J Hum Genet (2002) 47:325–329

SHORT COMMUNICATION

**Zhigang Guo · Akihiro Inazu · Wenxin Yu
Taeko Suzumura · Michiko Okamoto · Atsushi Nohara
Toshinori Higashikata · Ryuichi Sano
Kazuyoshi Wakasugi · Tetsuo Hayakawa
Koujiro Yoshida · Tadashi Suehiro · Gerd Schmitz
Hiroshi Mabuchi**

Double deletions and missense mutations in the first nucleotide-binding fold of the ATP-binding cassette transporter A1 (*ABCA1*) gene in Japanese patients with Tangier disease

Received: January 24, 2002 / Accepted: March 11, 2002

Abstract Tangier disease (TD) is a rare autosomal recessive disease characterized by plasma high-density lipoprotein deficiency caused by an ATP-binding cassette transporter A1 (*ABCA1*) gene mutation. We describe three different mutations in Japanese patients with TD. The first patient was homozygous for double deletions of 1221 bp between intron 12 and 14 and 19.9 kb between intron 16 and 31. The breakpoint sequence analyses suggest that it is a simultaneous event caused by double-loop formation through multiple *Alu*. The second patient was homozygous for a novel mutation of A3198C in exon 19, resulting in Asn935His. The third patient was homozygous for A3199G of exon 19 that leads to Asn935Ser, which is the same mutation found in German and Spanish families. Both Asn mutations involved Walker A motif of the first nucleotide-binding fold.

Z. Guo · A. Inazu (✉) · W. Yu · A. Nohara · T. Higashikata · H. Mabuchi
Molecular Genetics of Cardiovascular Disorders, Vascular Medicine, Division of Cardiovascular Medicine, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa 920-8641, Japan
Tel. +81-76-265-2251; Fax +81-76-234-4251
e-mail: inazua@mhs.mp.kanazawa-u.ac.jp

A. Inazu · T. Suzumura · M. Okamoto
Department of Laboratory Science, School of Health Sciences, Kanazawa University, Kanazawa, Japan

R. Sano
Sano Clinic, Yonezawa, Japan

K. Wakasugi
Wakasugi Clinic, Yamagata, Japan

T. Hayakawa · K. Yoshida
Toyama Municipal Hospital, Toyama, Japan

T. Suehiro
Second Department of Internal Medicine, Kochi Medical School, Kochi, Japan

G. Schmitz
Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany

Key words Tangier disease · *ABCA1* · Large deletion · Double deletions · Missense mutation · Walker A

Introduction

Tangier disease (TD) is characterized by severe deficiency or absence of high-density lipoprotein (HDL) in plasma and results in the accumulation of cholesterol ester in many tissues, including tonsils, liver, spleen, lymph nodes, thymus, gastrointestinal mucosa, and peripheral nerves (Assmann et al. 2001). Foam cells are present in affected tissues. Defective efflux of cellular cholesterol and phospholipids to HDL, which is the first step in reverse cholesterol transport, has been reported in TD. Autosomal recessive TD and autosomal codominant disease of familial HDL deficiency were caused by an ATP-binding cassette transporter A1 (*ABCA1*) gene defect, located in chromosome 9q31 (Bodzioch et al. 1999; Marcil et al. 1999).

This article concerns novel and previously reported missense mutations in Walker A motif and an event of double deletions in three Japanese patients with TD, with a probable mechanism of *Alu*-mediated double-loop formation and intragenic recombination in the *ABCA1* gene.

Patients and methods

Case 1 (a 57-year-old male) had angina pectoris with 90% stenosis of the left anterior descending artery, accompanied by heart failure, yellow tonsils, and hepatosplenomegaly. Foamy macrophages were observed in the tonsils and bone marrow, and stomatocytosis was also noted. Plasma lipid and apolipoprotein (apo) levels were as follows: cholesterol (CHOL), 22 mg/dl; triglyceride (TG), 88; HDL-C, 4; apo A-I, 3.2; and apo A-II, 3.5. The patient's sister (49 years old) had a history of splenectomy and low HDL-C (2 mg/dl). Case 2 was a 69-year-old male who also had yellow tonsils. Foamy macrophages were found in the gastric mucosa and

he had not only hepatosplenomegaly but also chronic hepatitis (HCV) and type 2 diabetes mellitus. Plasma lipid and apolipoprotein levels were as follows: CHOL, 34; TG, 187; HDL-C, 0.8; apo A-I, <5; and apo A-II, <2. Case 3 was a 20-year-old man who was diagnosed with an obsessive-compulsive disorder. He had mild splenomegaly, but no enlargement of tonsils. Plasma lipid and apolipoprotein levels were as follows: CHOL, 61; TG, 114; HDL-C, 0; apo A-I, 0; and apo A-II, 0.3. He had an almost normal electroencephalogram. The WAIS-R test showed his intelligence to be below −2 SD. None of the three indexed patients showed any peripheral neuropathy. Cases 2 and 3 did not have coronary artery disease (CAD). A relative increase in pro-apo AI was observed in the plasma of cases 1 and 2. Decreased cellular cholesterol efflux activity was found in case 2 (Hirano et al. 2000). Informed consent for all studies was obtained.

Fasting venous blood samples were collected in tubes containing Na_2 ethylene diamine tetra acetate (EDTA). The plasma lipid levels were determined with enzymatic methods. Plasma apolipoprotein levels were determined with immunoturbidimetric assays.

Genomic DNA was extracted from white blood cells. Each exon of the *ABCA1* gene was amplified with Taq DNA polymerase (Biotech International, Tokyo, Japan). Primer 3 software (Whitehead Institute for Biomedical Research, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) was used to design primers, which flank every exon and the adjacent splice junctions based on the published sequence (Santamarina-Fojo et al. 2000) (primer sequences available from AI upon request). Primers for exons 19 and 22–24 were synthesized according to the published sequences (Lapicka-Bodzioch et al. 2001). Long-range polymerase chain reaction (PCR) was used with LA Taq DNA polymerase (Takara Shuzo, Otsu, Japan).

DNA (5 μg) was digested with *Eco*RI and run on a 0.8% agarose gel. We used a pcDNA3.1/V5/His-TOPO vector containing partial *ABCA1* cDNA obtained from G. Schmitz, which consists of 6603 bp but is missing a 5′ sequence corresponding to exon 1 (Langmann et al. 1999). The 1055-bp and 3862-bp probes containing the sequence from exon 12–17 and exon 15–42 of the *ABCA1* cDNA were synthesized with PCR or the long-range PCR method and labeled with PCR DIG probe synthesis kit (Boehringer Mannheim, Mannheim, Germany) using *Xba*I digested plasmid as a template.

Single-strand conformation polymorphism (SSCP) analysis for each exon of *ABCA1* was carried out on 10% nondenaturing polyacrylamide gels. Two electrophoretic conditions were used for SSCP: 1 × Tris-borate + EDTA (TBE) buffer at 4°C and 1 × TBE buffer containing 10% glycerol at room temperature. The abnormal bands in SSCP analyses were purified with Microcon YM-30 (Millipore, Bedford, MA, USA). The purified double-strand DNA from the second PCR was sequenced with the dideoxynucleotide chain termination method, using a Thermo sequenase II (Amersham Pharmacia Biotech, Cleveland, OH, USA) in ABI 310 automated DNA sequencer (Perkin-Elmer, Foster City, CA, USA).

Amplified DNA of exon 19 in the second patient was incubated with *Nla*III (New England Biolabs, Beverly, MA, USA). The mutation in the third patient was identified by *Dde*I (TOYOBO, Tokyo, Japan) digestion of a new PCR product using a forward mismatch primer (5′-gatcacctccttcctgggcctc-3′) in exon 19 and a reverse primer (5′-gggatcagcatggtttccta-3′) in intron 19.

Results

We first used the PCR method to examine each exon of 1–50 of the *ABCA1* gene in three patients. No PCR products were amplified in exon 12, 13, or 17–31 in the first patient. Using long-range PCR, we confirmed double deletions in this patient, 1.2 kb from intron 12–14 and 19.9 kb from intron 16–31, which encodes the sixth transmembrane region — a linker region — the seventh transmembrane region of the putative secondary structure. Southern blot using *Eco*RI digestion and the probes of the cDNA encoding exon 12–17 and exon 15–42 confirmed the patient was homozygous for the double deletions (Fig. 1). By primer walking, we could determine the breakpoint sequences (Fig. 2).

In the second and third patients, abnormal patterns were revealed only in the PCR products from exon 19. The direct sequencing revealed different mutations in exon 19 in cases 2 and 3 (Fig. 3, left panel). The A3198C mutation in exon 19 found in case 2 had resulted in a change of Asn935His. This substitution created an *Nla*III cutting site that could be detected by RFLP analysis of the PCR product of exon 19, yielding 165- and 83-bp fragments in the normal allele and

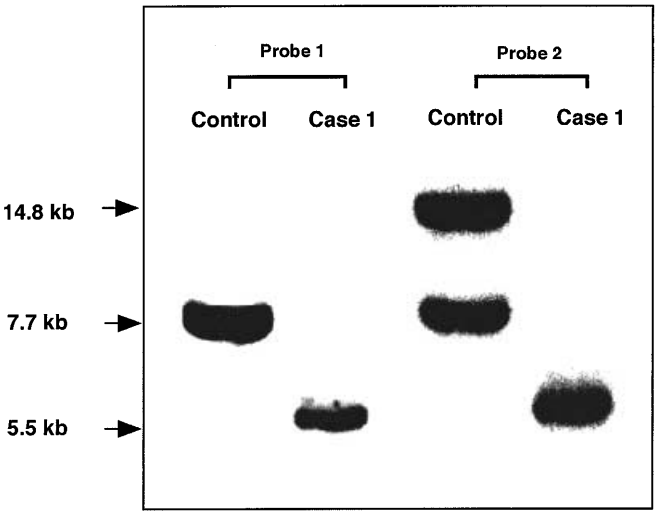

Fig. 1. Southern blot analysis of the first patient's genomic DNA. DNA was digested by *Eco*RI and hybridized with cDNA probe 1 containing sequences of exon 12–17 and cDNA probe 2 corresponding to exon 15–42. The result shows that case 1 was homozygous for the deletion in the *ABCA1* gene. The normal fragment of 14.8 kb was detected by probe 2, but it was not found in case 1. The normal fragment of 7.7 kb was detected by both probes, but the fragment of 5.5 kb was found in case 1

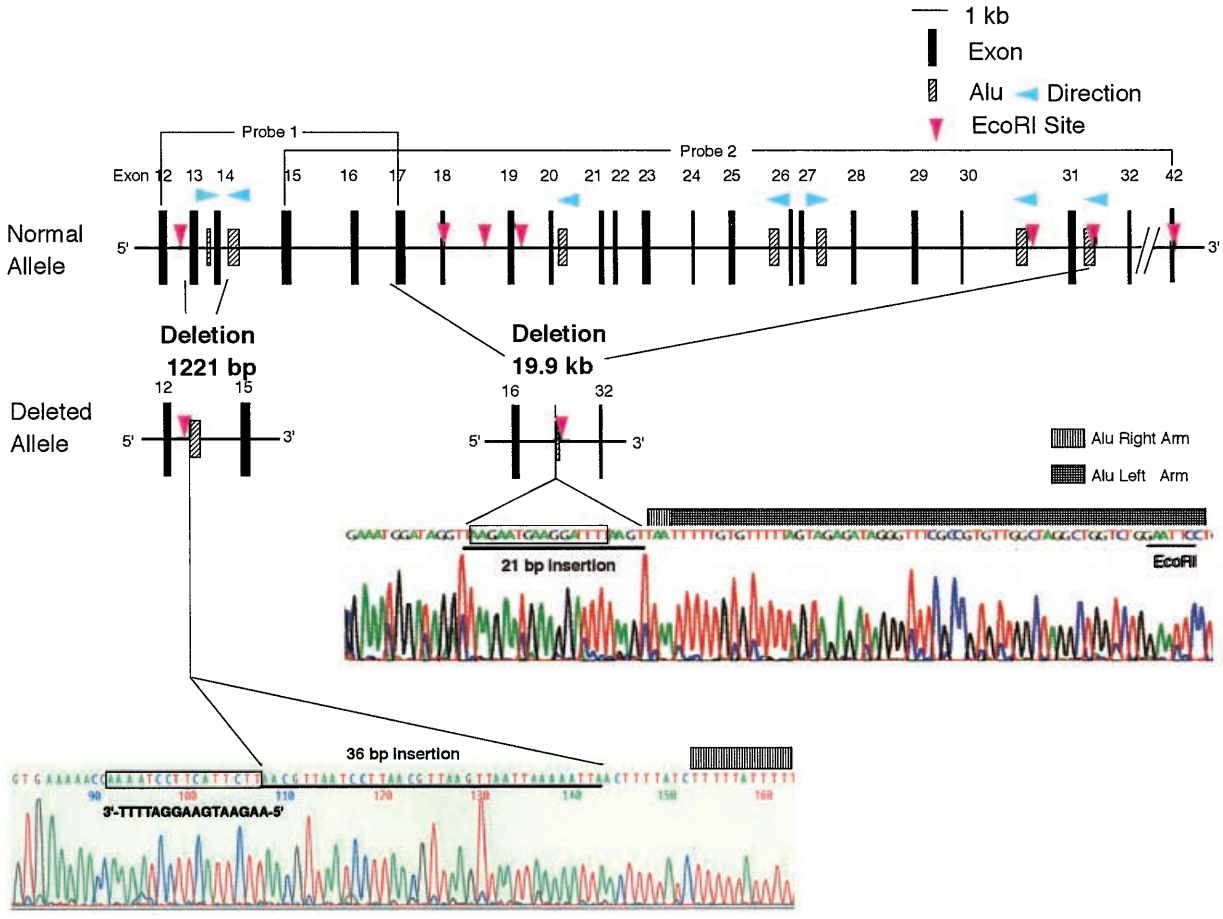

Fig. 2. Breakpoint sequences of doubly deleted *ABCA1* gene in the first patient with Tangier disease. Probe 1 detected a 1.2-kb deletion from intron 12 to intron 14, with an orphan 36-bp insertion in the breakpoint. The 19.9-kb deletion detected by probe 2 was also found from intron 16 to intron 31. The 3′ breakpoint was found within the right arm of *Alu* in intron 31, and had a small insertion of 21 bp in which the 16-bp sequence was complementary to the sequence found in front of the 5′ deleted region

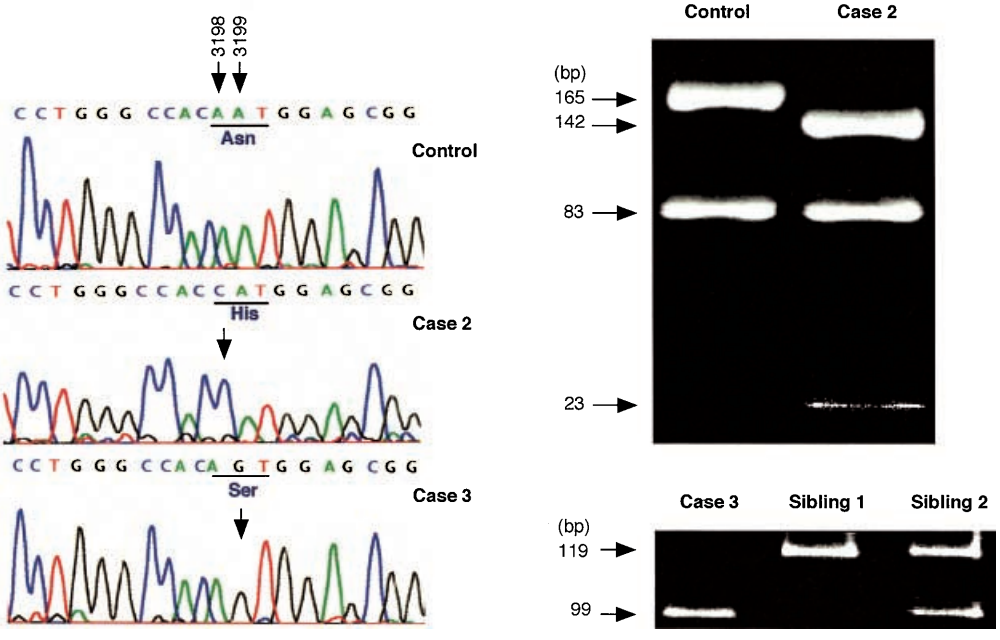

Fig. 3. Missense mutations in the first nucleotide-binding fold in the second and third patients by direct sequencing and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analyses. Case 2, homozygous for the A>C mutation at 3198 (Asn935His), as shown in the *left panel*, was detected by PCR-RFLP using *Nla*III digestion in the *right panel*. Case 3, homozygous for the A>G mutation at 3199 (Asn935Ser), as shown in the *left panel*, was confirmed by PCR-RFLP using *Dde*I digestion on 10% polyacrylamide gel in the *right panel*. Sibling 1 was normal for the mutation (high-density lipoprotein-C [HDL-C], 72 mg/dl). Sibling 2 was heterozygous for the mutation and had a low HDL-C of 39 mg/dl

142-, 83-, and 23-bp fragments from the mutant allele, showing case 2 was homozygous for the mutation (Fig. 3, right panel). The mutation in case 3 was A3199G of exon 19, resulting in a change of Asn935Ser. This mutation could be confirmed by the presence of a 99-bp fragment instead of a 119-bp fragment in the PCR-RFLP assay using a mismatch primer and *Dde*I digestion.

Discussion

This report concerns novel and previously reported missense mutations in the Walker A motif and large double deletions of the *ABCA1* gene in three Japanese patients with TD.

Since TD was initially discovered in two sibs from Tangier Island in Virginia, more than 60 cases have been reported from all over the world (Assmann et al. 2001). Among the Japanese, nine unrelated cases, including the three in this report, have been reported (Huang et al. 2001; Nishida et al. 2002).

Gene analysis further confirmed that all three patients were homozygous for the *ABCA1* gene mutation. The first patient had double deletions, which is the second largest deletion reported so far in the *ABCA1* gene. This deletion leads to a severe phenotype, including CAD. The second patient had a novel mutation of Asn935His. The Asn935Ser mutation in the third case showed atypical clinical signs, such as an obsessive-compulsive disorder and lower intelligence, in addition to the typical features. The mutation of Asn935Ser in the third patient was the same as that of German and Spanish families (Bodzioch et al. 1999; Utech et al. 2001), which suggests that it is a recurrent mutation and that they did not have cognitive disorders. Besides its accumulation in the arteries, cholesterol also accumulates within Schwann cells and causes peripheral neuropathy in approximately 50% of TD patients (Assmann et al. 2001). Case 3 may be afflicted with central nervous system (CNS) involvement. However, association between CNS involvement and TD is currently uncertain because of a lack of common CNS features.

The *ABCA1* gene consists of 50 exons and is 149 kb in length, with its protein consisting of two nucleotide-binding folds (NBFs) containing highly conserved Walker A and B motifs and one pair of six-transmembrane domains (Santamarina-Fojo et al. 2000). To date, 32 disease-causing mutations in the *ABCA1* gene have been reported, including 15 missense mutations, 7 small deletions, 4 small insertions, 3 nonsense mutations, 1 insertion and deletion mutation, 1 splicing mutation, and 1 large deletion (Bodzioch et al. 1999; Marcil et al. 1999).

Most missense mutations were found in the first extracellular loop and in or near the first NBF. In our patients, the two missense mutations involving Asn935 were found in Walker A motif (GHNGAGKTT) of the first NBF of the *ABCA1* gene. The Ala937Val mutation in Walker A motif (Bodzioch et al. 1999) and Ala1046Asp near Walker B motif (Wang et al. 2000) of the first NBF have been reported. However, only Arg2021Trp has been reported so far in the second NBF, but this case had another mutation of Asp1229Asn (Huang et al. 2001). Thus, the pathological significance of the mutation in the second NBF is unclear.

In the first large deletion, a patient had a genomic deletion of about 1.5 kb at the 3′ end of the *ABCA1* cDNA (Bodzioch et al. 1999). However, detailed gene arrangement has not been shown. Our first case had double deletions probably via a single event, as suggested by sequence analysis of the breakpoints (Fig. 2). The 3′ deletion junction had an insertion of 21 bp. The 16 bp within the 21-bp insertion was not found in the original sequence, but was complementary to the proximal sequence of the 5′ deletion junction. Indeed, the same oriented *Alu* sequence was found in both intron 14 and 31, facilitating the stabilization of the folding of the *ABCA1* gene to promote nonhomologous intragenic recombination.

There have been five cases with double deletions in the same gene, such as dystrophin, growth hormone, β-globin, and N-acetylgalactosamine-6-sulfatase. Although premutation was suggested in the dystrophin gene (Hoop et al. 1994), a simultaneous event of double deletions was proposed because of inversion between deletions in thalassemia patients (Jones et al. 1981; Jennings et al. 1985; Kulozik et al. 1992). A mechanism of double deletions was less clear in cases with isolated growth hormone deficiency type1A and mucopolysaccharidosis type IVA (Goossens et al. 1986; Hori et al. 1995). The case with TD suggests that, like the cases with thalassemia, a simultaneous event mediated by the *Alu*-mediated recombination is a mechanism of double deletions.

References

Assmann G, Von Eckardstein A, Brewer HB (2001) Familial analphalipoproteinemia: Tangier disease. In: Scriver CR, Beaydet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease, 8th edn. McGraw-Hill, New York, pp 2937–2960

Bodzioch M, Orso E, Klucken J, Langmann T, Bottcher A, Diederich W, Drobnik W, Barlage S, Buchler C, Porsch-Ozcurumez M, Kaminski WE, Hahmann HW, Oette K, Rothe G, Aslanidis C, Lackner KJ, Schmitz G (1999) The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. Nat Genet 22:347–351

Goossens M, Brauner R, Czernichow P, Duquesnoy P, Rappaport R (1986) Isolated growth hormone (GH) deficiency type1A associated with a double deletion in the human GH gene cluster. J Clin Endocrinol Metab 62:712–716

Hirano K, Matsuura F, Tsukamoto K, Zhang Z, Matsuyama A, Takaishi K, Komuro R, Suehiro T, Yamashita S, Takai Y, Matsuzawa Y (2000) Decreased expression of a member of the Rho GTPase family, Cdc42Hs, in cells from Tangier disease — the small G protein may play a role in cholesterol efflux. FEBS Lett 484:275–279

Hoop RC, Russo LS, Riconda DL, Schwartz LS, Hoffman EP (1994) Restoration of half the normal dystrophin sequence in a double-deletion Duchenne muscular dystrophy family. Am J Med Genet 49:323–327

Hori T, Tomatsu S, Nakashima Y, Uchiyama A, Fukuda S, Sukegawa K, Shimozawa N, Suzuki Y, Kondo N, Horiuchi T, Ogura S, Orii T (1995) Mucopolysaccharidosis type IVA: common double deletion in the N-acetylgalactosamine-6-sulfatase gene (*GALNS*). Genomics 26:535–542

Huang W, Moriyama K, Koga T, Hua H, Ageta M, Kawabata S, Mawatari K, Imamura T, Eto T, Kawamura M, Teramoto T, Sasaki J (2001) Novel mutations in *ABCA1* gene in Japanese patients with Tangier disease and familial high density lipoprotein deficiency with coronary heart disease. Biochim Biophys Acta 1537:71–78

Jennings MW, Jones RW, Wood WG, Weatherall DJ (1985) Analysis of an inversion within the human beta globin gene cluster. Nucleic Acids Res 13:2897–2906

Jones RW, Old JM, Trent RJ, Clegg JB, Weatherall DJ (1981) Major rearrangement in the human β-globin gene cluster. Nature 291:39–44

Kulozik AE, Bellan-Koch A, Kohne E, Kleihauer E (1992) A deletion/inversion rearrangement of the β-globin gene cluster in a Turkish family with $\delta\beta^0$-thalassemia intermedia. Blood 79:2455–2459

Langmann T, Klucken J, Reil M, Liebisch G, Luciani L-F, Chimini G, Kaminski WE, Schmitz G (1999) Molecular cloning of the human ATP-binding cassette transporter 1 (*hABC1*): evidence for sterol-dependent regulation in macrophages. Biochem Biophys Res Commun 257:29–33

Lapicka-Bodzioch K, Bodzioch M, Krull M, Kielar D, Probst M, Kiec B, Andrikovics H, Bottcher A, Hubacek J, Aslanidis C, Suttorp N, Schmitz G (2001) Homogenous assay based on 52 primer sets to scan for mutations of the *ABCA1* gene and its application in genetic analysis of a new patient with familial high-density lipoprotein deficiency syndrome. Biochim Biophys Acta 1537:42–48

Marcil M, Brooks-Wilson A, Clee SM, Roomp K, Zhang LH, Yu L, Collins JA, Dam MV, Molhuizen HOF, Loubster O, Ouellette BFF, Sensen CW, Fichter K, Mott S, Denis M, Boucher B, Pimstone S, Genest J Jr, Kastelein JJP, Hayden MR (1999) Mutations in the *ABC1* gene in familial HDL deficiency with defective cholesterol efflux. Lancet 354:1341–1346

Nishida Y, Hirano K, Tsukamoto K, Nagano M, Ikegami C, Roomp K, Ishihara M, Sakane N, Zhang Z, Tsuji K, Matsuyama A, Ohama T, Matsuura F, Ishigami M, Sakai N, Hiraoka H, Hattori H, Wellington C, Yoshida Y, Misugi S, Hayden MR, Egashira T, Yamashita S, Matsuzawa Y (2002) Expression and functional analyses of novel mutations of ATP-binding cassette transporter-1 in Japanese patients with high-density lipoprotein deficiency. Biochem Biophys Res Commun 290:713–721

Santamarina-Fojo S, Peterson K, Knapper C, Qiu Y, Freeman L, Cheng JF, Osorio J, Remaley A, Yang XP, Haudenschild C, Prades C, Chmini G, Blackmon E, Francois T, Duverger N, Rubin EM, Roiser M, Denefle MP, Fredrickson DS, Brewer HB Jr (2000) Complete genomic sequence of the human *ABCA1* gene: analysis of the human and mouse ATP-binding cassette A promoter. Proc Natl Acad Sci USA 97:7987–7992

Utech M, Hobbel G, Rust S, Reinecke H, Assmann G, Walter M (2001) Accumulation of RhoA, RhoB, RhoG, and Rac1 in fibroblasts from Tangier disease subjects suggests a regulatory role of Rho family proteins in cholesterol efflux. Biochem Biophys Res Commun 280:229–236

Wang J, Burnett JR, Near S, Young K, Zinman B, Hanley AJG, Connelly PW, Harris SB, Hegele RA (2000) Common and rare *ABCA1* variants affecting plasma HDL cholesterol. Arterioscler Thromb Vasc Biol 20:1983–1989