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Novel rare alleles of *ABCA1* are exclusively associated with extreme high-density lipoprotein-cholesterol levels among the Han Chinese

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Abstract

Background: High-density lipoprotein (HDL) is a major plasma lipoprotein directly associated with cholesterol metabolism. The ATP binding cassette transporter 1 gene (*ABCA1*) is one of the major genes modulating plasma levels of HDL-cholesterol (HDL-C). Rare alleles of *ABCA1* associated with extreme HDL-C concentrations have not been previously investigated in the Chinese.
Methods: Blood samples were collected from 470 subjects whose HDL-C concentrations were within the top 5% of the distribution, 335 subjects in the lowest 5%, and 220 within the range 5%–95%. First, we sequenced all exons of the *ABCA1* gene from 50 subjects from the group with extremely high HDL-C, and 50 from the group with extremely low HDL-C concentrations. Next, in the remaining subjects, we genotyped the non-synonymous variants identified exclusively with either extreme group.
Results: Four novel non-synonymous alleles were identified; all were rare. Alleles c.3029C>T (p.Ala1010Val) and c.5399A>G (p.Asn1800Ser) were found exclusively in the low group, c.2031C>A (p.Asp677Glu) and c.2660G>T (p.Cys887Phe) exclusively in the high group.
Conclusions: Our results show that some rare alleles of *ABCA1* are associated with marked phenotypes, supporting the ''rare-variant common-disease'' hypothesis. Certain alleles also provide tools for identifying individuals at high risk of dyslipidaemia, allowing for early therapeutic intervention.
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Keywords: ATP binding cassette transporter 1 gene (*ABCA1*); cholesterol efflux regulatory protein; high-density lipoprotein cholesterol; rare allele.

Introduction

High-density lipoprotein (HDL) is one of the major plasma lipoproteins directly associated with cholesterol metabolism. Low concentrations of HDL-cholesterol (HDL-C) are a strong and independent risk factor for coronary heart disease (1). About 50% of the variation in plasma level HDL-C is believed to be genetically determined (2, 3). ATP binding cassette transporter 1 gene (*ABCA1*, Genbank ID NM_005502, alias: *ABC1*; *CERP*; *ABC-1*) is one of the major genes modulating plasma HDL-C concentrations, and underlies two genetic diseases with low HDL-C: Tangier disease (MIM 205400) and familial HDL deficiency (MIM 604091) (4). *ABCA1* codes for cholesterol efflux regulatory protein (CERP, UniProtKB/Swiss-Prot O95477). This protein mediates the intracellular cholesterol pump, thereby transporting cellular cholesterol to the plasma membrane and incorporating it into plasma HDL particles.

The ''common-variant common-disease'' is the accepted hypothesis for the genetic contribution to common disease. Recently, several studies have shown that rare alleles also contribute to common diseases, and may have stronger phenotypic effects than common alleles (5–10). This has prompted efforts to identify rare alleles in common diseases. The importance of studying rare alleles lies not only

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in identifying individuals at high risk to allow for early intervention, but also in understanding disease mechanisms and gene function. Variations in *ABCA1* from white and black Americans, and Danish individuals have been reported (5, 6). Wang and co-workers (11) screened for *ABCA1* mutations in a Caucasian family with Tangier disease, aboriginal Canadians of Oji-Cree and Inuit origin with familial HDL deficiency and a subgroup of 37 Chinese normolipidaemic subjects selected from 223 subjects. They reported three common non-synonymous variants of *ABCA1*: p.Val825Ile, p.Ile883Met and p.Arg1587Lys (originally reported as V/I765, I/M823 and R/K1527) among the Chinese subjects (11). However, rare alleles of the gene associated with extreme HDL-C concentrations among the Han Chinese have not been previously investigated. It is not known whether there are population-specific alleles bearing strong phenotypic effects. In an attempt to further test the ''rare-variant common-disease'' hypothesis and study whether there are some rare alleles that may have strong phenotypic effects among the Han Chinese, we studied subjects showing extreme HDL-C concentrations and their associated rare alleles.

Materials and methods

Study subjects

A total of 1025 subjects were recruited from the general population and individuals who underwent health checks from April to November 2008 at laboratories in two centres (the Third Affiliated Hospital of Sun Yat-Sen University and Dongguan People's Hospital) in Guangdong Province, Southern China. All subjects were of Han Chinese ethnicity. The distribution of HDL-C concentrations among the Han Chinese and selection for genotyping were based on distribution data for Japanese subjects recommended by Daiichi Pure Chemicals (male 1.41 ± 0.33 mmol/L, female 1.58 ± 0.32 mmol/L; http://www.sekisuimedical.jp/). These ranges were adopted at the two centres used in this study. The study group included 470 subjects, in whom HDL-C concentrations were in the top 5% of the distribution (extremely high group); 335 in the lowest 5% (extremely low group); and 220 subjects in which HDL-C was within the 5% and 95% limits (middle-range group). Individuals with diseases or conditions that may affect plasma HDL-C, such as diabetes mellitus, dysthyroidism, liver dysfunction, and pregnancy, were excluded. All subjects gave informed consent. The study was conducted in accordance with the Declaration of Helsinki and was approved by the University Ethics Committee.

HDL-C measurement

Following a 12-h fast, blood was collected in tubes that promote coagulation. Blood samples were centrifuged at 1800 *g* for 10 min and the supernatant carefully separated. HDL-C was measured using a homogenous method (12), the Cholestest N HDL kit (Daiichi Pure Chemicals, Kyoto, Japan) with a Hitachi autoanalyzer (Hitachi, Tokyo, Japan) according to the manufacturer's instructions. Cholestest N calibrator (Daiichi Pure Chemicals) was used to calibrate the assay necessary. Liquid Assayed Multiqual (BioRad, Irvine, CA, USA) was used for quality control to monitor the precision of the HDL-C procedure. The quality control value for HDL-C for Level 2 was 1.39 ± 0.15 mmol/L, and for Level 3, 1.97 ± 0.15 mmol/L.

Detection of rare alleles of *ABCA1*

Direct sequencing of all exons in 50 subjects in the high and 50 subjects in the low HDL-C groups As phenotype-associated rare alleles are more likely to be harboured in extreme phenotypes, we first explored rare alleles in the low and high HDL-C groups. Fifty randomly selected subjects (31 males and 19 females, age 55 ± 18 years) in the low HDL-C group and 50 subjects (28 males and 22 females, age 46 ± 19 years) in the high HDL-C group were first screened for *ABCA1* variation. Genomic DNA was extracted from peripheral blood cells with Tiangen DNA blood Mini kits (Tiangen, Beijing, China) according to the manufacturer's instructions. Primers were designed for all the exons and intron-exon boundaries, with 100–200 bp extensions into intronic regions. PCR products were sequenced in both directions using the ABI Sequence Analyzer $3730 \times L$ (Applied Biosystems, Foster City, CA, USA). Results were compared with sequences retrieved from the UCSC Genome Browser (http://genome.ucsc.edu). Standard nomenclature, as described by den Dunnen and Antonarakis (www.hgvs.org/mutnomen/) (13), was used for description of DNA sequence variation, with +1 corresponding to the A of the ATG translation initiation codon of the GenBank mRNA sequence NM_005502.2. Genomic DNA (gDNA) numbering is also provided with the first nucleotide of GenBank reference sequence NG_007981 as +1.

TaqMan-based genotyping assays in the rest of the assembly Six rare non-synonymous variants, not shared in both groups, were identified using direct sequencing as described above, using high and low HDL-C groups. The variants were then tested for in the remaining individuals with extreme HDL-C (high and low groups) using the TaqMan-based genotyping assay with the ABI 7500 real-time PCR System (Applied Biosystems). In order to explore the phenotypic effects and further confirm the rarity of these alleles, we also tested for these same alleles in the subjects with HDL-C in the middle-range. Primer Express 2.0 (Applied Biosystems) was used to design primers and probes (listed in Table 1). Premix Ex TaqTM kit (TaKaRa, Otsu, Shiga, Japan) was used in the genotyping assay. The reaction mix contained 10 μL premix, 10 μM primers, 10 μM probes, 0.4 μL ROX reference dye, and 10 ng DNA template, in a total volume of 20 μL. Amplification was carried out with one 10 s cycle at 95°C, 50, 5 s cycles at 95°C and maintenance at 60°C for 34 s. The SDS v1.2× System Software (Applied Biosystems) was used to analyse the results. Sample genotypes confirmed by direct sequencing were used as positive controls and samples with no DNA as negative controls in each plate.

Cross-species alignment of *ABCA1* cDNA and protein sequences

The CLUSTAL X (1.81) program (http://www.clustal.org/) was used to compare the human *ABCA1* cDNA sequence (NM_005502) with nucleotide sequences of the mouse (*Mus musculus*) (NM_013454.3), rat (*Rattus norvegicus*) (NM_178095.2) and chicken (*Gallus gallus*) (NM_204145.1). The same program was used to compare amino acid sequences between these species (human UniProtKB/Swiss-Prot O95477; mouse P41233; rat Q80ZB2; and chicken Q8UVV4).

Table 1 Primers and probes used in the TaqMan-based genotyping assay.

Variations	Substitutions	Primer/probe	Sequence 5′→3′
p.Arg496Trp	c.1486C>T	Primers-F/R	TCCAGTCCAGTAATGGTTCTGTGTA/CCTGTGTTAGGCTTGAGGGATAGT
		Probe-C	FAM-CTAACCAGGCAATCCGGACCATATCTC-TAMRA
		Probe-T	HEX-CTAACCAGGCAATCTGGACCATATCTCG-TAMRA
p.Asp677Glu	c.2031C>A	Primers-F/R	TCACAAGAAGAGGAATGAGGCTACT/CTTTCTCCCCTTGTCCTTCCTT
		Probe-C	FAM-ATGCTGTTGTCCAGGCCCATGAT-TAMRA
		Probe-A	HEX-ATGCTGTTTTCCAGGCCCATGATC-TAMRA
p.Cys887Phe	c.2660G>T	Primers-F/R	GTGCTTTCTGTGGGTTCATTTCT/ACCTTCATCCCATCTCGGTAGA
		Probe-G	FAM-TCCTGTCACAGTCTGCATGGAGGAG-TAMRA
		Probe-T	HEX-CCTGTCACAGTCTTCATGGAGGAGGA-TAMRA
p.Ala1010Val	c.3029C>T	Primers-F/R	TTTTGCTTTTCAGCTTGCTTGA/AGGCTGACTGTCGAAGAACACAT
		Probe-C	FAM-TCCATCTCCGCCTTCACGTGC-TAMRA
		Probe-T	HEX-CTGCTCCATCTCCACCTTCACGTG-TAMRA
p.Val1096Ile	c.3286G>A	Primers-F/R	GAGGAGCCCACACAGCACA/TCCAGTGCTTACCCCTGCTAAT
		Probe-G	FAM-CCCCAGGACGTCCGCTTCATC-TAMRA
		Probe-A	HEX-CCCAGGATGTCCGCTTCATCCA-TAMRA
p.Asn1800Ser	c.5399A>G	Primers-F/R	ATGAATGACACCCGTTTCTTCTC/ATTGCCTGGTTTTTCACCATGT
		Probe-A	FAM-AATAATATCAATGATATCCTGAAGT-MGB
		Probe-G	HEX-ATAATATCAGTGATATCCTGAAG-MGB

Topological model of wild-type and mutant proteins

HMMTOP (14), TMHMM (15), TMpred (16), Tmap (17), DAS (18), Phobius (19), TOPpred (20) and SOSUI (21) softwares were used to model the transmembrane topology of the wild-type and mutant CERP proteins found to be exclusively associated with extreme HDL-C concentrations.

Results

HDL-C levels

A total of 335 subjects with extremely low HDL-C, 470 with extremely high HDL-C concentrations and 220 with middle-range HDL-C were recruited. Details of HDL-C concentrations, gender and age of the subjects are shown in Table 2.

Rare alleles identified and their association with HDL-C levels

Results of direct sequencing in the 50 high and 50 low HDL-C subjects A total of 21 variants were discovered using direct sequencing of the randomly selected subjects in the two groups with extreme HDL-C concentrations (Table 3), 17 coding and four non-coding. Among the coding variants, 11 were non-synonymous and six synonymous with respect to amino acid sequence; five were novel. Six of the 11 non-synonymous variants were identified exclusively in either the low or the high HDL-C groups, the other five were shared by these two groups (Table 3). The six exclusive alleles were rare in the subjects who were sequenced (Table 3).

Results of the TaqMan-based genotyping assays and comparisons between the different HDL-C groups Genotyping of the six exclusive alleles in the remaining subjects comprising the groups with high and low HDL-C, and the subjects with HDL-C in the middle range (middle-range HDL-C group), further confirmed the rarity of the six alleles (Table 4). The c.1486C>T (p.Arg496Trp) allele initially identified in the low HDL-C group only, was also found in the middle HDL-C group. Allele c.3286G>A (p.Val1096Ile), initially identified only in the high HDL-C group, was alsofj found in the middle HDL-C group. Therefore, the alleles associated exclusively with high HDL-C were c.2031C>A (p.Asp677Glu) and c.2660G>T (p.Cys887Phe). The alleles associated exclusively with low HDL-C were c.3029C>T (p.Ala1010Val) and c.5399A>G (p.Asn1800Ser) (Table 4, Figure 1). These four exclusive alleles are all novel (Table 4).

Across-species comparisons

Alleles c.2031C>A (p.Asp677Glu), c.2660G>T (p.Cys887Phe) and c.5399A>G (p.Asn1800Ser) occurred in the highly conserved nucleotide and

Table 2 Plasma concentrations of HDL-C, gender and age of the study population.

	Low HDL-C		High HDL-C		Middle-range HDL-C	
	Male	Female	Male	Female	Male	Female
HDL-C, mmol/L	0.60±0.17	0.72±0.18	2.25±0.26	2.39±0.23	1.39±0.14	1.64±0.15
Age, years	53±18	52±19	47±16	54±14	51±15	47±12
Number	192	143	290	180	91	129
Total number	335		470		220	

Table 3 Variants of *ABCA1* identified by direct sequencing.

Gene region	Nucleotide change	Amino acid	References or dbSNP ID	No. of subjects			
				Low (n=50)		High (n=50)	
				H[a]	Q[b]	H[a]	Q[b]
Untranslated exonic region							
Exon 2	g.24399_24400insG	–	rs1799777	11	0	10	0
Exon 2	g.24459G>C	–	rs1800978	11	0	10	0
Exon 50	g.146152A>G	–	rs4149341	27	2	24	4
Exon 50	g.146738_146740delGTT	–	rs41474449	23	0	20	0
Translated exonic region							
Non-synonymous							
Exon 7	c.656G>A	p.Arg219Lys	rs2230806	24	7	39	1
Exon 12	**c.1486C>T**	**p.Arg496Trp**	**Ref.** (5)	**1**	**0**	**0**	**0**
Exon 15	**c.2031C>A**	**p.Asp677Glu**	**Novel**	**0**	**0**	**1**	**0**
Exon 16	c.2311G>A	p.Val771Met	rs2066718	5	0	2	0
Exon 17	c.2473G>A	p.Val825Ile	rs2066715	25	7	37	4
Exon 18	c.2649G>A	p.Met883Ile	rs2066714	19	7	19	3
Exon 19	**c.2660G>T**	**p.Cys887Phe**	**Novel**	**0**	**0**	**1**	**0**
Exon 21	**c.3029C>T**	**p.Ala1010Val**	**Novel**	**1**	**0**	**0**	**0**
Exon 23	**c.3286G>A**	**p.Val1096Ile**	**rs13306073**	**0**	**0**	**1**	**0**
Exon 35	c.4760G>A	p.Arg1587Lys	rs2230808	24	6	31	4
Exon 40	**c.5399A>G**	**p.Asn1800Ser**	**Novel**	**1**	**0**	**0**	**0**
Synonymous							
Exon 6	c.474G>A	p.Leu158	rs2230805	13	16	13	21
Exon 9	c.936C>T	p.Pro312	rs2274873	1	0	6	0
Exon 9	c.948G>A	p.Gly316	rs2246841	3	0	2	0
Exon 15	c.2040A>C	p.Ile680	rs2853579	19	7	20	3
Exon 16	c.2148C>T	p.Ser716	Novel	0	0	1	0
Exon 31	c.4281G>A	p.Thr1427	rs2066716	22	8	22	7

[a]H, heterozygote of the minor alleles; [b]Q, homozygote of the minor allele. Rare variants associated with extreme HDL-C concentrations are indicated in bold font.

amino acid sequences in all four species studied. Nucleotide C in c.3029C>T (p.Ala1010Val) was conserved in humans, mice and rats, but not in chickens (Figure 2).

Transmembrane topology of wild-type and mutant proteins

Gene *ABCA1* contains 50 exons, translation of CERP being initiated in exon 2, terminating in exon 50 (http://genome.ucsc.edu). The wild-type protein has two membrane-integral domains, each containing six membrane-spanning α-helices and two ATP-hydrolysing domains (22). Transmembrane topology simulation showed no predictable differences between wild-type and mutant proteins. However, all the exclusively-associated alleles were located in the functionally important regions, implying potential modes of action of these alleles. These are p.Asp677Glu in the first intracellular loop; p.Cys887Phe in the seventh

Table 4 Rare alleles identified in the whole study population.

Allele	HDL-C, mmol/L (Male/female)	No. of subjects[a]		Domain
		In this group	In the whole assembly (n=1025)	
Low HDL-C group		n=335		
c.3029C>T (p.Ala1010Val)	0.74/–[b]	1	1	1st ABC[c]
c.5399A>G (p.Asn1800Ser)	–[b]/0.74	1	1	5th intracellular loop
High HDL-C group		n=470		
c.2031C>A (p.Asp677Glu)	–[b]/2.13	1	1	1st intracellular loop
c.2660G>T (p.Cys887Phe)	2.09/2.27	2	2	H7[d]
Low HDL-C group+middle group		n=555		
c.1486C>T (p.Arg496Trp)	–[b]/1.03±0.79	2	2	Extracellular *N*-term loop
High HDL-C group+middle group		n=690		
c.3286G>A (p.Val1096Ile)	2.14±0.06/2.23±0.21	6	6	1st ABC[c]
Other combinations of HDL-C groups[e]				
No rare variation				

[a]All subjects were heterozygotes of the minor allele; [b]no rare variation was found with respect to gender of the subject; [c]ABC, ATP-hydrolysing domain; [d]H7, the 7th hydrophobic segment; [e]other combinations of HDL-C groups includes the ''high''+''low'' and ''high''+''low+middle'' groups.

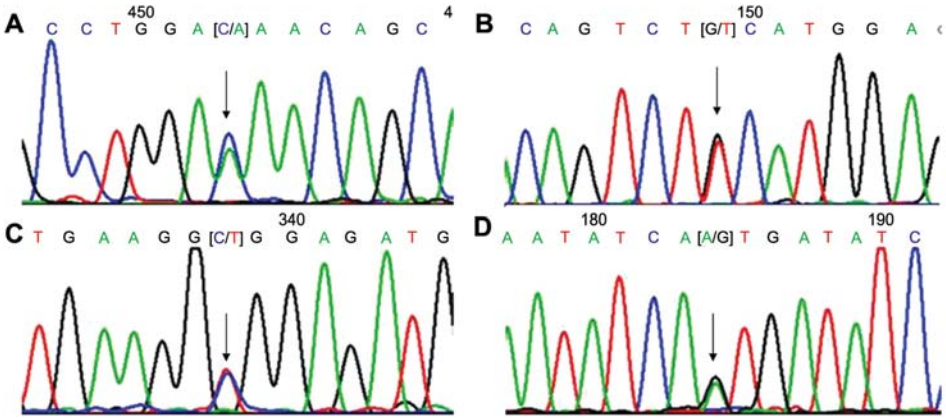

Figure 1 Partial sequencing results of the forward strand of the four rare alleles.
Arrows denote the alleles. (A) c.2031C>A; (B) c.2660G>T; (C) c.3029C>T; (D) c.5399A>G.

hydrophobic segment; p.Ala1010Val in the first ATP-hydrolysing domain, known to be necessary for transporter activity (23), and p.Asn1800Ser in the fifth intracellular loop.

Discussion

The *ABCA1* gene belongs to the large *ABC* membrane transporter superfamily. The encoded proteins transport various molecules, including metabolites, cholesterol, steroids and drugs across intra- and extra-cellular membranes. In humans, there are 49 paralogues of the superfamily, and 13 of these have been identified as being responsible for Mendelian diseases, including cystic fibrosis (MIM 219700), Startgardt disease (MIM 600110), Dubin-Johnson syndrome (MIM 237500), progressive familial intrahepatic cholestasis-3 (MIM 602347), X-linked sideroblastic anaemia and ataxia (MIM 301310). The *ABCA1* encoded protein, CERP, modulates cholesterol metabolism by mediating the ''reverse transport'' of cholesterol by pumping intracellular cholesterol to the plasma membrane and its subsequent incorporation into plasma HDL particles. The liver is the major organ where cholesterol is degraded and secreted. *ABCA1*, therefore, plays a crucial role in regulating plasma cholesterol concentrations. This has been clearly demonstrated in Tangier disease (MIM 205400) and familial HDL deficiency (MIM 604091). The classical phenotypes of Tangier disease are the near absence of HDL-C in plasma and clinical symptoms caused by intracellular cholesterol accumulation including enlarged yellow tonsils, splenohepatomegaly, and peripheral neuropathy (24). In familial HDL deficiency, plasma HDL-C is greatly reduced, accompanied by a higher incidence of premature coronary heart disease and atherosclerosis (25).

Plasma HDL-C concentrations are determined by both genetic and environmental factors. Inheritance is generally considered to be polygenic in the majority of subjects, but, as stated above, there are also Mendelian conditions associated with extreme HDL-C concentrations like Tangier disease and familial HDL deficiency. Cohen and colleagues demonstrated that rare alleles of *ABCA1* are associated with extreme HDL-C concentrations in Caucasians and African Americans in the US (5). This was confirmed by a large population-based study of subjects from Denmark that showed that both rare alleles and common variants also contribute to HDL-C concentrations in the general population (6). These and other recent studies (5–10) demonstrate that rare alleles not only contribute to common diseases, but can also exert stronger phenotypic effects than common variants. To prioritise our efforts on the strong phenotype-bearing alleles, the present study was restricted to identify rare alleles. Our results show that alleles of c.3029C>T (p.Ala1010Val) and c.5399A>G (p.Asn1800Ser) are exclusively associated with low HDL-C, c.2031C>A (p.Asp677Glu) and c.2660G>T (p.Cys887Phe) with high HDL-C in Han Chinese. These alleles were rare, but the frequencies obtained from the whole assembly may not be representative of true population frequencies. This is because there were more subjects tested in the extreme HDL-C groups than in the middle-range group, and the ratio of males and females was not equivalent. The three common non-synonymous variants reported by Wang et al. (11) were also detected in our subjects, but none was associated exclusively with extreme HDL-C concentrations. These results not only support the ''rare-variant common-disease'' hypothesis, but also demonstrate that rare alleles can have strong phenotypic effects in different populations. The four extreme phenotype-associated alleles are novel. More importantly from a clinical point of view, our results provide a novel tool for identifying individuals at high risk, with the potential for early intervention. In the present study, allele c.1486C>T (p.Arg496Trp), previously shown to be associated with high HDL-C in American subjects, was identified in Han Chinese subjects from the groups with extremely low- and middle-range HDL-C concentrations. The reason for this discrepancy is currently unknown and further studies are needed to address this question.

The *ABCA1* gene is located on 9q31 and consists of 50 exons. Its protein product is translated from within

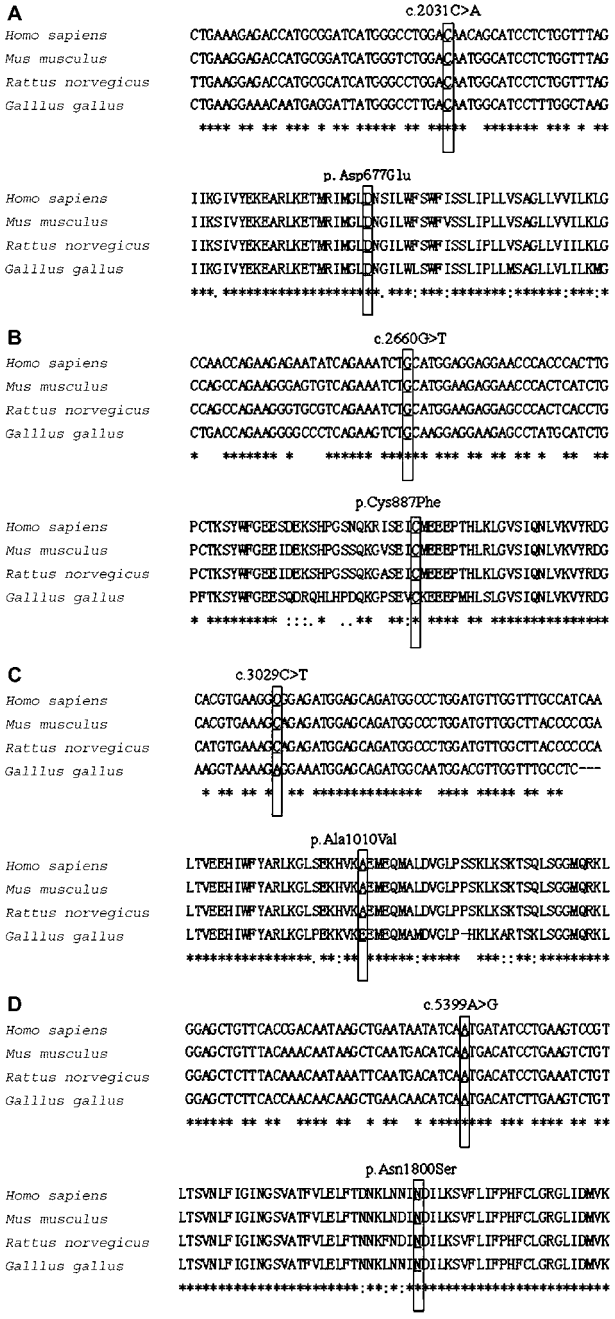

Figure 2 Multiple sequence alignment of *ABCA1* across species.
The CLUSTAL X (1.81) program was used to align the cDNA and protein sequences between species. The nucleotides and amino acids corresponding to the four rare variants are framed. (A) c.2031C>A (p.Asp677Glu); (B) c.2660G>T (p.Cys887Phe); (C) c.3029C>T (p.Ala1010Val); (D) c.5399A>G (p.Asn1800Ser). Identical bases and amino acids in the four species are denoted with asterisks.

exon 2 and terminates in the part of exon 50 that consists of 2261 amino acids (http://www.uniprot.org/uniprot/). Topological models of wild-type and mutant proteins show that all the extreme phenotype-associated mutant sites occur in functionally important regions. None occurred in the transmembrane section, consistent with previous findings in a Danish population (6). Rare alleles are important, although the present study demonstrates that not all rare alleles have significant phenotypic effects. In the present study, alleles c.1486C>T (p.Arg496Trp) and c.3286G>A (p.Val1096Ile) were not associated with any phenotypic changes. Some rare alleles, even coding variations, may therefore be neutral, as also found in the Danish population (6).

Plasma HDL-C concentrations and studies of the genetic contributions underlie that it serves as a good example to show that most clinical conditions can be caused by mutations in more than one gene, as well as by a whole spectrum of frequencies of alleles that may be common or rare within each gene. Some rare alleles are more often associated with extreme phenotypes. Re-sequencing and deep-sequencing efforts are therefore important for revealing these rare but significant alleles. This is important not only for identification of individuals at high risk who may benefit from early intervention, but also for our basic understanding of mechanisms of disease.

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