Specific Mutations in ABCA1 Have Discrete Effects on ABCA1 Function and Lipid Phenotypes Both In Vivo and In Vitro

Roshni R. Singaraja, Henk Visscher, Erick R. James, Angeliki Chroni, Jonathan M. Coutinho, Liam R. Brunham, Martin H. Kang, Vassilis I. Zannis, Giovanna Chimini, Michael R. Hayden

Abstract—Mutations in ATP-binding cassette transporter A1 (ABCA1) cause Tangier disease (TD) and familial hypoalphalipoproteinemia (FHA),1–3 characterized by low to absent plasma high-density lipoprotein cholesterol levels. However, wide variations in clinical lipid phenotypes are observed in patients with mutations in ABCA1. We hypothesized that the various lipid phenotypes would be the direct result of discrete and differing effects of the mutations on ABCA1 function. To determine whether there is a correlation between the mutations and the resulting phenotypes, we generated in vitro 15 missense mutations that have been described in patients with Tangier disease and familial hypoalphalipoproteinemia. Using localization of ABCA1, its ability to induce cell surface binding of apolipoprotein A-I, and its ability to elicit efflux of cholesterol and phospholipids to apolipoprotein A-I we determined that the phenotypes of patients correlate with the severity and nature of defects in ABCA1 function. (Circ Res. 2006;99:389-397.)

Key Words: ABCA1 ■ basic research ■ cell culture ■ cholesterol homeostasis ■ HDL cholesterol ■ mutation ■ tissue culture

Mutations in ATP-binding cassette transporter A1 (ABCA1) lead to Tangier disease (TD) and familial hypoalphalipoproteinemia (FHA),1–3 characterized by low to absent levels of plasma high-density lipoprotein (HDL) cholesterol (HDL-C). The defect in patients with TD and FHA leads to an inability to efflux intracellular cholesterol and phospholipids to apolipoprotein A-I (ApoA-I).

Despite longstanding recognition that the hallmark of TD is exceedingly low HDL levels, careful assessment reveals significant variation in HDL in patients with mutations in ABCA1.1–13 In patients diagnosed with TD caused by mutations in both ABCA1 alleles, plasma HDL-C levels ranged from <1% to >13% of normal HDL-C values. Of those with FHA, the variability was even greater, with plasma HDL-C levels ranging from 30% to 83% of age- and sex-matched controls.

The aim of this study was to investigate the functional basis for varying lipid phenotypes in patients with different ABCA1 mutations. ABCA1 is synthesized in the endoplasmic reticulum and is transported via vesicles to the plasma membrane, which is essential for its function in lipid efflux and cellular cholesterol homeostasis.14 We hypothesized that patients with the most severe decrease in plasma HDL-C would have nonfunctional ABCA1, occurring either because of impaired trafficking of ABCA1 to the plasma membrane or because of absent transport activity. Those with milder decreases in plasma HDL-C may have ABCA1 that is present at the plasma membrane with partial function.

We generated stable cell lines expressing 15 missense mutations described in the ABCA1 gene. For each mutant we determined intracellular localization by immunofluorescence and glycosylation status to assess defects in transport. Additionally, we assessed their ability to induce binding of ApoA-I and to induce efflux of both cholesterol and phospholipids. These experiments reveal a spectrum of functional defects that provide a mechanism for the observed variability in patient phenotypes in both TD and FHA and also provide further insights into the function of specific sequences of ABCA1.

Materials and Methods

Generation of ABCA1 Variant Constructs and Cell Lines

pcDNA3-ABCA1 was generated by RT-PCR amplification of human liver ABCA1. C-terminal enhanced green fluorescent protein (EGFP; Clontech) tagging did not mislocalize or alter lipid efflux capabilities (data not shown). All ABCA1 variants were generated by PCR-based site-directed mutagenesis,15 sequenced, and cloned into pcDNA5-
FRT (Invitrogen); polyclonal ABCA1 stable cell lines were generated in 293 Flp-In cells (Invitrogen).

**Cell Surface Biotinylation Assays**
Cells were washed with cold PBS containing 1 mmol/L MgCl₂ and 0.1 mmol/L CaCl₂. EZ-link-sulfoly-NHS-LC biotin (2 mg/mL) was added for an hour on ice, and the reaction was quenched with 10 mmol/L glycine. Immunoprecipitation was performed using the AC10 antibody and probed with streptavidin (BioRad).

**Glycosylation Status**
According to manufacturer’s specifications, 20 μg of ABCA1 lysates were digested with either EndoH or PNGase (NEB), separated on 3% to 8% gels (Invitrogen), and analyzed by Western blotting with AC10.

**Statistics**
All statistical analyses were performed with the 2-tailed Student t-test using GraphPad Prism (GraphPad Software).

**Results**

**Variation of Plasma HDL-C Levels in Patients With ABCA1 Missense Mutations**
We obtained plasma HDL-C levels from every TD and FHA patient that has been described that harbors 15 specific missense mutations (Figure 1). Correlation of genotypes with other phenotypic markers, such as incidence of atherosclerosis, was not possible because there was no consistency in the reporting of other phenotypes to provide sufficient sample sizes for comparison.

We compared the reported HDL-C levels of the patients to those of age- and sex-matched Lipid Research Clinic (LRC) controls. These mutations were chosen because they were localized in the extracellular, NBD, Walker motif, transmembrane domain, intracellular and C-terminal domains of ABCA1, thus providing insight into the different functional domains of ABCA1. In ABCA1 heterozygotes, 3 distinct phenotypic groups emerged, one in which HDL-C levels were ~50% of those of age- and sex-matched controls, one in which HDL-C levels were at least 70% of controls (A255T, W590S, T929I), and one in which HDL-C levels were significantly below the expected 50% of the levels for controls (30.4% of controls) (M1091T) (Table).

In patients defined by missense mutations on both alleles, 2 clear groups were observed: those showing negligible plasma HDL-C (R587W, N935S, A1046D, C1477R, and R2081W) and those with HDL-C levels that were ~10% of HDL-C in controls (A255T).

**Heterozygotes With Nonfunctional ABCA1 Have ~50% of Normal HDL-C Levels**
We reasoned that mutations in ABCA1 that abolish all activity would result in HDL-C levels that are ~50% of those of controls because the remaining wild-type allele would be sufficient to produce close to 50% of normal HDL. Indeed, patients heterozygous for the mutations R587W, Q597R, ΔL693, N935S, A1046D, C1477R, and R2081W had between 47% and 69% of HDL-C levels of controls (Table).

**Trafficking of ABCA1**
We first determined the intracellular localization of the GFP-tagged ABCA1 mutants. Six mutants, R587W, Q597R, ΔL693, N935S, N1800H, and R2081W, showed no localization at the plasma membrane and instead accumulated intracellularly (Figure 2A), indicating that these mutations severely affect ABCA1 function by preventing its migration to the plasma membrane, thus diminishing its ability to efflux lipids and generate HDL.

In contrast, C1477R, D1289N, and P2150L showed similar distribution to wild-type ABCA1, indicating that these mutants cause defects despite their normal localization at the plasma membrane. Two mutants, A1046D and S1506L, showed an intermediate phenotype where plasma membrane localization was reduced (Figure 2A).

**Glycosylation Status**
ABCA1 is normally glycosylated. At the endoplasmic reticulum (ER), proteins are sensitive to endoglycosidase H (EndoH) digestion. In the Golgi, proteins are EndoH insensitive. Proteins localized at both the ER and the Golgi are sensitive to PNGase digestion. Thus we would predict that wild-type ABCA1, which is found at the ER and at the plasma membrane, would show both EndoH resistant and a sensitive band, whereas ABCA1 mutants that accumulate exclusively in the ER would show only the lower molecular weight EndoH sensitive band.

Indeed, following EndoH treatment, wild-type ABCA1 shows a shift in molecular weight, indicating partial EndoH sensitivity (Figure 2B). Of the mutants not localizing to the plasma membrane, R587W, Q597R, ΔL693, and N935S are all EndoH sensitive, indicating that they do not exit the ER. R2081W, along with the mutants localizing to the plasma membrane by fluorescence, D1289N, C1477R, and P2150L,
showed both EndoH resistant and sensitive bands, indicating that they are distributed at the ER and at the Golgi, thus confirming the GFP localization data. S1506L, which appeared to have partial plasma membrane localization, showed a digestion pattern essentially similar to wild-type ABCA1, supporting the notion that a portion of S1506L is localized at the plasma membrane.

**Cell Surface Biotinylation**

To assess cell surface localization, we performed cell surface biotinylation assays. R587W, Q597R, ΔL693, S1506L, and R2081W showed significantly reduced cell surface ABCA1 expression, confirming our previous localization data. By contrast, D1289N, C1477R, and P2150L were at the plasma membrane in similar quantities as wild-type ABCA1 (Figure 2C), also confirming our previous localization data. Mutant A1046D showed an intermediate phenotype with some ABCA1 localized at the plasma membrane (Figure 2C).

**ApoA-I Binding**

We hypothesized that ABCA1 mutants not at the plasma membrane will not induce ApoA-I binding. Alternately,
ABCA1 mutants that localize to the plasma membrane but still lack ApoA-I binding may have specifically disrupted binding sites or alterations in the conformation necessary for interaction with ApoA-I.

All 6 mutants showing no plasma membrane localization elicited significantly reduced cell surface ApoA-I binding (R587W, 33.0±8.9%, n=3, P=0.006; Q597R, 17.4±14.0%, n=3, P=0.009; ΔL693, 32.6±10.6%, n=3, P=0.008; N935S, 26.4±37.5%, n=3, P=0.01; N1800H, 36.9±15.5%, n=3, P=0.01; R2081W, 34.6±16.6%, n=3, P=0.02) (Figure 3A), indicating that although plasma membrane localization of ABCA1 is essential, it is not sufficient for ApoA-I binding. The A1046D and S1506L mutants displayed a partial ability to induce cell surface ApoA-I binding (56.3±16.4%, n=3, P=0.02 and 61.0±12.7%, n=3, P=0.004, respectively; Figure 3A), suggesting that in these mutants, some of the protein is localized at the plasma membrane.

A direct head-to-head comparison of the ability of all 15 mutants to bind ApoA-I was performed to determine whether the a priori grouping of the severity of the mutants using patient HDL-C levels was in agreement with the functional data. Indeed, the predicted loss of function mutants showed an intermediate ability to elicit cell surface ApoA-I binding (19.0±3.9%, n=3, P=0.0008) (Figure 3A), indicating that although plasma membrane localization of ABCA1 is essential, it is not sufficient for ApoA-I binding. The A1046D and S1506L mutants displayed a partial ability to induce cell surface ApoA-I binding (56.3±16.4%, n=3, P=0.02 and 61.0±12.7%, n=3, P=0.004, respectively; Figure 3A), suggesting that in these mutants, some of the protein is localized at the plasma membrane.

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**Phosphocholine and Cholesterol Efflux**

We next evaluated the ability of the mutants to efflux lipids. All 6 mutants with defective plasma membrane localization
showed significantly reduced phosphocholine and cholesterol efflux (Figure 3B and 3C), indicating that plasma membrane localization and ApoA-I binding are essential for ABCA1-mediated lipid efflux. Of the 3 mutants showing plasma membrane localization, C1477R showed significantly reduced lipid efflux. Because C1477R showed diminished cell surface ApoA-I binding, this finding indicates that ApoA-I binding is required for ABCA1-mediated lipid efflux. However, D1289N and P2150L showed normal cholesterol efflux and close to normal phosphocholine efflux, suggesting that both could be variants rather than mutants.

In addition, direct head-to-head comparisons of the cholesterol and phospholipid efflux capabilities of the 15 missense mutants were performed to determine whether the a priori classification of the severity of the mutants based on patient phenotypes was in agreement with the functional data (online Figure IB and IC). Indeed the loss of function mutations showed an intermediate phenotype compared with the mutants with partial function, and the putative dominant negative mutant M1091T showed the most severe phenotype.

In addition, to predict the functional consequence of the selected 15 missense mutations in this gene, we used PANTHER, a program that determines site-specific variation among evolutionarily related proteins. This program predicted that 2 of the 15 missense mutations would be benign (subPSEC score $<-3$) (online Table I), and that 5 of 8 putative loss-of-function mutations would result in a severe phenotype.

Near-Complete Absence of Plasma HDL-C in Homozygotes for Mutations in ABCA1 Implies the Presence of Null Alleles of ABCA1

Patients homozygous for R587W mutations showed 6.3% of normal HDL-C levels, those with N935S showed 2.62%, and those with N1800H showed 3.4% of normal plasma HDL-C levels. These findings confirmed our previous hypothesis that these mutations result in a complete lack of function of the ABCA1 protein.

<50% of Normal HDL-C Levels in ABCA1 Heterozygotes Imply a Dominant-Negative Function for the Mutant Allele

Of the ABCA1 heterozygotes described thus far, only 1 missense mutation, M1091T, results in HDL-C levels that are
significantly below the 50th percentile of age- and sex-matched controls. ABCA1 harboring this mutation is localized to intracellular regions and excluded from the plasma membrane (Figure 4A). Only an EndoH sensitive band was present, implying that the protein did not exit the ER (Figure 4B). A significant reduction in biotinylated M1091T was observed, thus corroborating the intracellular localization and EndoH data (Figure 4C). The lack of localization at the plasma membrane corresponded to a significantly reduced ability to induce ApoA-I binding (15.2 ± 9.4%, n = 3, P = 0.004) (Figure 4D). A significant reduction in biotinylated M1091T was observed, thus corroborating the intracellular localization and EndoH data (Figure 4C). The lack of localization at the plasma membrane corresponded to a significantly reduced ability to induce ApoA-I binding (15.2 ± 9.4%, n = 3, P = 0.004) (Figure 4D) and markedly reduced efflux of phosphocholine (8.6 ± 6.6%, n = 6, P < 0.0001) and cholesterol (6.9 ± 10.4%, n = 5, P = 0.0005) (Figure 4E and 4F). Patients heterozygous for this mutation have only 36% of normal relative efflux levels.\

**Heterozygotes With >70% HDL-C Levels Have Mutant ABCA1 That Retains Partial Activity**

We next addressed biochemical defects in heterozygous patients with >70% of normal HDL-C levels and hypothesized that these mutant alleles would retain partial activity. Three mutations fit this criteria, with patients harboring A255T showing 76%, W590S showing 83%, and T929I showing 76% of normal HDL-C levels.

**Intracellular Localization**

All 3 mutants A255T, W590S, and T929I, were localized by immunofluorescence to the plasma membrane and to intracellular regions in a manner indistinguishable from wild-type ABCA1 (Figure 5A). All three mutants also showed both EndoH-resistant and EndoH-sensitive bands (Figure 5B), further confirming their presence at the plasma membrane. In addition, the mutants were assessed for cell surface biotinylation and were indistinguishable from wild type (Figure 5C).

**ApoA-I Binding**

All mutants showed normal ApoA-I binding compared with wild-type ABCA1 (A255T, 98.0 ± 10.2%, n = 4; W590S, 94.9 ± 26.7%, n = 3; T929I, 83.6 ± 14.5, n = 3) (Figure 5D).

**Cholesterol and Phosphocholine Efflux**

All 3 mutants displayed defects in both cholesterol (Figure 5E) and phosphocholine (Figure 5F) efflux (A255T, cholesterol 49.2 ± 7.7%, n = 5, P = 0.0001, choline 41.5 ± 22.5%, n = 8, P = 0.0002; W590S, cholesterol 47.1 ± 13.1%, n = 5, P = 0.0008, choline 44.7 ± 21.1%, n = 3, P < 0.05; and T929I,
cholesterol 69.9±11.1%, n=5, P=0.004, choline 59.9±9.4%, n=7, P<0.0001). Thus localization at the plasma membrane and normal ApoA-I binding are essential but insufficient to efflux lipids. Mutant W590S has been previously shown to induce reduced annexin V binding, which is a measure of plasma membrane flipping.18 Plasma membrane flipping is observed in wild-type ABCA1-expressing cells and may be a critical factor for efflux to occur. Recent work has shown that W590S associates normally with ApoA-I. However, the ApoA-I released from wild-type ABCA1 was bound to lipids, whereas the ApoA-I released from W590S was lipid-free, indicating a defect in the lipidation of ApoA-I.24 When the ability of the ABCA1 mutants to promote αHDL formation was assessed (Figure 5G), wild-type ABCA1 was able to form αHDL of 10.4 to 12.2 nM diameter, whereas A255T and W590S formed only lipid-free ApoA-I and T929I formed lipid-free and ApoA-I of 7.1 to ∼9-nM diameter.

Increased Plasma HDL-C Levels (>10th Percentile) in Homozygotes Confirm the Retention of Partial Activity by Mutant Alleles

Data generated thus far would predict that mutant alleles that retain partial activity in patients homozygous for mutations in ABCA1 would confer higher plasma HDL-C levels than those in whom both mutant alleles lack complete function. We had previously hypothesized and confirmed in heterozygous FHA patients that the mutant A255T allele retained partial activity. Similarly, patients with TD who were homozygous for A255T show 13.3% of age- and sex-matched control HDL-C levels.

Discussion

We sought to delineate how different mutations in ABCA1 influence its function to provide insights into the phenotypic variability in patients with TD and FHA. In the past,
ascertained of these diseases was based purely on phenotype. The discovery of the mutation has led to a range of phenotypes being observed in both FHA and TD patients, with varying symptoms and severity.1–13

In general, 3 phenotypic groups were evident when we compared patient HDL-C levels to age- and sex-matched controls, those with >50% HDL-C, those with ~50% HDL-C, and those with <50% HDL-C. We hypothesized that patients with HDL-C >50th percentile represented a group in whom ABCA1 mutant alleles retained partial activity. By contrast, ABCA1 mutant alleles that generate inactive ABCA1 would result in HDL levels accounted for completely by the wild-type allele. Patients with HDL significantly <50th percentile would be predicted to have mutants that suppressed the activity of the wild-type allele (a dominant-negative mutation). Those homozygous for a particular mutation in ABCA1 were classified into 2 groups: those in whom the ABCA1 alleles showed partial retention of activity (HDL-C>10%), and those in whom the ABCA1 alleles showed a complete loss of activity (HDL-C<10%).

We then performed detailed biochemical analysis on the mutations in each group to determine how they affected the functions of ABCA1. As expected, almost all mutations resulted in defects in both phosphocholine and cholesteryl efflux, although different biochemical defects were identified as the underlying cause for this.

Of the mutants showing complete loss of function, most showed no plasma membrane localization, reduced ApoA-I binding, and no lipid efflux, implying that trafficking to the plasma membrane is essential for ABCA1 function. C1477R, however, did reach the plasma membrane, similar to wild-type ABCA1. However, it induced negligible cell surface ApoA-I binding,18,19,25 resulting in significant reductions in lipid efflux capacity. The defect in the C1477R mutant suggests that although plasma membrane localization is critical for lipid efflux, clearly other mechanisms must result in the failure to recruit ApoA-I. C1477R is localized within the second large extracellular loop in ABCA1, and studies in the related transporter ABCA4 revealed that its 2 large extracellular loops interact with each other through disulfide bonds.26 Because ABCA4 and ABCA1 are closely related, it is possible that the disrupted cysteine in the second large extracellular loop in ABCA1 may also prevent interaction between the 2 large extracellular loops, which could be essential for cell surface ApoA-I binding and lipid efflux.

Both A1046D and S1506L were partially localized at the plasma membrane and showed significantly reduced ApoA-I binding. The data for S1506L agree with previous data.25 The S1506L mutation also occurs in the second large extracellular portion of ABCA1 and may potentially destroy a domain of interaction for the extracellular loops necessary for ApoA-I binding. The mutation A1046D, however, occurs in the intracellular domain of ABCA1 and is localized between the first Walker A and B motifs. That this intracellular mutation would cause a similar biochemical defect as those in the second large extracellular loop suggests that some intracellular sites may significantly influence the 3-dimensional folding efficiency of ABCA1 that is vital for its ability to induce ApoA-I binding. Alternately, this mutation could affect other processes such as phosphorylation that are important for proper ABCA1 function.

Both P2150L and D1289N are functionally undistinguishable from wild-type ABCA1. Thus, despite their description as mutations, our data are more consistent with these being variants. P2150L has only been described in patients who also have the R587W variant (M.R.H., unpublished data, 2000). In our assays R587W clearly shows biochemical defects, implying that in these patients with R587W/P2150L, the defects in ABCA1 function are more appropriately ascribed to the R587W variant. In addition, TD has been diagnosed in patients homozygous for the R587W mutation,8,27 presumably without P2150L, making it more likely that P2150L is a nonfunctional variant. D1289N has been described as a variant in patients that are homozygous for R2081W.11 Biochemical characterization of R2081W results in defects in subcellular localization and lipid efflux, suggesting that D1289N is another variant. In addition, the bioinformatics analyses by PANTHER indicated that these were putative nonfunctional residues.

All 3 missense mutations (A255T, W590S, and T929I) that showed residual function were localized to the plasma membrane and induced cell surface ApoA-I binding at levels similar to wild-type ABCA1. Our findings for the mutant W590S therefore agree with previous findings that W590S does localize normally and shows normal ApoA-I binding18,19,25 These data imply that although mutant ABCA1 localizes to the plasma membrane and induces ApoA-I binding, these events are insufficient for normal lipid efflux to occur. Previous studies showed that W590S, which has defective lipid efflux, cross-links efficiently to ApoA-I, and its rate of dissociation from ApoA-I was similar to wild-type ABCA1.24 However, the ApoA-I released from wild-type ABCA1 was bound to lipids, whereas the ApoA-I released from W590S was lipid-free.24 Mutants A255T, W590S, and T929I were normal in their binding to lipid-free ApoA-I. However, A255T and W590S failed completely to lipitate ApoA-I, and T929I produced lipitated species with abnormal size.

One mutant, M1091T, when present in heterozygous patients, results in significantly lower than 50% of normal HDL-C and efflux levels. This mutant displayed the most severe defects, with almost complete lack of cholesterol and choline efflux. It did not localize to the plasma membrane and also showed negligible ApoA-I binding. ABCA1 has previously been hypothesized to dimerize or form part of a higher order complex based on the observations that truncation mutants in ABCA1 suppress the activity of wild-type ABCA1,16 suggesting a dominant-negative role for these mutants. Using chemical cross-linking, ABCA1 dimers and tetramers were found in isolated plasma membranes and in intracellular compartments.26 ApoA-I was found to be associated with multimeric, but not monomeric forms of ABCA1. Thus M1091T may act in a dominant-negative manner by an as of yet unknown mechanism.

Overall, the biochemical data presented in this study explain the varying HDL-C levels in patients with mutations in ABCA1 and provide novel insights into genotype/phenotype correlations between various ABCA1 mutants and plasma HDL-C levels.
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Disclosures
None.

References
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Supplementary Figure 1: Head-to-head comparison of the functional consequences of the ABCA1 mutants. The 15 missense mutants were compared directly for their ability to (A) induce cell surface binding of ApoA-I, (B) their ability to induce cholesterol efflux, and (C) their ability to induce phospholipid efflux. The mutants A255T, W590S and T929I which were hypothesized to have residual function based on patient HDL-C levels showed the mildest phenotypes, and the mutant M1091T, hypothesized to act in a dominant negative manner based on patient HDL-C data showed the most severe phenotype in these assays.
Supplementary Table 1: Bioinformatic predictions of the functional consequences of the 15 missense mutations in ABCA1 as determined by PANTHER

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