Abnormal splicing of ABCA1 pre-mRNA in Tangier disease due to a IVS2 +5G>C mutation in ABCA1 gene

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Abstract Two point mutations of ABCA1 gene were found in a patient with Tangier disease (TD): *i*) G>C in intron 2 (IVS2 +5G>C) and *ii*) c.844 C>T in exon 9 (R282X). The IVS2 +5G>C mutation was also found in the brother of another deceased TD patient, but not in 78 controls and 33 subjects with low HDL. The IVS2 +5G>C mutation disrupts ABCA1 pre-mRNA splicing in fibroblasts, leading to three abnormal mRNAs: devoid of exon 2 (Ex2⁻/mRNA), exon 4 (Ex4⁻/mRNA), or both these exons (Ex2⁻/Ex4⁻/mRNA), each containing a translation initiation site. These mRNAs are expected either not to be translated or generate short peptides. To investigate the in vitro effect of IVS2 +5G>C mutation, we constructed two ABCA1 minigenes encompassing Ex1–Ex3 region, one with wild-type (WTgene) and the other with mutant (MTgene) intron 2. These minigenes were transfected into COS1 and NIH3T3, two cell lines with a different ABCA1 gene expression. In COS1 cells, WTgene pre-mRNA was spliced correctly, while the splicing of MTgene pre-mRNA resulted in Ex2⁻/mRNA. In NIH3T3, no splicing of MTgene pre-mRNA was observed, whereas WTgene pre-mRNA was spliced correctly. These results stress the complexity of ABCA1 pre-mRNA splicing in the presence of splice site mutations.—Altilia, S., L. Pisciotta, R. Garuti, P. Tarugi, A. Cantafora, L. Calabresi, J. Tagliabue, S. Maccari, F. Bernini, I. Zanotti, C. Vergani, S. Bertolini, and S. Calandra. **Abnormal splicing of ABCA1 pre-mRNA in Tangier disease due to a IVS2 +5G>C mutation in ABCA1 gene.** *J. Lipid Res.* 2003. **44:** 254–264.

Supplementary key words ABCA1 minigenes • in vitro splicing • donor splice site mutation

Tangier disease (OMIM 600046) is a rare recessive disorder characterized by a severe deficiency or absence of HDL in plasma and an accumulation of cholesteryl esters in macrophages and other reticuloendothelial cells in many tissues (1). Cholesteryl esters accumulation accounts for some of the clinical features of Tangier disease (TD), including orange-yellow tonsils, hepatosplenomegaly, peripheral nerve neuropathy, and corneal opacifications (1, 2). The biochemical features of TD reflect a defect in the efflux of cholesterol and phospholipids from the cells due to a defective function of a membrane transporter designated ABCA1 (2, 3). This transporter plays a key role in apolipoprotein A-I (apoA-I)-mediated cholesterol efflux from the cells, which represents one of the first steps in the reverse cholesterol transport (3). Mutations in the ABCA1 gene leading to the loss of function or deficiency of ABCA1 protein are the underlying defects in TD and in the codominant disorder, familial HDL deficiency (FHD) (1–3). Since the cloning of ABCA1 cDNA (GenBank no. AF285167), more than 40 mutations of this gene have been reported in patients with TD and FHD (4–17).

ABCA1 gene (GenBank no. AF275948) is located on chromosome 9 (9q31) and comprises 50 exons (18). The open reading frame of the ABCA1 transcript is of 6,783 bp (18, 19), as opposed to the 6,603 described in an earlier report (20). This transcript contains an in-frame ATG codon in exon 2, predicted to be the correct translation start site. This ATG codon is in a strong context, consistent with Kozak's rules for initiation of translation (21), in contrast with the ATG in exon 4 (a potential translation initiation site), which is in a weaker context with regard to these rules. The translation product of the full-length ABCA1 mRNA is a protein of 2,261 amino acids (18, 19), 60 amino acids more than originally reported (1). This 60-

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amino-acid extension contains a potentially cleavable signal sequence, with cleavage predicted to occur between amino acids 45 and 46 (18). The functional role of this extension has been recently elucidated in vitro using mutant ABCA1 cDNAs deleted at the 5′ end (22). These studies showed that: *a)* the 60-amino-acid extension is required for the stable protein expression of transporter constructs containing any downstream transmembrane domains, and *b)* the putative signal sequence contained in this extension is not cleaved from the protein. It apparently serves as an anchor sequence that positions the N-terminus of ABCA1 in a type II orientation, leading to the extracellular presentation of a 600-amino-acid loop (22). These in vitro observations suggest that mutations in ABCA1 gene, which eliminate (or disrupt) the 60 amino acid extension, are likely to prevent the synthesis of ABCA1 protein, leading to a complete deficiency of this transporter in the cells.

There are some indications that under normal physiological conditions, ABCA1 pre-mRNA is subject to alternative splicing. Costet et al. (23) reported three alternative transcripts of ABCA1 gene in HepG2 cells involving the exon 1–exon 5 region. We demonstrated the presence of an alternatively spliced ABCA1 mRNA lacking exon 4 in several human cell types, including normal skin fibroblasts (24). A recent study conducted in human ABCA1 transgenic mice showed the presence of a transcript containing an alternative exon 1, followed by correctly spliced exons 2–4 (25). This newly discovered exon, named exon 1A, is located 2,210 bp upstream from exon 2.

In the present study, we report the effects of a point mutation in intron 2 of ABCA1 gene found in a patient with Tangier disease. This mutation disrupts the normal splicing, causing the formation of several abnormal ABCA1 mRNAs in cultured skin fibroblasts. One of these mRNAs does not contain exon 2; therefore, it is predicted to encode an ABCA1 protein devoid of the 60-amino-acid extension involved in anchoring the transporter to the plasma membrane. Another of these abnormal mRNAs is devoid of both exon 2 and exon 4, which contain the two translation initiation sites.

MATERIALS AND METHODS

Subjects

Family 1. The proband was a 66-year-old female (I.1 in **Fig. 1**) with severe HDL deficiency (**Table 1**). She had had tonsillectomy at the age of 8. Since the age of 20, she had secondary amenorrhea caused by primary hypogonadism with ovary and uterine hypoplasia. She was a heavy smoker (40 cigarettes/day). At the age of 57, she was found to have a thoracoabdominal aortic aneurysm with aortic valve insufficiency. At 62, the diameter of the descending thoracic aorta was 6.5 cm. The patient was not surgically treated because of the exceptionally high operative risk. At 67, the thoracoabdominal aortic diameter was 9.0 cm and the patient died from aortic dissection and rupture. The proband's apparently healthy brother (I.2) had hypoalphalipoproteinemia. The proband's sister (I.3) had normal lipid values (Table 1).

Family 2. The subject (I.1 in Fig. 1) was investigated at the age of 62. The presence of orange-yellow tonsils, splenomegaly, pe-
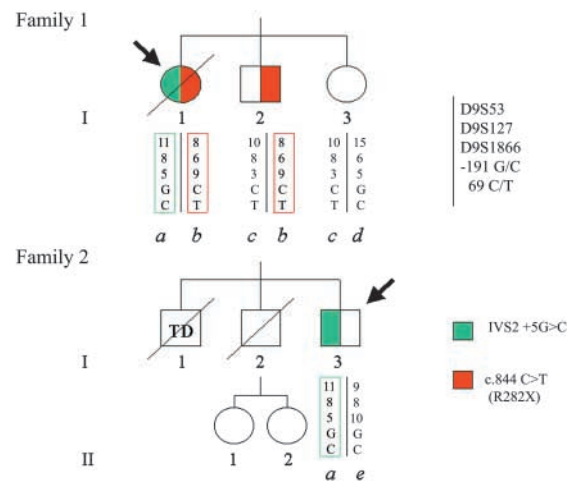

Fig. 1. Pedigrees of the two probands investigated in this study. Subject I.1 of Family 1 and subject I.1 of Family 2 were affected by Tangier disease. The probands investigated are indicated by an arrow. The haplotypes (*a–e*), constructed with five genetic markers located close to or within the ABCA1 gene, are indicated below the genotyped family members. The alleles corresponding to microsatellites D9S53, D9S127, and D9S1866 are indicated by the repeat numbers of each allele. The alleles corresponding to SNPs at position −191 and 69 of the ABCA1 gene (19) are indicated by the nucleotide found in each allele. The IVS2 +5G>C mutation and the cosegregating haplotype are indicated by green color. The R282X mutation and the cosegregating haplotype are indicated by red color (see sequence analysis in Fig. 3).

ripheral nerve neuropathy, corneal infiltrations, and ischemic heart disease associated with analphalipoproteinemia (Table 1) suggested the diagnosis of Tangier disease. He died from myocardial infarction at the age of 64. Full details of his clinical manifestations were reported previously (26). The proband of the present study (subject I.3 in Fig. 1) was the 94-year-old brother of the Tangier patient (Table 1). He had suffered from myocardial infarction at the age of 92. He had low HDL cholesterol (HDL-C) levels but no clinical manifestations of Tangier disease. Two other members of family 2 (subjects II.1 and II.2 in Fig. 1) we investigated were healthy and had normal HDL-C concentrations (Table 1).

Family 1 and Family 2 were apparently unrelated. However, they have been living in the same geographical area in northern Italy.

Biochemical analyses

Plasma cholesterol and triglycerides were measured enzymatically and apoA-I, apoA-II, and apoB by immunoturbidimetry (Roche Diagnostics GmbH, Mannheim, Germany) using an automated analyzer (Hitachi model 912, Hitachi, Ltd., Tokyo). HDL-C was measured in plasma supernatant after precipitation of apoB-containing lipoproteins by phosphotungstate-MgCl$_2$. Plasma lipoproteins were separated by density gradient ultracentrifugation (27). Two-dimensional gel electrophoresis of apoA-I was performed as reported previously (28).

Fibroblast culture and blood lymphocyte separation

Skin fibroblasts from proband I.1 (Family 1) and healthy control subjects were grown and maintained in 75 cm^2 flasks in Dulbecco's Modification of Eagle's Medium (DMEM) (Gibco-Invitrogen, Paisley, UK), 100 IU/ml of penicillin, and 50 μg/ml of streptomycin, 2 mM L-glutamine, 10% FBS (Gibco), and 95% air/5% CO$_2$. Total RNA was isolated by extraction with Eurozol (Euroclone Ltd, Paignton, Devon, UK).

TABLE 1. Clinical characteristics of the subjects investigated

	Family 1			Family 2			
Subject (sex)	I.1 (F)	I.2 (M)	I.3 (F)	I.1 (M)	I.3 (M)	II.1 (F)	II.2 (F)
Age (years)	66	65	57	62	94	57	54
Cholesterol (mmol/l)	2.82	2.79	5.94	2.0	2.79	5.92	5.22
HDL-cholesterol (mmol/l)	0.08	0.52	1.47	0.05	0.44	1.06	1.11
Triglycerides (mmol/l)	2.18	1.48	2.00	1.6	1.81	1.24	0.87
ApoA-I (mg/dl)	<10	62	151	<10	64	142	150
ApoA-II (mg/dl)	5	19	38	—	25	44	47
ApoB (mg/dl)	93	63	103	—	66	104	84

Peripheral blood mononuclear cells were isolated from proband I.3 (Family 2) (29). RNA was extracted using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Cell cholesterol efflux in fibroblasts

The assay of the ABC1-mediated cholesterol efflux was performed as previously described (30). Control and Tangier fibroblasts (proband I.1 in Family 1) between the 9th and the 11th split were grown in DMEM medium with 10% FBS. Cells were incubated at 37°C in 5% CO_2, seeded in 24-well plate, and then grown to 80–90% confluence before use. Cell monolayers used for cholesterol efflux studies were washed with phosphate-buffer saline (PBS) and then incubated for 24 h in medium containing [1,2-^{3}H]cholesterol (2 μCi/ml) (31) and 1% FBS. Following 24 h labeling period, cells were washed and incubated overnight in media containing 0.2% BSA in the presence or absence of 9-*cis*-retinoic acid (10 μM) and 22-hydroxycholesterol (10 μM) (32). [1,2-^{3}H]cholesterol labeled monolayers were then washed with PBS and incubated for 6 h (efflux time) in the presence and absence of human apoA-1 (25 μg/ml). Cholesterol efflux was quantified by measuring the radioactivity of the incubation medium after the removal of floating cells by centrifugation. Cell lipids were extracted with isopropanol, and lipid radioactivity was measured in an aliquot of the extract. The radioactivity released into the medium was expressed as the fraction of the total radioactive cholesterol present in each well. The percent of radiolabeled cholesterol released (% efflux) was calculated as: (cpm in medium at 6 h/cpm in the monolayer) × 100. All the efflux assays were performed in triplicate. The results are reported as mean ± SD. Statistical significance was evaluated by two-tailed Student's *t*-test.

Genotyping of polymorphic markers of chromosome 9q31 region

Genomic DNA was extracted from peripheral blood leukocytes by a standard procedure (33). The genotyping of individuals belonging to the families included in the study was carried out using the following CA dinucleotide variable repeats (present on chromosome 9q31, near or inside the ABCA1 gene region): D9S53, D9S1866, and D9S127. The D9S53 is a highly polymorphic marker (11 alleles, heterozygosity 0.838) located in intron 36 of ABCA1 gene (8). Primers and conditions for the amplification of the DNA segment that includes this marker were as reported for Mfd135 amplimer in GDB database (http://www.gdb.org accession #180704). The D9S1866 is a polymorphic marker (8 alleles, heterozygosity 0.630) that is less than 1 cM telomeric the ABCA1 gene and co-segregates with this gene (5). Primers and conditions for the amplification of the DNA segment including this marker were as reported for AFM064xe5 amplimer in GDB database (http://www.gdb.org accession #612606). The D9S127 is a polymorphic marker (6 alleles, heterozygosity

0.720) that is about 1.6 cM centromeric the ABCA1 gene. Primers and conditions for the amplification of the DNA segment including this marker were as reported for 9CMP2 amplimer in GDB database (http://www.gdb.org accession #181966).

The PCR amplifications of these microsatellites were performed using forward primers end-labeled with Cy5.5 fluorescent dye (MWG-Biotech, Ebersberg, Germany). The sizing was evaluated under standard conditions with denaturing capillary electrophoresis and laser-induced fluorescence (LIF) detection as previously described (34) using accurately sized homozygote samples as internal standards. The alleles of each microsatellite were reported as variable repeat numbers that were calculated from the range of sizes of 100 analyzed alleles.

Genotyping of biallelic polymorphisms

The members of families 1 and 2 were also genotyped for two ABCA1 single-nucleotide polymorphisms (SNPs), −191 C/G (*Hga*I), and 69 C/T (*Bsm*AI), which are located in the 5′ untranslated region and in exon 1, respectively. These SNPs were numbered considering +1 the first nucleotide of exon 1 (19). The methods used for genotyping were previously described (19). The heterozygosity of these SNPs, in our population, turned out to be 0.475 (−191 C/G) and 0.478 (69 C/T), respectively.

Sequence analysis of candidate genes

The apoA-I and LCAT genes were sequenced using the primers previously described (35, 36). The promoter and the 50 exons, as well as the splice junctions, of the ABCA1 gene were amplified using primers described by Pullinger et al. (19) and Wang et al. (11). For the promoter and exon 1, the conditions were 94°C for 3 min, 33 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 7 min. For the other exons, the conditions were: 94°C for 3 min; 30 cycles at 94°C for 15 s, 52°C for 30 s (exons 21, 26, 30, 33, 39) or 58°C for 30 s (other exons), 72°C for 1 min, and a final extension at 72°C for 7 min. The amplification products were purified by QIAquick kit (Qiagen), quantified on 1% agarose gel and sequenced by automatic sequencer CEQ2000 DNA Analysis System (Beckman Coulter, Fullerton, CA).

Screening of ABCA1 gene mutations

The IVS2 +5G>C transversion (see Results) introduces a new *Sty*I restriction site in the ABCA1 gene. To screen for the presence of this mutation, genomic DNA was amplified using the following primers: 5′-GCTGGATTAGCAGTCCTCATTG-3′ (forward primer in exon 2) and 5′-CCCCAACTCAAAACCACAAAG-3′ (reverse primer in intron 2). DNA was amplified in a 50 μl volume containing 0.2 mM of each dNTP, 20 pmol of each primer, 1 U Taq DNA polymerase in 1× PCR buffer, and 2 mM $MgCl_2$. The conditions were 94°C for 3 min, followed by 30 cycles of 94°C for

15 s, 58°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 7 min. An aliquot of the amplification product was incubated with 8 units of *Sty*I (Amersham Biosciences, Europe GmbH, Milan, Italy) for 4 h at 37°C. The digestion products were separated on 2% agarose gel.

The c.844 C>T transition at codon 282 (see Results) introduces a new *Afl*III restriction site in exon 9. To screen for this mutation, a 515 bp region encompassing exon 9 was amplified using the primers reported previously (11) and the amplification conditions adopted for IVS2 +5G>C mutation. The amplification product was incubated with 6 units of *Afl*III (Amersham Biosciences) for 4 h at 37°C. The digestion products were separated on 2% agarose gel.

Reverse transcription and sequencing of ABCA1 cDNA

Five micrograms of total RNA extracted from fibroblasts or lymphomonocytes was reverse transcribed in a 20 µl reaction mixture using SuperScript™ II RT Kit (Gibco-Invitrogen) according to manufacturer's instructions. PCR amplifications were performed immediately after the RT reaction in a mixture containing 5 µl of cDNA, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9, and 1.5 mM MgCl$_2$), 0.5 mM of each dNTP, 50 pmol of each primer, and 1.5 units of Taq Expand High Fidelity DNA polymerase (Roche GmbH, Mannheim, Germany). To amplify the exon 1–exon 3 region, the following primers were used: 5′-GGAGAAAGAGACGCAAACACA-3′ (forward primer in exon 1) and 5′-GTTGTTCATAGGGTGGGTAGCTC-3′ (reverse primer in exon 3). The amplification conditions were 29 cycles at 95°C for 40 s, 58°C for 30 s, and 68°C for 3 min. PCR products were purified using High pure PCR Purification Kit (Roche). Electrophoresis was performed on 1% agarose gel. To amplify the exon 1–exon 5 region, the following primers were used: 5′-GGAGAAA GAGACGCAAACACA-3′ (forward primer in exon 1) and 5′-TC TCAGAACTTTGCGCATGTCCT-3′ (reverse primer in exon 5). To amplify the exon 2–exon 5 region, the following primers were used: 5′-CTCAGCTGAGGTTGCTGCTGTG-3′ (forward primers in exon 2) and 5′-TCTCAGAACTTTGCGCATGTCCT-3′ (reverse primer in exon 5). The amplification reaction was performed for 29 cycles under the conditions specified above. PCR products were electrophoresed on 2–3% agarose gel. To amplify the exon 5–exon 10 region, the following primers were used: 5′-TGTTCT CAGATGCTCGGAGGCTT-3′ (forward primer in exon 5) and 5′-TACAGGATCTTCCCAACGAGCAG-3′ (reverse primer in exon 10). The amplification conditions were 10 cycles at 95°C for 3 min, 95°C for 40 s, 65°C for 40 s, and 68°C for 7 min, followed by 20 cycles at 95°C for 40 s, 65°C for 40 s, and 68°C for 7 min. PCR products were electrophoresed on 2% agarose gel.

In the case of proband I.1 (Family 1), the RT-PCR products (exon 1–exon 5 region) were cloned in the pTargeT expression vector (Promega, Madison, WI). Plasmids were sequenced directly using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 3100 genetic analyzer (Applera Europe B.V, Nieuwerkerk, The Netherlands).

Construction of the expression vector

To investigate the effects of the IVS2 +5G>C mutation on ABCA1 pre-mRNA splicing, we constructed two minigenes designed MTgene (mutant) and WTgene (wild-type) containing exon 1 followed by the abridged intron 1 (392 bp), exon 2, abridged intron 2 (265 bp), exon 3, and a truncated intron 3 (66 bp). The minigene constructs were generated from genomic DNA of proband I.1 (Family 1), by ligating three PCR fragments designated FRG1, FRG2, and FRG3, using *Eco*RI and *Bam*HI sites. FRG1 contained exon 1 followed by the 5′ end of intron 1; FRG2 contained the 3′ end of intron 1 followed by exon 2 and the 5′ end of intron 2; FRG3 contained the 3′ end of intron 2 followed

by exon 3 and the 5′ end of intron 3. FRG1 was amplified using the following primer pairs: 1F (forward primer) 5′-GAGCACAG GCTTTGACCGATAGTA-3′ and 1Rm (reverse primer) 5′-GCG AATTCAGGACATCGTCTCCCCA-3′, with the first eight nucleotides modified to introduce an *Eco*RI site. FRG2 was amplified using primer pairs: 2Fm (forward primer) 5′-CGGAATTCTGA TGCAGAGCTGTGTGTTG-3′, with the first eight nucleotides modified to introduce an *Eco*RI site, and 2Rm (reverse primer) 5′-CGGGATCCTCCATCAATCCCTGTGTGAAAAC-3′, with the first eight nucleotides modified to introduce a *Bam*HI site. FGR3 was amplified using the following primer pairs: 3Fm (forward primer) 5′-CGGGATCCGTGGTGGGAGAGACTGGAG-3′, with the first eight nucleotides modified to introduce a *Bam*HI site, and 3R (reverse primer) 5′-GCCCACGTTAATTGCCTGGATT-3′. The PCR amplifications were performed for 30 cycles under the following conditions: 95°C for 40 s, 60°C for 40 s, and 68°C for 2 min. These fragments were ligated and cloned into the pTargeT expression vector that contains the Neomycin resistance gene (Neo^R gene) under the control of the SV40 early promoter. Plasmids of the minigene constructs were prepared using Qiagen Plasmid maxi kit (Qiagen) and sequenced. The two minigenes had the same sequence with the exception of the IVS2 +5G>C transversion.

Expression of ABCA1 minigenes in transfected cells

COS1 and NIH3T3 cells were maintained in the DMEM containing 5–10% FBS and used for transfection. Cells 6–7 × 10^5 were plated in 60 mm ∅ dishes. After 24 h, 4 µg of plasmids of the wild-type and mutant minigene constructs were transfected using LipofectaMINE Plus reagent (Invitrogen, Life Technologies, Paisley, UK). Forty-eight hours after transfection, total RNA was extracted with Eurozol and treated with RNase-free DNase (Promega). RT-PCR amplification was performed using primers 1F (forward primer in exon 1) and 3RA (reverse primer in exon 3) (see above) under the conditions specified above. The RT-PCR products were sequenced as described above. To provide an internal control for transfection efficiency, the mRNA of neomycin-resistance gene (Neo^R) was reverse transcribed and PCR amplified using primers AKS-249 (5′-AAGCGG GAAGGGACTGGC-3′) and AKS-250 (5′-AGGCGATAGAAGGCG ATGC-3′) (37).

RESULTS

Proband I.1 (Family 1 in Fig. 1)

This proband had none of the classic phenotypic manifestations of TD, apart from the very low HDL-C and apoA-I levels (Table 1). To the best of our knowledge, the presence of a large aneurysm of thoracoabdominal aorta has not been reported previously in TD patients.

ABCA1-mediated cholesterol efflux was evaluated in fibroblasts incubated in the presence of 9-*cis*-retinoic acid and 22-hydroxycholesterol using apoA-I as cholesterol acceptor. This treatment induced a 5-fold increase of cholesterol efflux in control fibroblasts but had no effect in proband's fibroblasts. The cholesterol efflux in proband's fibroblasts was similar to that observed in another patient with TD (homozygous for R587W in ABCA1 gene) we have previously reported (13) (**Fig. 2**).

The density profile of plasma lipoproteins separated by density gradient ultracentrifugation was characterized by the almost complete absence of HDL peak (data not

shown). Two-dimensional gel electrophoresis of plasma HDL revealed the presence of trace amounts of mature apoA-I with a relative increase of proapoA-I (data not shown). HDL peak was found to be reduced in proband's brother, but to be normal in proband's sister (data not shown).

Proband I.3 (Family 2 in Fig. 1)

The proband of Family 2 was the brother of a deceased patient with all the classic manifestations of TD, whose clinical and biochemical phenotype had been reported previously by our group (26). Despite a low HDL-C level (Table 1), proband I.3 had been free of major health problems until the age of 92.

Analysis of candidate genes

The sequence of apoA-I and LCAT genes, performed before the discovering of the ABCA1 gene, did not reveal sequence variations in either of the two probands. The sequence of ABCA1 gene revealed that proband I.1 (Family 1) was a compound heterozygote for: *i*) a G>C transversion at the 5th nucleotide of intron 2 (IVS2 +5G>C), and *ii*) a c.844 C>T transition in exon 9, which converts the arginine codon at position 282 into a termination codon (R282X) (**Fig. 3**). The IVS2 +5G>C transversion introduces a new *Sty*I restriction site. The *Sty*I digestion of a PCR product encompassing exon 2/intron 2 boundary (**Fig. 4**, upper panel) generates a single fragment (301 bp) in controls (lane 2) and two fragments (242 and 59 bp) in the presence of the mutation (lanes 3 and 4). The c.844 C>T mutation (R282X) introduces a new *Afl*III restriction site. The *Afl*III digestion of PCR-amplified exon 9 (Fig. 4, lower panel) results in a single fragment of 515 bp in controls (lane 4), and in two fragments of 333 bp and 182 bp in the presence of the mutation (lanes 2 and 3). The proband's brother was heterozygous for the nonsense

mutation in exon 9 (R282X). Neither of the mutations of the ABCA1 gene were found in the proband's sister.

The proband I.3 (Family 2) was found to be heterozygous for the mutation in intron 2 (IVS2 +5G>C) found in proband I.1 (Family 1); no other mutations of ABCA1 gene were detected in this subject. The two other members of this family (II.1 and II.2) had normal ABCA1 gene sequence.

Haplotype analysis

The genotyping of five polymorphic markers close to or within the ABCA1 gene demonstrated: *i*) the IVS2 +5G>C mutation co-segregates with haplotype *a*; *ii*) the probands of Family 1 and Family 2 share this haplotype; *iii*) the nonsense mutation (R282X) cosegregates with haplotype *b* (Fig. 1).

Mutation screening

To ascertain whether the IVS2 +5G>C transversion was a rare mutation or a common polymorphism, we applied the *Sty*I restriction analysis (see above) to 78 randomly selected control subjects and to 33 individuals with primary hypoalphalipoproteinemia (HDL-C <0.85 mmol/l). None of them were found to be carriers of this mutation.
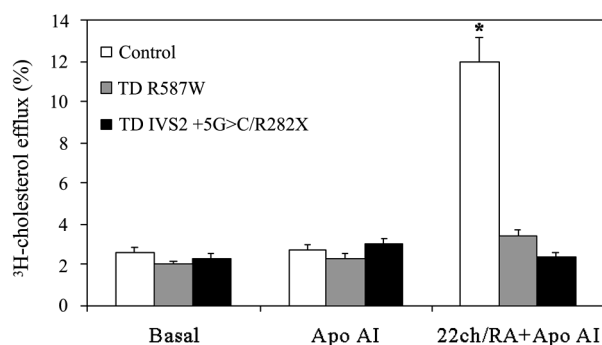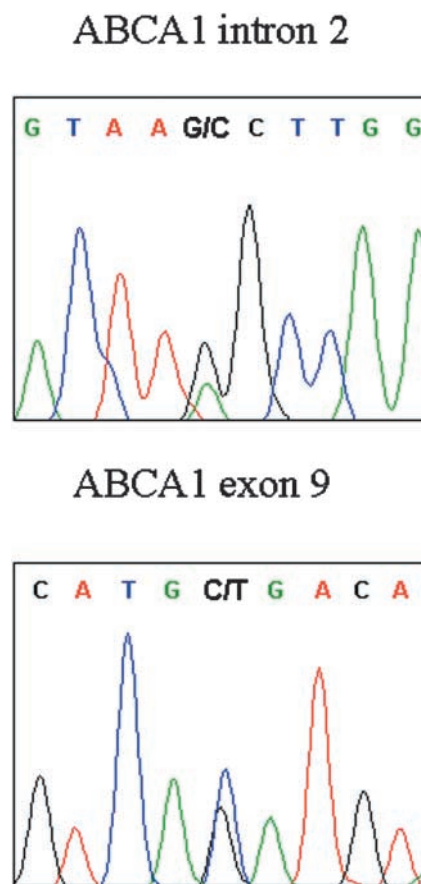

Fig. 3. Mutations of the ABCA1 gene found in proband I.1 (Family 1). This proband was found to be a compound heterozygous for a G>C transversion in intron 2 (IVS2 +5G>C) (upper panel) and for a C>T transition in exon 9 (lower panel), which converts the arginine codon (CGA) at position 282 into a termination codon (TGA).
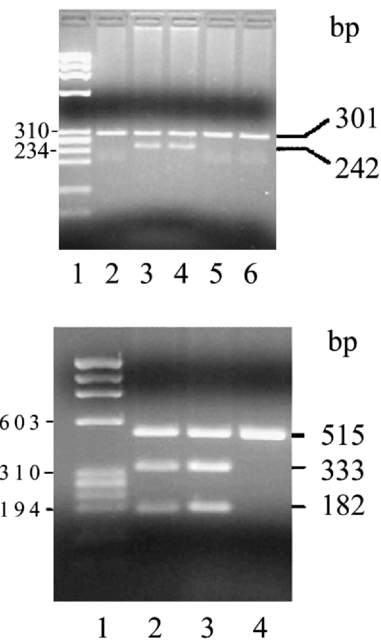

Fig. 2. ABCA1-mediated cholesterol efflux from human fibroblasts incubated with human apolipoprotein A-I (apoA-I). Skin fibroblasts from a control subject, proband I.1 of Family 1 (IVS2 +5G>C/R282X), and another TD patient (homozygous for R587W) reported previously (13) were labeled with [³H]cholesterol and then incubated in the presence or in the absence of 22-hydroxycholesterol and 9-*cis*-retinoic acid (22ch/RA). Cholesterol efflux was measured by incubating the cells with 25 μg/ml of human apoA-I for 6 h. Cholesterol efflux is expressed as percent (±SD) of total cell radioactivity present in the incubation media. * $P < 0.001$ versus efflux in cells incubated in the absence of 22ch/RA.

Fig. 4. Screening of the two mutations of the ABCA1 gene shown in Fig. 3. Upper panel: PCR amplification of exon 2-intron 2 boundary (301 bp) followed by digestion with the restriction enzyme Sty I. Lane 1: mw markers; lane 2: control subject; lanes 3 and 4: proband I.1 (Family 1) and proband I.3 (Family 2); lanes 5 and 6: siblings of proband I.1 (Family 1). Lower panel: PCR amplification of exon 9 (515 bp) followed by digestion with the restriction enzyme *Afl*III. Lane 1: mw markers; lane 2: proband I.1 (Family 1); lane 3: proband's brother; lane 4: proband's sister.
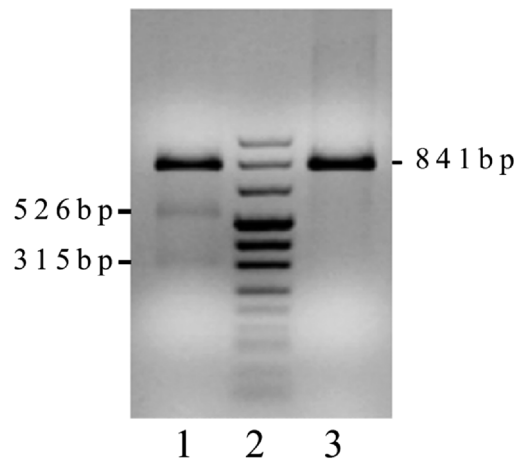
Fig. 5. Analysis of ABCA1 cDNA in fibroblasts of proband I.1 (Family 1). ABCA1 cDNA region spanning from exon 5 to exon 10 was amplified by PCR and digested with *Afl*III to detect the c.844 C>T mutation (R282X) in exon 9. Lane 1: proband I.1; lane 2: mw markers; lane 3: control subject.

Analysis of cDNA

Unexpectedly, the sequence of the cDNA region encompassing exons 5–10 failed to confirm the presence of the c.844 C>T (R282X) mutation in exon 9 in proband I.1 (Family 1), suggesting that only one mutant allele was expressed in fibroblasts. In order to ascertain whether minute amounts of the transcript harboring the c.844 C>T mutation were present in proband's fibroblasts, we performed a restriction analysis of the cDNA fragment spanning from exon 5 to exon 10. This cDNA fragment was digested with the restriction enzyme *Afl*III, whose site in exon 9 is introduced by the c.844 C>T mutation (as indicated in the lower panel of **Fig. 4**). **Figure 5** shows that *Afl*III digestion generated a single 841 bp fragment in control (lane 3) and three fragments in the proband, a major fragment of 841 bp, and trace amounts of two fragments of 526 and 315 bp respectively (lane 1). The latter arise from the transcript of the mutant allele harboring the c.844 C>T mutation in exon 9. These findings indicate that ABCA1 mRNA species present in proband's fibroblasts mostly derived from the mutant allele harboring the splice site mutation in intron 2 (IV2+5G>C).

To define whether the IVS2 +5G>C transversion affects the splicing of ABCA1 pre-mRNA, we performed the analysis of cDNA generated by RT-PCR of total RNA isolated from the fibroblasts of proband I.1 (Family 1) and from the lymphomonocytes of proband I.3 (Family 2). **Figure 6** (upper panel) shows the separation of the RT-

PCR amplification products of the region encompassing exons 1–3 in a series of control fibroblasts (negative for the presence of the IVS2 +5G>C mutation) and in fibroblasts, of proband I.1 (Family 1). In control fibroblasts, we observed the expected band of 281 bp (exon 1–exon 3 amplification) and trace amounts of a minor band of 123 bp, clearly detectable only in some cell lines (Fig. 6, lanes 4–10). In proband's fibroblasts, the intensity of the 281 bp band was markedly reduced, whereas that of the 123 bp was increased (lanes 2 and 3). The nucleotide sequence showed that in the 281 bp band, exon 1 was followed by exons 2 and 3 (correctly spliced mRNA), whereas in the 123 bp band, exon 1 was followed by exon 3 with the complete skipping of exon 2 (Ex2⁻/mRNA) (**Table 2**). The same pattern was observed in lympho-monocytes of proband I.3 (Family 2) (data not shown). Since we had previously shown that in normal human fibroblasts ABCA1 premRNA is subject to an alternative splicing with the formation of a minor RNA species devoid of exon 4 (24), we amplified the cDNA region spanning from exon 2 to exon 5 to specifically evaluate this process in proband's cells. Figure 6 (lower panel) shows that, as expected, control cells (lanes 3–9) contained, besides the RT-PCR product of 377 bp (corresponding to the correctly spliced mRNA), minute amounts of a minor band (235 bp) corresponding to the mRNA devoid of exon 4 (Ex4⁻/mRNA) (Table 2) (24). The proportion of this minor band with respect to the 377 bp band was higher in proband's fibroblasts (lane 2). **Figure 7** shows the results of the amplification of the exon 1-exon 5 region of ABCA1 cDNA. In this experiment we used two types of control fibroblasts: *i*) cells with no expression of Ex2⁻/mRNA (see Fig. 6, upper panel); *ii*) cells containing trace amounts of both Ex2⁻/ and Ex4⁻/mRNA (see Fig. 6). In control fibroblasts with no expression of Ex2⁻/mRNA, we detected a major band of 517 bp and trace amounts of a minor band of 375 bp (Fig. 7, lane 3). In control cells containing Ex2⁻/ and Ex4⁻/mRNA besides
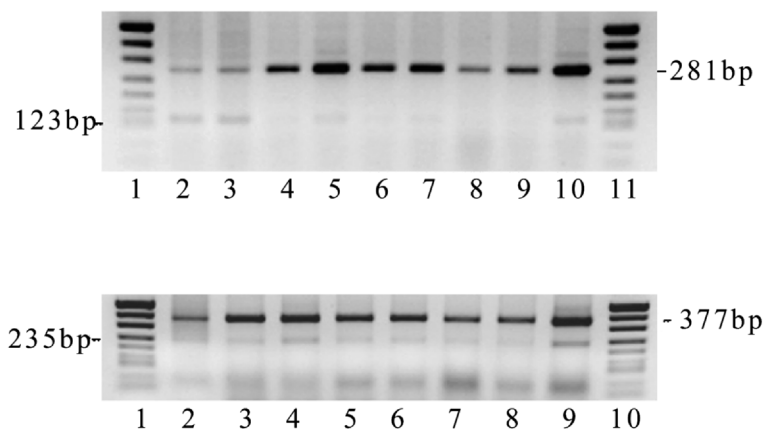
Fig. 6. Analysis of ABCA1 cDNA in fibroblasts of proband I.1 (Family 1). Upper panel: products of RT-PCR amplification of exon 1–exon 3 region (281 bp). Lanes 1 and 11: mw markers; lanes 2 and 3: fibroblasts of proband I.1 of Family 1 (10 μg and 20 μg of cDNA respectively); lanes 4–10: fibroblasts of control subjects (7–10 μg of cDNA). The 123 bp band corresponds to the ABCA1 mRNA devoid of exon 2 (Ex2⁻/mRNA) (see Table 2). Lower panel: products of RT-PCR amplification of exon 2-exon 5 region (377 bp). Lanes 1 and 10: mw markers; lane 2: proband 1.1 (family 1); lanes 3–9: fibroblasts of control subjects. The 235 bp band corresponds to the ABCA1 mRNA devoid of exon 4 (Ex4⁻/mRNA) (see Table 2).

the 517 bp band, we observed a doublet (375 bp and 359 bp bands) (Fig. 7, lane 4). In the fibroblasts of proband I.1 (Family 1), four bands of 517 bp, 375/359 bp (doublet), and 217 bp were present (Fig. 7, lane 2). The same pattern was observed in lympho-monocytes of proband I.3 (Family 2) (Fig. 7, lane 5). The nucleotide sequence (Table 2) indicated that the 517 bp corresponded to the normally spliced mRNA, the 375 bp to Ex4⁻/mRNA, the 359 bp to Ex2⁻/mRNA, and the 217 bp to an mRNA devoid of both exon 2 and exon 4 (Ex2⁻/Ex4⁻/mRNA).

Splicing of ABCA1 mRNA in transiently transfected cells

To ascertain whether the mutation in intron 2 was the cause of abnormal ABCA1 premRNA splicing observed in fibroblasts, we transfected two ABCA1 minigenes (see methods), cloned in an expression vector, into COS1 cells that have a measurable expression of ABCA1. The minigenes contained the wild-type intron 2 (WTgene) or the intron 2 harboring the IVS2 +5G>C mutation (MTgene). **Figure 8** shows the results of RT-PCR of ABCA1 mRNA isolated from transfected COS1 cells. The splicing of the premRNA of the WTgene (lane 4) generates a major fragment of 281 bp that contains the correctly spliced mRNA (exons 1–3) (see Table 2). This fragment superimposes on that of endogenous ABCA1 mRNA present in COS1 cells (lane 7). The splicing of the pre-mRNA of MTgene

(lane 5) resulted in two fragments, one (281 bp) corresponding to the wild-type mRNA present in COS1 cells and in control fibroblasts (lanes 6 and 7) and the other (123 bp) to an abnormal mRNA in which exon 1 joins directly to exon 3 with the complete skipping of exon 2 (Ex2⁻/mRNA) (see Table 2). It should be pointed out that a faint 123 bp band was also detectable in control fibroblasts (as documented in Fig. 6, upper panel) and in the untransfected COS1 cells (Fig. 8, lanes 6 and 7). We also performed transfection studies using NIH3T3 cells, which have a negligible expression of ABCA1 (30). **Figure 9** shows that in these cells the splicing of the WTgene generates a single band of 281 bp (lane 4) that contains exon 1 followed by exon 2 and 3 (Table 2). In sharp contrast, no bands were detected in the case of the MTgene (lane 5). Since the construct containing the MTgene was efficiently transfected and presumably transcribed (as indicated by the RT-PCR amplification of Neoᴿ mRNA) (lane 2), these results suggest that in NIH3T3, the primary transcript product of MTgene is rapidly degraded before splicing.

DISCUSSION

In this study, we report the characterization of two novel mutations of ABCA1 gene discovered in a patient with severe

TABLE 2. Nucleotide sequence of RT-PCR fragments of ABCA1 gene in carriers of the IVS2 +5G>C mutation

|	Exon Junctions		Exon Sequence
RT-PCR products of exon 1-exon 3 region			
281 bp	Normal junctions		Exons : 1-2-3
123 bp	. . . ACAG/TGTC . . .		Exons : 1-3
	Exon 1-Exon 3		(Ex2⁻/mRNA)
RT-PCR products of exon 2-exon 5 region			
377 bp	Normal junctions		Exons : 2-3-4-5
235 bp	...GAAT/TGTG . . .		Exons : 2-3-5
	Exon 3-Exon 5		(Ex4⁻/mRNA)
RT-PCR products of exon 1-exon 5 region			
517 bp	Normal junctions		Exons : 1-2-3-4-5
375 bp	. . . GAAT/TGTG . . .		Exons : 1-2-3-5
	Exon 3-Exon 5		(Ex4⁻/mRNA)
359 bp	. . . ACAG/TGTC . . .		Exons : 1-3-4-5
	Exon 1-Exon 3		(Ex2⁻/mRNA)
217 bp	. . . ACAG/TGTC . . .	. . . GAAT/TGTG . . .	Exons : 1-3-5
	Exon 1-Exon 3	Exon3-Exon 5	(Ex2⁻/Ex4⁻/mRNA)
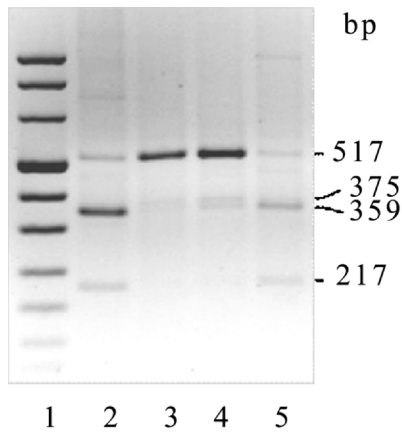
Fig. 7. Analysis of ABCA1 cDNA in fibroblasts of proband 1.1 (Family 1) and lympho-monocytes of proband 1.3 (Family 2). The figure shows the products of RT-PCR amplification of exon 1–exon 5 region (517 bp). Lane 1: mw markers; lane 2: proband 1.1 (Family 1); lane 3: control fibroblasts with no detectable Ex2$^-$/mRNA (Fig. 5, upper panel); lanes 4: control fibroblasts with trace amounts of Ex2$^-$/mRNA and Ex4$^-$/mRNA (see Fig. 5); lane 5: lympho-monocytes of proband 1.3 (Family 2). The 375 bp and 359 bp bands correspond to Ex4$^-$/mRNA and Ex2$^-$/mRNA respectively; the 217 bp band corresponds to the Ex2$^-$/Ex4$^-$/mRNA (see Table 2).

hypoalphalipoproteinemia (proband I.1, Family 1) who was suspected to have Tangier disease even though she had none of the classic phenotypic manifestations of this disease. Her major clinical problem was a thoracoabdominal aneurism, a condition not reported previously in TD patients (1, 38).
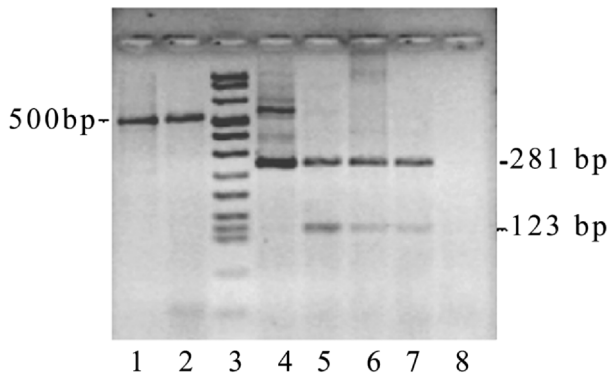
Whether the thoracoabdominal aneurism of the patient was due to Tangier disease, other cardiovascular risk factors (e.g., heavy smoking), or some intrinsic defect of the aortic wall cannot be established on the basis of the available data.

Despite this unusual clinical presentation, the apoA-I-mediated cholesterol efflux in the fibroblasts of this patient was found to be negligible and similar to that of another TD patient homozygous for a missense mutation in ABCA1 gene we previously genotyped (13).

One of the mutations found in proband I.1 (Family 1) is a nonsense mutation in exon 9 (R282X) that involves a CpG dinucleotide, a well-recognized hot spot for base substitution (39). The transcript of this mutant allele is hardly detectable in proband's fibroblasts (Fig. 5). This low expression is most likely the result of a rapid degradation of an mRNA harboring a premature stop codon (nonsense-mediated mRNA decay), a condition reported to occur in other genetic disorders (40). The translation product (if any) of this mutant allele is a truncated peptide of 281 amino acids, which is devoid of all critical domains (transmembrane and NBD domains) of ABCA1 protein. This peptide is presumably degraded shortly after its synthesis.

The other mutant allele of proband I.1 is a G>C transversion at the 5th position of intron 2 (IVS2 +5G>C). This substitution involves a nucleotide that is highly conserved in the donor splice sites of mammalian genes, being present in 82–85% of the cases (41), whereas C or T are present in only 5–6% of the cases. As a matter of fact,
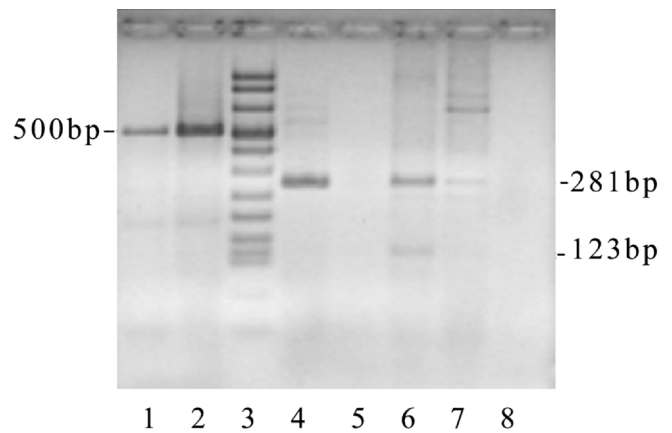

Fig. 8. Splicing of the transcripts of ABCA1 minigenes transfected into COS1 cells. Expression vector constructs, containing the wild-type or the mutant ABCA1 minigene (harboring the IVS2 +5G>C mutation), were transfected into COS1 cells. Total RNA extracted from these cells was retro-transcribed and PCR amplified using oligonucleotide primers complementary to exon 1–exon 3 region of ABCA1 mRNA. Lanes 1 and 2: RT-PCR amplification of neomycin resistance (Neo^R) gene present in the constructs of the wild-type and the mutant minigene, respectively, transfected into COS1 cells; lane 3: mw markers; lane 4: splicing product of the wild-type ABCA1 minigene in COS1 cells; lane 5: splicing product of the mutant ABCA1 minigene in COS1 cells; lane 6: splicing products of endogenous ABCA1 gene in control fibroblasts; lane 7: splicing products of endogenous ABCA1 gene in COS1 cells; lane 8: mock RT-PCR. The 281 bp band corresponds to the exon 1–3 region; the 123 bp band corresponds to an mRNA devoid of exon 2 (Ex2$^-$/mRNA) (see Table 2).

Fig. 9. Splicing of the transcripts of ABCA1 minigenes transfected into NIH3T3 cells. Expression vector constructs, containing the wild-type or the mutant ABCA1 minigene (harboring the IVS2 +5G>C mutation), were transfected into NIH3T3 cells. Total RNA extracted from these cells was retro-transcribed and PCR amplified using oligonucleotide primers complementary to exon 1–exon 3 region of ABCA1 mRNA. Lanes 1 and 2: RT-PCR amplification of Neo^R gene present in the constructs of the wild-type and the mutant minigene, respectively, transfected into NIH3T3 cells; lane 3: mw markers; lane 4: splicing product of the wild-type ABCA1 minigene in NIH3T3 cells; lane 5: splicing product of the mutant ABCA1 minigene in NIH3T3 cells; lane 6: splicing products of endogenous ABCA1 gene in control fibroblasts; lane 7: splicing products of endogenous ABCA1 gene in NIH3T3 cells; lane 8: mock RT-PCR. The 281 bp band corresponds to the exon 1–3 region; the 123 bp band corresponds to an mRNA devoid of exon 2 (Ex2$^-$/mRNA) (see Table 2).

in the donor splice sites of human ABCA1, gene G is present in position +5 in 80% of the introns. Since the IVS2 +5G>C was not found in 78 normolipidaemic subjects and in 33 subjects with primary hypoalphalipoproteinemia, we assume that this sequence variant is not a common polymorphism but a rare and possibly deleterious mutation. We provide some line of evidence that IVS2 +5G>C disrupts the splicing of ABCA1 pre-mRNA. We reported previously that some alternative splicing of ABCA1 pre-mRNA occurs in mammalian cell lines. In normal human fibroblasts, we have detected minute amounts (<10% of total mRNA) of an aberrant mRNA devoid of exon 4 (Ex4⁻/mRNA), which is predicted to encode a short peptide of 74 amino acids probably devoid of function (24). Here we show that control fibroblasts may occasionally contain trace amounts of another aberrant mRNA that is devoid of exon 2 (Ex2⁻/mRNA). This abnormal minor mRNA species is also detectable in COS1 cells, which express the ABCA1 gene under normal culture conditions. The presence of the IVS2 +5G>C transversion renders the donor splice site in intron 2 apparently much less efficient and is the cause of the formation of larger amounts of Ex2⁻/mRNA (Figs. 6, 7). A more extensive analysis of the mRNA species in TD fibroblasts revealed another abnormally spliced mRNA lacking both exon 2 and exon 4 (Ex2⁻/Ex4⁻/mRNA) (Fig. 7).

The presence of multiple aberrantly spliced mRNAs in our TD patient raises the question of the type of translation products that can be generated under these circumstances. The Ex2⁻/mRNA lacks the primary translation initiation site that is located in exon 2 (18, 19). This could lead to either a complete block in the translation process (22) or, possibly, to an activation of the alternative translation initiation site in exon 4 (which has a suboptimal context for translation initiation with respect to exon 2). If this were the case, we expect the formation of a peptide devoid of the first 60 amino acids but otherwise "normal" in terms of membrane spanning and ATP binding domains. Indeed, such a protein corresponds to the original ABC transporter deduced from the available cDNA sequence in the earliest report (20). A recent study, however, demonstrated that constructs dependent on initiation of translation from the ATG present in exon 4 (Met 61) are either poorly translated in vitro or the translation product is rapidly degraded upon its formation (22). In the absence of exon 2, two putative ATG codons present in exon 3 might become initiation sites. They, however, are in a context that does not allow the translation to proceed because of the presence of a termination codon in exon 5. Finally, we expect the Ex2⁻/Ex4⁻/mRNA, the other transcript present in TD cells, not to be translated at all, being devoid of the two translation initiation sites present in exons 2 and 4. However, if putative ATG codons in exon 3 were activated, the translation would proceed from the 3′ end of exon 3 to exon 5 and 6 until the occurrence of a stop codon in the latter exon. On the basis of these considerations, we may assume that the allele carrying the IVS2 +5G>C mutation is, in all respects, a "null allele."

To further investigate the effect of IVS2 +5G>C trans-

version, we constructed two ABCA1 minigenes (spanning from exon 1 to exon 3), which were inserted into an expression vector and transfected into two cell lines, COS1 and NIH3T3. These two cell lines were chosen as they differ in terms of endogenous expression of ABCA1 gene, which is present in COS1 cells but is virtually absent in NIH3T3. The results shown in Fig. 8 indicate that the transcript of the wild-type minigene is normally spliced. In contrast, the splicing of the transcript of the mutant minigene generates an mRNA that is devoid of exon 2. In the case of NIH3T3, the primary transcript of the wild-type minigene was spliced correctly, whereas we were unable to detect any spliced or unspliced form of the transcript resulting from the mutant minigene. Since the analysis of Neo^R transcript indicates that the mutant minigene was efficiently transfected and, indirectly, also transcribed, we interpreted the results obtained with NIH3T3 as indirect evidence of a complete block of the splicing process and of a rapid degradation of the transcript of the mutant minigene. Taken together, these findings confirm that, as observed in human fibroblasts, the presence of IVS2 +5G>C mutation disrupts the splicing of the primary ABCA1 transcript.

During the course of this study, we found that the IVS2 +5G>C mutation was present in the brother of another TD patient we reported on several years ago (26). This subject (proband I.3 in Family 2) had reduced plasma HDL but no signs of Tangier disease. Detectable amounts of abnormal ABCA1 mRNAs (Ex2⁻/mRNA, Ex4⁻/mRNA and Ex2⁻/Ex4⁻/mRNA) were found in peripheral blood lympho-monocytes of this subject, as found in fibroblasts of proband I.1 of Family 1 (Fig. 1).

We showed that both the two unrelated subjects carrying the IVS2 +5G>C mutation shared the same haplotype of the ABCA1 locus, a condition consistent with a common ancestor. They were apparently not related but came from the same geographical district.

In conclusion, we have characterized two new mutations of ABCA1 gene in a patient with Tangier disease. We provided evidence that one of these mutations (IVS2 +5G>C) disrupts premRNA splicing, leading to the formation of several types of abnormally spliced mRNAs. These mRNAs are predicted not to be translated at all or to encode short truncated peptides. The study of this naturally occurring mutation in intron 2 emphasizes the complexity of the ABCA1 pre-mRNA splicing.∎

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