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Ala519Thr Mutation in Exon 11 of *LDL Receptor* Gene in Members of a Malaysian Family with Hypercholesterolaemia

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Abstract. We report a case of a Familial Hypercholesterolaemic (FH) patient (FH1) and her family members. They are Malaysian of Indian origin with evidence of consanguinity in the parents. We characterised the *LDL receptor* gene mutation in FH1, her brother (S1) and their mother, P2. The father (P1) died of coronary heart disease (CHD) in his early 40's. Our investigation reveals that FH1 and her family do not carry the two of the major known *Familial Defective ApoB* mutations, Arg3500Gln and Arg3531Cys. Sequencing analysis of the LDL receptor gene demonstrated that FH1 is homozygous for a G to A substitution at nucleotide position 1618, which causes the amino acid to change from alanine to threonine at position 519 (A519T). Both the mother and the eldest brother (S1) of FH1 are heterozygous for the A519T mutation. The A519T mutation had been previously reported in Western ethnicity of the United Kingdom, German and Icelandic origin but this is the first to be identified in the Asian region.

Keywords. Familial Hypercholesterolaemia, LDL Receptor, RT-PCR, Gene sequencing

INTRODUCTION

Mutations in the low-density lipoprotein (LDL) receptor gene on chromosome 19 result in an autosomal dominant disorder, familial hypercholesterolaemia (FH) (Brown *et al.,* 1986). This disorder of defective LDL clearance gives rise to increased plasma cholesterol 2 to 5 times higher than normal and an increased risk of premature coronary heart disease. The genetic mutation in this inherited disease may occur in one (heterozygous FH) or both (homozygous FH) LDL receptor alleles with frequencies of about 1 in 500 and 1 in a million respectively (Bertolini *et al.,* 1992). Despite conventional diet and drug therapy, most FH heterozygotes acquire CHD by the age of 35 while FH homozygotes normally die of myocardial infarction (MI) within their first 2 decades of life (Brown *et al.,* 1986; Hobbs *et al.*, 1990). Early detection of such patients is therefore necessary in order to delay the onset of clinical symptoms.

Familial defective apoB (FDB), another inherited disease that causes hypercholesterolaemia and premature development of CHD (Goldstein *et al*, 1983), is clinically indistinguishable from FH. Genetically, it is caused by mutation(s) in the *apo B-100* gene, specifically in the 3' end that is not present in the *apo B-48* gene. Two major mutations in the *apo B* gene have been associated with FDB: i) Arg3500Gln (Gaffney *et al.*, 1995); and ii) Arg3531Cys (Pullinger *et al.*, 1995) that can both be detected by PCR assays of the genomic DNA. A variety of different mutations in the *LDL receptor* gene that result in FH have been characterized that include gross deletions, major gene rearrangements, small deletions and point mutations. A database of *LDL receptor* gene mutations (http://www.ucl.ac.uk/fh/genebook.html) indicates that there are over 600 different mutations characterized worldwide demonstrating a high degree of allelic heterogeneity at this locus (Varret *et al.*, 1997). The majority of the LDL receptor mutations characterised are among the European, American and Afrikaner populations. In this study, we attempted to characterise the *LDL receptor* gene mutation(s) in a Malaysian familial hypercholesterolaemic patient and her family members.

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MATERIALS AND METHODS

Patient and family. The Paediatrics Clinic at University Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia had identified and clinically diagnosed a homozygous FH patient (FH1) based on (i) high total cholesterol (TC) level of 14.6 mmol/L, (ii) high plasma LDL level of 13.44 mmol/L, (iii) the presence of tendon xanthomas on both knees and elbows (iv) family history of coronary heart disease (CHD). The patient, FH1 is a 20 year-old Malaysian female of Asian-Indian origin who was 16 year-old at the time of diagnosis. FH1 initially presented with a concern about small lumps on both elbows and knees. These lumps were first detected as small nodules when she was 10 years old and grew bigger as she got older. Upon inspection, the lumps appeared to be tendon xanthomas (Figure 1a). Apart from her knees and elbows (Figure 1b), these xanthomas were also present on her Achilles' tendon on both legs (Figure1c). For her initial treatment, she received cholestyramine and atorvastatin that subsequently reduced her cholesterol level down to 9.0 mmol/L over a six-month period. There was evidence of consanguinity in the family whereby the mother of FH1 (P2) was married to her uncle (P1). P1 died of CHD in his early 40's. P2 was also hypercholesterolaemic with total cholesterol level of 7.1 mmol/L but did not have any xanthomas. FH1 had a brother (S1), aged 18 who was also hypercholesterolaemic with a total cholesterol level of 8.2 mmol/L. Her two other siblings, S2 and S3, aged 16 and 15 years old respectively, were normocholesterolaemic and did not show any clinical symptoms of FH.

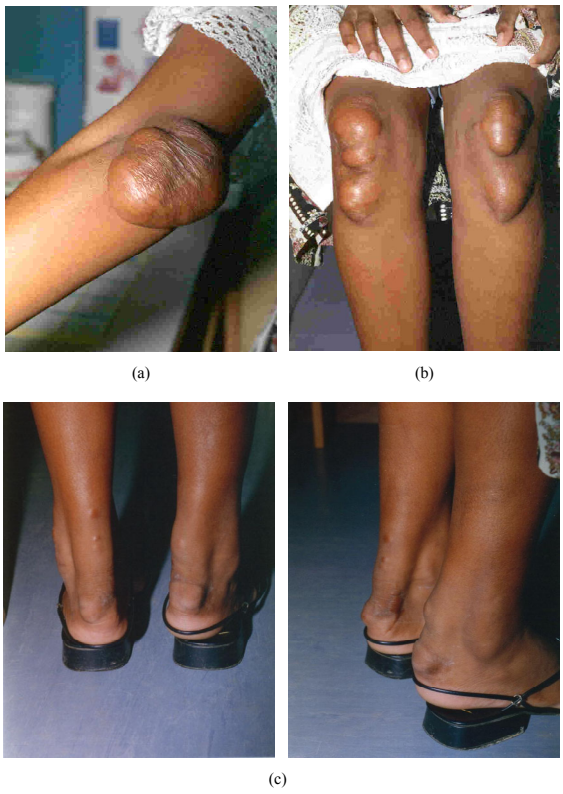
The purpose of the study was explained to the patient's mother, members of the family and other volunteers. Informed consent was obtained from all to use their DNA samples for this study.

RNA isolation. Peripheral blood mononuclear cells (PBMN) were isolated from fresh whole blood using Histopaque®-1077 (Sigma Chemicals Corporation Ltd) according to the manufacturer's instructions. Total cellular RNA (tcRNA) was isolated from PBMN using the Acid Guanidinium Thiocyanate Phenol Chloroform Extraction method as initially described by Chomczynski and Sacchi, 1987.

FDB analysis. Two different non-radioactive PCR reactions were performed on genomic DNA to exclude Familial Defective ApoB (FDB) as the cause for the clinical symptoms. Two known apoB mutations, FDB Arg3500Gln and Arg3531Cys were investigated by PCR-based methods as previously described by Gaffney *et al*, 1995 and Pullinger *et al*, 1995 respectively.

RT-PCR amplification of the cDNA for gene sequencing. Total cellular RNA (tcRNA) was reverse-transcribed to cDNA before the LDL-R cDNA could be PCR-amplified. Specific oligonucleotide primers were used to reverse transcribe LDL-R mRNA in two separate tubes. Exons 1 to 9 were reversed transcribed using primer RTa: 5' GCG ACC ACG TTC CTC AGG TTG GGG ATG AGG and exons 5 to 18, RTb: 5' ACT TCC TGG AGA GAA ATG GAG GTG T). TcRNA (1000ng) was made up to 10.2 µl with sterile distilled water in a thin-walled 0.5 ml microcentrifuge tube. The tubes containing RNA were heated at 70°C for 10 minutes. A mastermix (MM1) containing 1X first strand buffer (50 mM Tris-HCl pH 8.3 at 25°C, 75 mM KCl and 3 mM $MgCl_2$), 0.1 M DTT and 10 mM dNTP mix was prepared. After heating the RNA, the tubes were then quick-chilled in ice. RNasin (32 units) was added followed by 7 µl of the MM1 and 1 µl (1.25 µM) of the appropriate primer. Both the tubes were then heated at 42°C for 2 minutes before 200 units (1 µl) of SUPERSCRIPT™ II enzyme was added to the tube. The incubation for the reaction was carried out at 42°C for 50 minutes.

The two sets of cDNA were then PCR amplified in three overlapping segments using 3 sets of oligonucleotide primers: PCR1 (5'primer-220 – 5'AAC GAG TTC CAG TGC CAA GAC GGG AAA TGC ATC3' and 3'primer-225 – 5'TTG TAG CCA CCC TCC AGG TTC ACG CAG AGC TG3'); PCR2 (5'primer-224 – 5'GGA CCC AAC AAG TTC AAG



(a) (b) (c)

Figure 1. Pictures of subcutaneous tendon xanthomas in FH1. (a) A huge tendon xanthoma in the elbow. (b) Several xanthomas found in the knees. (c) Xanthomas of the Archilles tendon

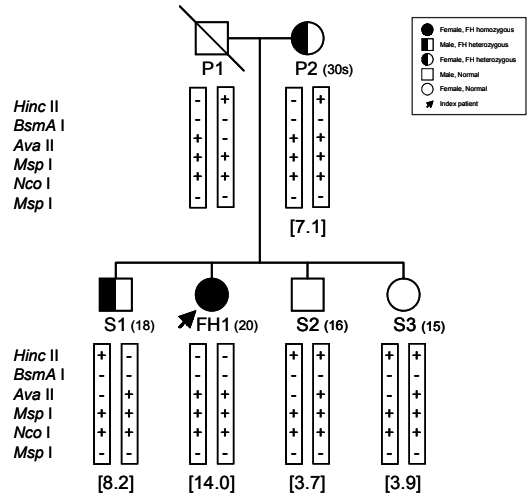


Figure 2. Family pedigrees of the index patient (FH1) and her family members, showing the haplotypes derived from the *BsmA* I site in exon 10, *Hinc* II in exon 12, *Ava* II in exon 13, *Msp* I in exon 15, *Nco* I in exon 18 and *Msp* I in exon 18 polymorphisms. The haplotype for P1 was predicted based on haplotypes observed from FH1, P2, S1, S2 and S3. The information for *BsmA* I and *Hinc* II were extracted from sequencing analysis. Also shown are their age and total cholesterol levels.
(+) denotes the presence of a restriction site and (-) denotes the absence of a restriction site. Numbers in brackets denote the age of individuals in years. Numbers in square brackets denote total cholesterol level measured in mmol/L.

TGT CAC AGC GGC3' and 3'primer-231 – 5'CCA TGG CTC AGG GTG GTC CTC TCA CAC CAG TT3'); PCR3 (5'primer-184 – 5'AAA CCT ACT GTC CCC AGA GGA TAT GG3' and 3'primer-183 – 5' GCA GGG GCG GGA CTC CAG GCA GA3').

PCR1, PCR2 and PCR3 amplify exons 2-8, 6-14 and 13-18 respectively. The cDNA from tube RTa was used in PCR1 while that from tube RTb was used in both PCR2 and PCR3. In a thin-walled PCR microcentrifuge Eppendorf tube, 5 µl of cDNA was mixed with 44.5 µl of mastermix (MM2) which contains 1X Fermentas PCR buffer (containing 20 mM ammonium sulfate, 75 mM Tris-HCl pH 8.8 at 25°C and 0.1% Tween 20), 1 mM $MgCl_2$, 200 µM dNTPs, 10% DMSO and 0.5 µM of each 5' and 3' primers. *Taq* DNA Polymerase (2.5 units) was added to each tube and the reaction mixture was overlaid with two drops (40 µl) of light mineral oil. The amplification was performed in an Eppendorf Thermocycler and the PCR program was optimised as follows: 35 cycles of 94°C for 1 minute for initial denaturation, 65°C for 1 minute for annealing of primers and 72°C for 3 minutes for extension of primers. The initial heating was adjusted to 94°C for 3 minutes. The PCR product was then purified using a QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instruction. After purification, DNA was quantified using a GeneQuant spectrophotometer.

Sequencing the PCR product/DNA. Sequencing of the LDL receptor gene was carried out using an automated ABI Prism Gene Sequencer (Model 377, Version 2.1.1) at AMCAL, University of Malaya, Kuala Lumpur. Seven overlapping fragments of the gene were sequenced using 3 forward (54, G and 77B) and 4 reverse (55, Fn, Jn and 183) primers. Primers 54 and 55 were used to sequence PCR1 product while primers G, 77B and Fn for PCR2 product and primers Jn and 183 for PCR3 product. The primer sequences are; 54: 5' CCC CAG CTG TGG GCC TGC GAC AA; G: 5' CCT GAG GAA CGT GGT CGC TCT; 77B: 5' CTA CTC GCT GGT GAC TGA; 55: 5' CAT CCG AGC CAT CTT CGC AGT C; Fn: 5' AGA CCA GTA GAT TCT ATT GCT G; Jn: 5' GGA GGT GTC GGG AAC AGG CCG GGT GG; 183: as above.

The sequenced fragments of LDL receptor gene were analyzed using Apple Macintosh SeqEd Software. Parallel visual comparison of the sequencing chromatography profiles was also made between FH1, P2, S1, a normal individual (N3) and the published LDL receptor gene sequence.

Analysis of the* Ava *II (exon 13),* Msp *I (exon 15 and 18) and* Nco *I (exon 18) polymorphisms. PCR amplification of the LDL receptor gene from genomic DNA was carried out using the following primers; SP78: 5'GTC ATC TTC CTT GCT GCC TGT TTA G and SP79: 5'GTT TCC ACA AGG AGG TTT CAA GGT T for exon 13; SP82 : 5' GAA GGG CCT GCA GGC ACG TGG CAC T and SP83: 5' GTG TGG TGG CGG GCC CAG TCT TTA C for exon 15; N1: 5' CAA TCT TGT CGT TGA TGG and N2: 5' CAAACG ATG CAG ACT GGA GG for exon 18. The PCR product was then digested with *Ava* II (exon 15), *Msp* I (exon 18), *Nco* I and *Msp* I (exon 18) restriction endonuclease following the manufacturer's instruction (New England Biolabs). The digested products were separated using 2% agarose mini-gel, pre-stained with EtBr.

RESULTS

Identification of the LDL receptor gene mutation in FH1. Two known Familial Defective ApoB mutations, Arg3500Gln and Arg3531Cys were not found in any of the family members (data not shown). Comparison of nucleotide sequencing results of the amplified RT-PCR product from FH1 with that of the published sequence and normal individual (N3) revealed a single base substitution at nucleotide position 1618 of the gene (Figure 3). This base substitution was found in the PCR2 product, sequenced with primer-G, which revealed nucleotide sequence from the middle of exon 9 to the end of exon 12. The substitution is from Guanine (G) to Adenine (A), resulting in a codon change from GCC to ACC, and an amino change at position 519 from alanine to threonine (A519T).

For FH1's mother, P2, the sequence generated at codon 1618 was found to be 50% G and 50% A, indicating

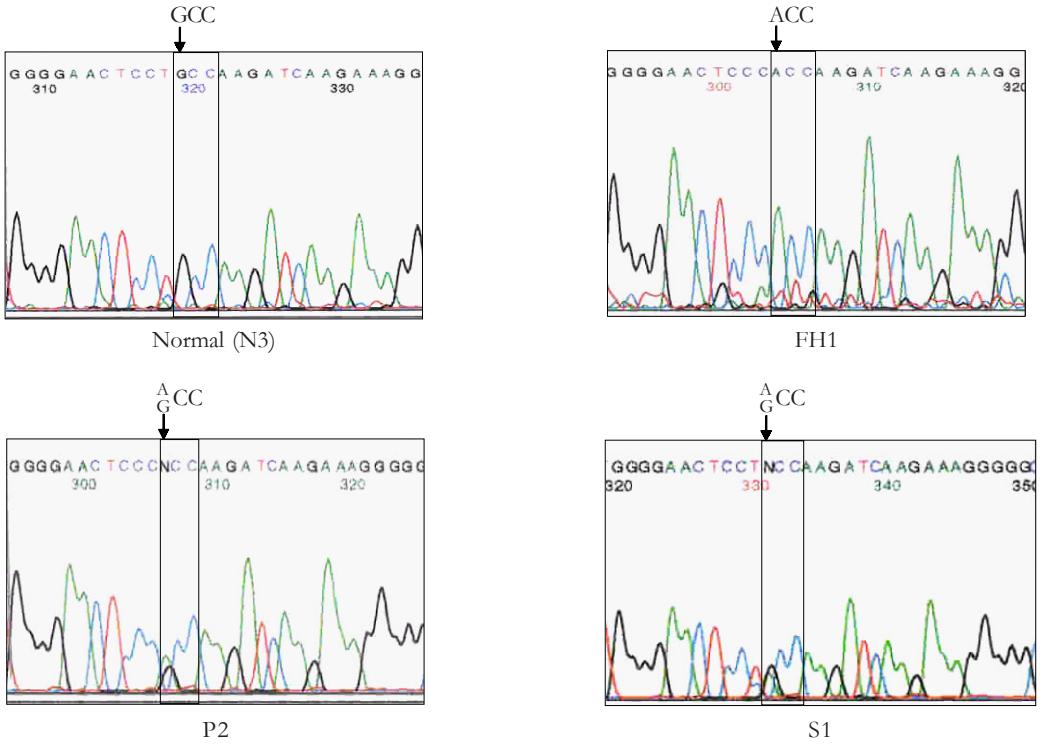


Figure 3. Nucleotide sequence of the coding strand of normal and mutant fragments of the LDL receptor mRNA of the PCR2 products sequenced with primer-G for N3, FH1, P2 and S1. The point of interest of each sample is indicated by the box. The nucleotide for N3 at position 1618 is G while that of FH1 sample is A. P2 and S1 chromatography profiles show twin-peak with 50% G and 50% A.

heterozygosity for the mutation (Figure 3). The sequencing profile of this particular mutation site in FH1's siblings, S1 when compared with the sequence in FH1 and P2 is shown in Figure 3. Similar to P2, analysis of the sequence at nucleotide position 1618 of the LDL receptor gene also showed two peaks that were 50% G and 50% A.

The variation in sequence seen at nucleotide 1617 in these subjects (C in FH1 and P2, but T>C in S1 and N3) is a reflection of the relatively rare exon 11 *Aci* I polymorphism, previously reported in the LDL receptor gene mutation database (http://www.ucl.ac.uk/fh/genebook.html., see also Heath and Humpries, 1999). Sequence inspection of RT-PCR products revealed the genotype of two further polymorphic sites in these individuals: for *BsmA* I site (exon 10): FH1 (-/-), P2 (-/-), S1 (-/-) and for *Hinc* II site (exon 12): FH1 (-/-), P2 (+/-), S1 (+/-). The base sequence at nucleotide 1617 and 1618 and these two polymorphic sites in individual family members indicates that FH1 is homozygous for A519T mutation having inherited the same allele from both parents. This conclusion was corroborated by investigating the genotype of a number of polymorphic sites in genomic DNA from family members by restriction analysis. Both FH1 and P2 were homozygous for the following sites: *Ava* II, exon 13; *Msp* I, exon 15; *Nco* I, exon 18; *Msp* I, exon 18, whereas siblings S1, S2 and S3 were all heterozygous for the *Ava* II site. Taken together with the sequence data on the *Aci* I site (Figure 3) this argues strongly that FH1 has inherited the same LDL receptor alleles from both parents. The haplotype of the mutant allele is summarized in Figure 2.

DISCUSSION

In this study, uncultured PBMN were used as the source for RNA. All subjects were shown not to carry the two known FDB mutations, Arg3500Gln and Arg3531Cys. Comparison of the nucleotide sequencing results of the amplified RT-PCR product from FH1 with that of P2, S1, N3 and the published sequences revealed the presence of a Guanine (G) to Adenine (A) substitution at position 1618 of the LDL receptor gene (Figures 3). This single base substitution (G1618A), results in a codon change from GCC to ACC and an amino acid change from Alanine (Ala) to Threonine (Thr) at position 519 (A519T). The G1618A mutation lies at

nucleotide position 23 in exon 11 of the gene, which is part of the second, EGF precursor domain of the LDL receptor. This domain is responsible for the acid-dependent dissociation of lipoproteins from the LDL-receptor in the endosome during receptor recycling to the membrane (Brown and Goldstein, 1986).

The A519T mutation is a Class V mutation of FH. Amino acid A519 lies in one of the YTWD repeat domains that form the central part of the EGF precursor domain. This residue is completely conserved amongst vertebrate LDL receptor and VLDL/vitellogenin receptors, but not within the corresponding YTWD domains of the related LRP family receptors (Mehta *et al.*, 1991; Webb *et al.*, 1994; Herz *et al.*, 1988). The crystal structure of the EGF-YTWD domain of LDL receptor has recently been solved and shows A519 positioned between ß-sheets 2 and 3 of blade 4 of the ß-propellor structure of the YTWD (Jeon *et al.*, 2001). Prediction of the effect of the A519T mutation on the structure and cycling of the receptor are unclear, but it can be postulated that the mutant A519T LDL receptors can be synthesized, they may be able to bind to LDL to form an LDL/LDL receptor complex, and the complex may be internalised or endocytosed into the cell. However, the mutation may lead to failure of dissociation of lipoproteins from the receptors in the acidic environment of the endosome, whereby the receptor fails to release the ligand. Consequently, the receptors are degraded within the cell without being recycled to the membrane. The essential event for the receptor-cycling therefore fails to take place. Alternatively, receptor structure may be sufficiently disrupted for it to be degraded during the secretory process so that it never reaches the cell surface. As a result, the number of receptors on the membrane is reduced and consequently, the ability to clear LDL from circulation is also reduced. Subsequently, both plasma TC and LDL-C levels increase and this will lead to atherosclerosis and finally CHD. Sun *et al.*, (1997) reported that a heterozygous FH with A519T mutation when compared to normal, had 42% of the normal LDL receptor activity and 47% of the LDL receptor protein in lymphoblast. If there were no effect of genetic background, one would predict that FH1, a homozygote for the A519T mutation would have very little LDL receptor activity or protein. However, treatment of FH1 with cholestyramine and atorvastatin reduced the LDL–cholesterol levels to similar extent to that achieved by combined statin/bile acid sequestrant treatment of the A519T heterozygote patient described by Sun *et al.*, 1998. This once again demonstrates the effectiveness of statin treatment in homozygous FH patients.

Analysis of the gene sequence from the mother of FH1 (P2) showed that at nucleotide position 1618, there is a 50% G and 50% A indicating that she is a heterozygous for the G1618A mutation; with one normal allele and one mutated allele. Therefore, it is predicted that whilst producing normal number of LDL receptors, half of them are defective, unable to be recycled to the surface and are degraded. This is consistent with the fact that she is already hypercholesterolaemic in her 30's (TC=7.1 mmol/L) because the LDL can only be cleared at half of the normal rate. FH1's father, P1, who died due to coronary heart disease in his early 40's, is suspected to be a heterozygote for the A519T mutation. Therefore, FH1 must have received defective alleles from each parent. Since there is consanguinity in the family, the A519T mutation must be carried through successive generations of the family.

Further analysis of the nucleotide sequence from the predicted heterozygous brother of FH1 (S1) with TC level of 8.2 mmol/L, also showed that at nucleotide position 1618 there is a 50% G and 50% A confirming that he is a heterozygous for the A519T mutation. S1 must have received a defective allele from one of the parents. FH1's two other siblings, S2 and S3 were normocholesterolaemic with TC=3.7 mmol/L and 3.9 mmol/L respectively (Figure 2), could have received normal alleles from both parents. Further investigation by identifying the presence of this A519T mutation in the rest of the FH1's siblings and distant family members would be beneficial, as this would encourage early prophylactic measures such as diet therapy before the onset of clinical symptoms that may lead to CHD. However, the majority of the paternal family members are living in India, hence the difficulty of obtaining blood samples for analysis.

According to the LDL receptor mutation database http://www.ucl.ac.uk/fh/mutab.html, the A519T mutation identified in FH1 has already been found in other Western ethnicities (United Kingdom, German and Icelandic). However, this is the first to be identified in an FH patient in Malaysia as well as in the Southeast Asia region. The haplotype deduced for this allele will allow assessment of its relationship to European alleles described.

CONCLUSIONS

We have identified the mutation in the LDL receptor gene as a single base substitution at position 1618 of the gene that results in amino acid change from Alanine to Threonine. It is postulated that the LDL receptors can be synthesized, they can bind to LDL to form LDL/LDL receptor complex, and the complex can be internalised into the cell. However, the mutation may lead to failure of dissociation of lipoproteins from the receptors in the endosome hence the receptors are degraded without being recycled to the membrane. As a result, the number of receptors on the membrane is reduced and the ability to clear LDL from the circulation will also be reduced. Consequently, both plasma TC and LDL-C levels increase and this will lead to atherosclerosis and finally CHD.

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LDL Receptor mutation database: http://www.ucl.ac.uk/fh/mutab.html