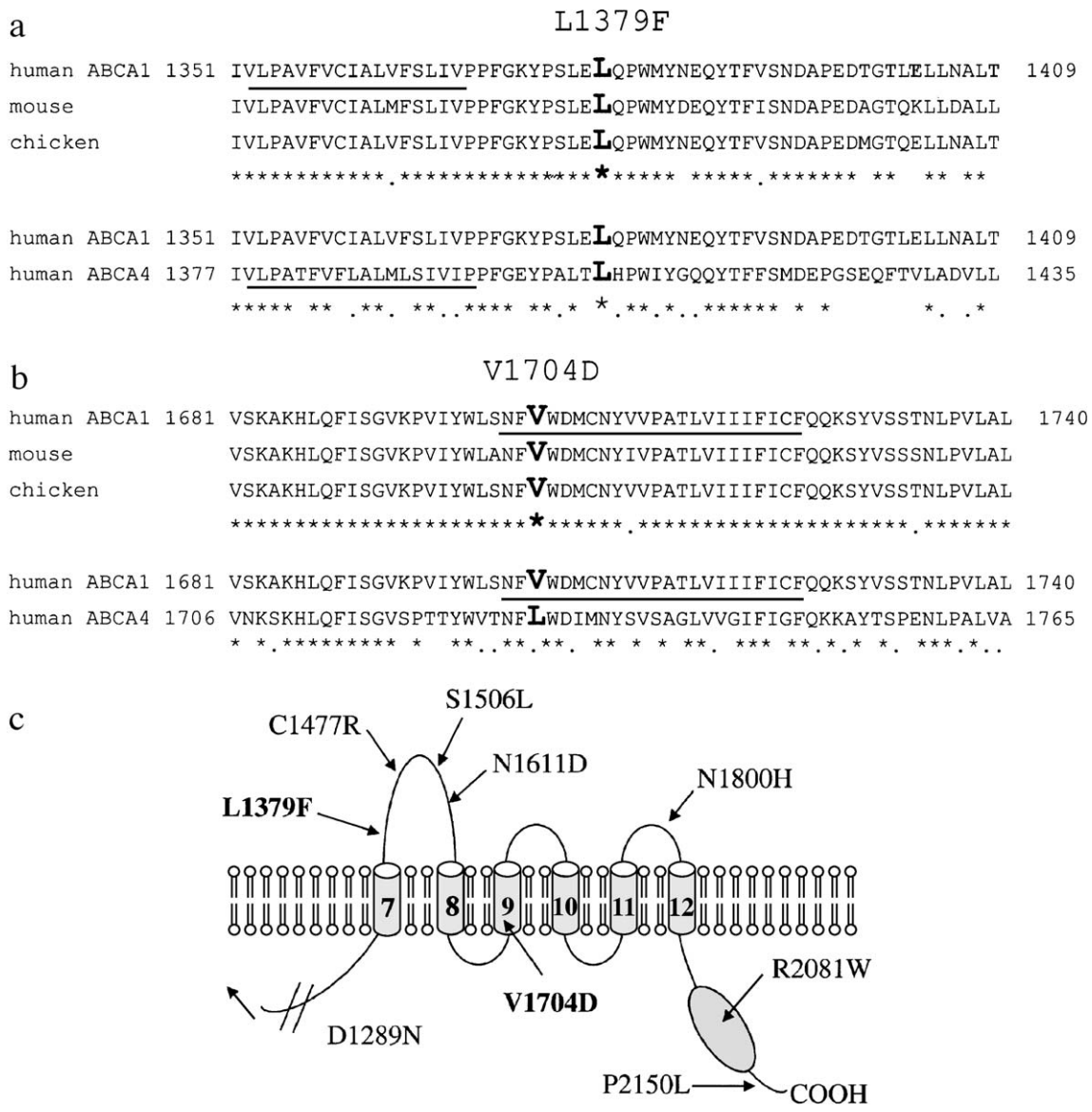

Fig. 5. Diagram showing alignment of human, murine and chicken ABCA1, and human ABCA1 and ABCA4. (a) Above, alignment of residues 1351–1409 of human ABCA1 (NM_005502) with residues 1291–1349 of mouse (GenBank accession: NM_013454) and chicken ABCA1 (AF363377). Below, alignment of the same residues of ABCA1 with residues 1377–1435 of human ABCA4 (NM_000350). The position of the L1379F substitution in ABCA1 is indicated in bold; the residues predicted by Simple Modular Architecture Research Tool (SMART; http://www.smart.embl-heidelberg.de) analysis of the amino acid sequence of ABCA1 to form membrane-spanning segment 7 in ABCA1 and ABCA4 are underlined. (b) Above, alignment of human, mouse and chicken ABCA1 and below, human ABCA1 with human ABCA4, showing the position of the V1704D mutation in ABCA1 (indicated in bold). Residues predicted by SMART to form membrane-spanning segment 9 in the carboxy-terminal half of ABCA1 and ABCA4 are underlined. (c) Diagram showing the predicted topology of the carboxy-terminal half of ABCA1; membrane-spanning segments and the nucleotide binding domain (shaded oval) were predicted by SMART. The two novel amino acid substitutions in the patient are indicated in bold; other single amino acid substitutions in the region in Tangier/HDL deficient individuals are also shown (reviewed by Singaraja et al. [31]).

Despite these insights into ABCA1 structure, the molecular mechanism for ABCA1-mediated cholesterol efflux is still poorly understood [40]. Various models are proposed, but each remains controversial, making it difficult to interpret the structure–function relationship of the two new mutations we describe. In our transfection studies both mutants found in the patient clearly disrupted the trafficking of the ABCA1 protein to the plasma membrane as visualised by confocal microscopy. However, V1704 is predicted to lie within membrane-spanning segment 9 and thus it is not surprising that replacement of the hydrophobic valine residue with negatively charged aspartate in this position disrupts trafficking of the protein. Interestingly, only one mutation in the first set of transmembrane segments of ABCA1 (residues 636–908) has been observed previously and none in the second set where V17104 is located. The known mutant protein has a deletion of L693 from transmembrane segment 3 (equivalent to transmembrane seg-

ment 9 in the carboxy-half of ABCA1), and also fails to be transported from the ER [39].

The other amino acid substitution, L1379F, is predicted to lie in the extracellular loop between transmembrane segments 7 and 8 that may constitute the binding site for apoAI [41]. This mutation predicts a relatively conservative substitution of one bulky hydrophobic residue for another and it is less clear why it should disrupt trafficking of ABCA1, but other amino acid substitutions in HDL deficient patients have been found to lie in the same predicted extracellular loop. Two of these, C1477R and S1506L, do not appear to disrupt transport of the protein to the cell surface, as judged by the accessibility of the protein in non-permeabilised cells, but the mutant proteins are unable to mediate cholesterol efflux [38]. However, the subcellular localisation of these proteins was not visualised by microscopy.

Skin fibroblasts from our proband contained approximately three times more ABCA1 mRNA than normal cells, showing that the defect in ABCA1-mediated cholesterol efflux resulted in up-regulation of the *ABCA1* gene. There was surprisingly little, less than 10%, variation in ABCA1 mRNA expression between different control cells. Preincubation with cholesterol increased ABCA1 mRNA levels in all cells, but more so in the patient's cells. Despite this difference in mRNA content, the amount of ABCA1 protein detected by Western blotting of membranes from the proband's or normal cells was similar. However, in localisation studies we were able to demonstrate that, although highly expressed, both mutant proteins display markedly disrupted trafficking to the plasma membrane suggesting severely impaired function. Failure of the mutant ABCA1 proteins to reach the surface and bind apoAI may also make them more susceptible to proteolytic degradation [40,42].

Both the proband's parents were alive and well at 80 years of age; clearly, the inheritance of both mutant alleles of ABCA1 was necessary for the almost total HDL deficiency and the unusually severe and progressive premature coronary disease seen in this female patient of 42 years. However, despite the almost complete absence from plasma of HDL, there was no evidence of the cholesterol accumulation in the liver, spleen or tonsils that is characteristic of Tangier disease. Although Tangier patients without all these clinical signs have been observed previously [5,43], there is currently no explanation for this phenotypic variation. The relationship between particular ABCA1 mutations, the reduction in HDL-cholesterol levels and defective lipid efflux has been discussed in a recent review [31], but why some Tangier disease patients have obvious cholesterol deposition while others do not was not raised. One possibility is suggested by emerging evidence that ABCA1 also mediates intracellular trafficking of substrate lipids, as well as of apoAI acceptor [44]. It is tempting to speculate that the ABCA1 proteins in our patient and that described by Bertolini et al. [43] retain some residual intracellular function that somehow reduces the cholesterol accumulation seen in macrophages in liver, spleen or tonsils from Tangier patients with null alleles.

Acknowledgements

We are grateful to Mandy Bennett (Imperial College) for assistance with WAVE analysis, Carol Wooding (MRC Clinical Sciences Centre) for membrane preparations and Bruce Pottinger (MRC Clinical Sciences Centre) for tissue culture. We would also like to thank the MRC Clinical Sciences Centre Imaging Facility. This work was supported by the Swiss National Science Foundation (C.A., 823A-056551), the Wellcome Trust (S.S., PG 0544130), and the British Heart Foundation (E.E., PG 98062). We would also like to thank the families for their cooperation.

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