# DETECTING FAMILIAL DEFECTIVE APOLIPOPROTEIN B-100 R3500Q IN VIETNAMESE PATIENTS BY PCR-SEQUENCING

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**ABSTRACT**

*Familial defective apolipoprotein B-100 (FDB) is an autosomal codominant disorder associated with hypercholesterolemia, caused by mutations in and around codon 3500 of the Apolipoprotein (Apo) B gene, which encodes Apo B-100. The first mutation occurred in Arginine codons to be described, and the most characterized, is caused by a G→A transition at nucleotide 10,708 and results in the substitution of Arginine by Glutamine at codon 3500 (ApoB R3500Q). In this study, we have identified 27 R3500Q mutations in known FDB patients using PCR-Sequencing method. As the result, most of the patients carried heterozygous mutation R3500Q. PCR-Sequencing method that we have applied in this study proved consistent and so easily identified mutations correctly.*

***Keywords:*** *Apoliprotein B-100; familial defective; ApoB R3500Q.*

## 1. Introduction

Familial defective apolipoprotein B-100 (FDB) is an autosomal codominant disorder associated with hypercholesterolemia (Innerarity *et al*, 1990; Myant, 1993; Tybjærg-Hansen, Humphries, 1992), caused by mutations in and around codon 3500 of the Apolipoprotein (Apo) B gene, which encodes Apo B-100. This is the main protein of low-density lipoprotein (LDL) and is the ligand through which LDL binds to its receptor in the process of receptor-mediated endocytosis (Brown, Goldstein, 1986).

The mutations all occur in Arginine codons and result in an Apo B-100 molecule that exhibits defective binding to the LDL receptor, leading to impaired uptake of LDL into the cell and consequently, hypercholesterolemia. The first to be described, and the most characterized, is caused by a G→A transition at nucleotide 10,708 and results in the substitution of Arginine by Glutamine at codon 3500 (*ApoB* R3500Q) (Table 1 and references therein). The other two, both recent discoveries, are each caused by a C→T transition, one at nucleotide 10,800 and the other at nucleotide 10,707. These result, respectively, in the substitution of Arginine by Cysteine at codon 3531 (*ApoB* R3531C) (Table 1 and references therein) and Arginine by Tryptophan at codon 3500 (*ApoB* R3500W) (Table 1 and references therein). We selected total of 21 referent studies in database with period lasted until 2015 concerning in FDB and found out that *ApoB* gene point mutations related to hypercholesterolemia with 12 major categories, namely: A3426V, A3527A, E3405Q, L3350L, L3517L, R3500Q, R3500W, R3527Q, R3531C, T3540T,

T3552T, R50W (Futema *et al*, 2012; Choong *et al*, 1997; Fisher *et al*, 1999; Dedoussis *et al*, 2004; Friedl *et al*, 1991; García-García *et al*, 2001; Heath *et al*, 2001; Henderson *et al*, 1997; Horvath *et al*, 2001; Pullinger *et al*, 1995; Real *et al*, 2003; Tybjaerg-Hansen *et al*, 1998; Wang *et al*, 2005; Tai *et al*, 1998; Tai *et al*, 2001; Real *et al*, 2003; Futema *et al*, 2013; Marduel *et al*, 2010; Rabès *et al*, 2000; Thomas *et al*, 2013; Thiart *et al*, 2000), of which, only rare mutation R50W positioned at exon 3, all remained mutations positioned at exon 26. In detail, R3500Q mutation was announced at the most, accounting for 34.4% (Futema *et al*, 2012; Choong *et al*, 1997; Fisher *et al*, 1999; Dedoussis *et al*, 2004; Friedl *et al*, 1991; García-García *et al*, 2001; Heath *et al*, 2001; Henderson *et al*, 1997; Horvath *et al*, 2001; Pullinger *et al*, 1995; Real *et al*, 2003; Tybjaerg-Hansen *et al*, 1998; Wang *et al*, 2005; Tai *et al*, 1998; Tai *et al*, 2001; Real *et al*, 2003; Futema *et al*, 2013; Marduel *et al*, 2010; Rabès *et al*, 2000; Thomas *et al*, 2013; Thiart *et al*, 2000). The frequency of R3500Q was range from 0.02% to 57.14% (Futema *et al*, 2012; Choong *et al*, 1997; Fisher *et al*, 1999; Dedoussis *et al*, 2004; Friedl *et al*, 1991; García-García *et al*, 2001; Heath *et al*, 2001; Henderson *et al*, 1997; Horvath *et al*, 2001; Pullinger *et al*, 1995; Real *et al*, 2003; Tybjaerg-Hansen *et al*, 1998; Wang *et al*, 2005; Tai *et al*, 1998; Tai *et al*, 2001; Real *et al*, 2003; Futema *et al*, 2013; Marduel *et al*, 2010; Rabès *et al*, 2000; Thomas *et al*, 2013; Thiart *et al*, 2000). The detection of FDB was conducted from various sources such as whole blood, fibroblast, peripheral blood leukocyte, buccal, saliva, …, ect, in which, the predominant kind of sample was whole blood. For method detection, several specific methods, such as PCR-Sequencing, PCR-SSCP, PCR-RFLP, AS-PCR, etc…, were applied in detection FDB (Futema *et al*, 2012; Choong *et al*, 1997; Fisher *et al*, 1999; Dedoussis *et al*, 2004; Friedl *et al*, 1991; García-García *et al*, 2001; Heath *et al*, 2001; Henderson *et al*, 1997; Horvath *et al*, 2001; Pullinger *et al*, 1995; Real *et al*, 2003; Tybjaerg-Hansen *et al*, 1998; Wang *et al*, 2005; Tai *et al*, 1998; Tai *et al*, 2001; Real *et al*, 2003; Futema *et al*, 2013; Marduel *et al*, 2010; Rabès *et al*, 2000; Thomas *et al*, 2013; Thiart *et al*, 2000). Among them, PCR-Sequencing was the most common method for detection of FDB.

**Table 1. Categorize *ApoB* gene mutations from published studies**

| Name | Publication [n (%)] ∑ = 32 | References |
|---|---|---|
| A3426V | 1 (3,1%) | Futema *et al*, 2012 |
| A3527A | 1 (3,1%) | Choong *et al*, 1997 |
| E3405Q | 1 (3,1%) | Fisher *et al*, 1999 |
| L3350L | 1 (3,1%) | Fisher *et al*, 1999 |
| L3517L | 1 (3,1%) | Choong *et al*, 1997 |
| R3500Q | 11 (34,4%) | Fisher *et al*, 1999; Dedoussis *et al*, 2004; Friedl *et al*, 1991; García-García *et al*, 2001; Heath *et al*, 2001; Henderson *et al*, 1997; Horvath *et al*, 2001; Pullinger *et al*, 1995; Real *et al*, 2003; Tybjaerg-Hansen *et al*, 1998 ; Wang *et al*, 2005 |

| Name | Publication [n (%)] ∑ = 32 | References |
|---|---|---|
| R3500W | 4 (12,5%) | Choong et al, 1997; Fisher et al, 1999 ; Tai et al, 1998 ; Tai et al, 2001 |
| R3527Q | 4 (12,5%) | Futema *et al*, 2012; Real *et al*, 2003; Futema *et al*, 2013; Marduel *et al*, 2010 |
| R3531C | 5 (15,6%) | Heath *et al*, 2001; Henderson *et al*, 1997; Pullinger *et al*, 1995; Tybjaerg-Hansen *et al*, 1998; Rabès *et al*, 2000 |
| R50W | 1 (3,1%) | Thomas *et al*, 2013 |
| T3540T | 1 (3,1%) | Thiart *et al*, 2000 |
| T3552T | 1 (3,1%) | Thiart *et al*, 2000 |

We have presented the most significant results of the data mining. Through this step, obviously toward screening for familial defective apolipoprotein or for familial hypercholesterolemia, in general, for Vietnamese patients, the first approach is to focus survey are some hot-spots, such as *ApoB* gene R3500Q. Therefore, the aim at the present study was to analyze the presence of the most common caused FDB, R3500Q mutation, in Vietnamese patients by using PCR-sequencing method.

## 2. Materials and methods

### Primer designed

*ApoB* gene was collected from Genbank (NCBI) by accession number NC_000002.11. Subsequently, primers for PCR-Sequencing were designed by Primer3 version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/). Physical characteristics of primers were analyzed by OligoAnalyzer 3.1 (Integrated DNA Technologies, http://sg.idtdna.com/calc/analyzer), Annhyb (http://bioinformatics.org/annhyb/), and BLAST (NCBI) (blast.ncbi.nlm.nih.gov/Blast.cgi). SNPCheck3 was used to check SNPs of primer sequences.

### Samples collection, DNA extraction

32 blood samples were collected from unrelated hyperlipidemic patients, attending the lipid clinic of Xuyen A Hospital and Thu Duc Hospital, Vietnam. These patients had cholesterol concentrations >5.2 mmol/L (range: 5.33–17.46 mmol/L) without tendon xanthomas. The procedures followed were in accordance with the current revision of the Helsinki Declaration of 1975.

DNA was extracted from clinical sample by means of an enzyme digestion using 700 µl lysis buffer (NaCl 5M, Tris-HCl 1M, EDTA 0.5M, SDS 10% and Proteinase K 1 mg/ml). The samples were incubated at 56$^{o}$C overnight. Then, DNA obtained and purified by Phenol/Chloroform extraction and ethanol precipitation. The quality and purity of DNA extraction was measured by the proportion of $A_{260}/A_{280}$. Then, the DNA solution was stored at EDTA 0.5M, -20$^{o}$C for further used.

### Detection of R3500Q

R3500Q detection was carried out by PCR-Sequencing method. The forward (ABOP-F) and reverse primer (ABOP-R) sequences were
5’-GACCACAAGCTTAGCTTGG-3’,
5’-GGGTGGCTTTGCTTGTATG-3’,
respectively. The amplification was done in a total volume of 15 µl, containing 10 ng DNA template. PCR reaction was subjected to initial at 95$^{o}$C for 5 minutes, followed by 35 cycles at 95$^{o}$C for 30 seconds, 54$^{o}$C for 30 seconds, 72$^{o}$C for 30 seconds, and finally 72$^{o}$C for 10

minutes. PCR products were directly loaded onto a 2.0% agarose gel, stained with Ethidium bromide, and directly visualized under UV illumination. Then, PCR products were sent to Nam Khoa Biotect for sequencing.

## 3. Results and discussion

### Primer designed

Primer3.0 program was used to design the primer to amplify a partial of *ApoB* regions. According to table 2, primers' several physical characteristics such as length, %GC, melting temperature ($T_m$), ΔG were almost corresponded to standard parameters of primer designed, such as 50-65% GC, melting temperature (Tm) rising between 50 and 65°C, dimerization capability (ΔG) is in the range of -9 Kcal/mole – +9 Kcal/mole, except the value of self-dimer structure forming by APOB-F (-10.23 Kcal/mole). The target-specificity of chosen primer was accessed by BLAST, as the results, APOB-F and APOB-R were specific to *ApoB* gene region containing *ApoB* R3500Q (G/A) with the same E-value = 0.66, ident = 100%.

**Table 2. The physical characteristic of primers**

| Primer | Sequence | Length (bp) | GC (%) | Tm ($^oC$) | (1) | (2) | (3) | Product (bp) |
|---|---|---|---|---|---|---|---|---|
| **APOB-F** | GACCACAAGC TTAGCTTGG | 19 | 52,6 | 53.6 | -3.79 | -10.23 | -5.09 | 334 |
| **APOB-R** | GGGTGGCTTT GCTTGTATG | 19 | 52,6 | 54.3 | 0.1 | -3.14 | | |

*Note: (1) Free energy for hair-spin structure forming (Kcal/mole); (2) Free energy for self-dimer structure forming (Kcal/mole); (3) Free energy for heterodimer structure forming (Kcal/mole).*

SNPCheck3 was used to check SNP on the primer sequences. As the result, we did not detect any SNP on two designed primers (Data not shown), so the pairing between each primer on target gene sequences should be specific.

### PCR and Sequence analysis of the *ApoB* gene R3500Q

Total samples were enrolled in PCR for detection of R3500Q. The APOB forward and reverse primers yielded a PCR product of 334 bp as shown in table 2. As the results, the PCR products were observed by electrophoresis in correctly sizes and easily identified (Fig.1).

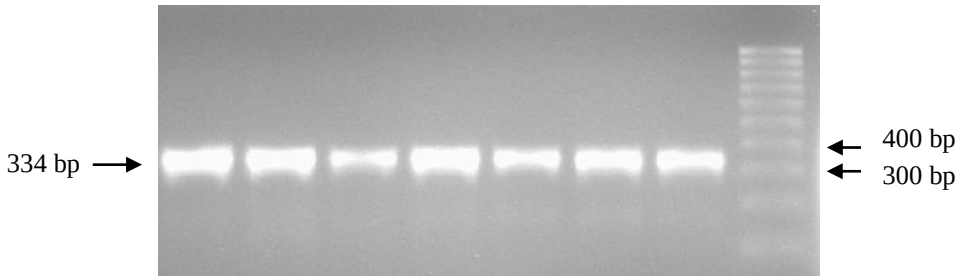


**Figure 1. Agarose gel electrophoresis of some representative samples.**

Consequently, PCR products was sequenced in order to detect R3500Q mutation. At first, the signal of peaks in PCR product sequencing was very good for reading nucleotide (Data not shown). Then, 32 double sequences were used to search for

the similarity by Blast. According to Blast results, all sequences were similar to *ApoB* gene sequences within Total score = 334, Ident = 100% and E-value < 2e-33 (Data not shown).

At position c10708, Genbank nucleotide sequence (NC00002.11) is G, while its location in the patient TD10 appeared two peaks, corresponding to two alleles, one allele sequence is G and another is A. So, TD10 patient carried R3500Q mutation (G→A transition), heterozygous (Fig. 2).

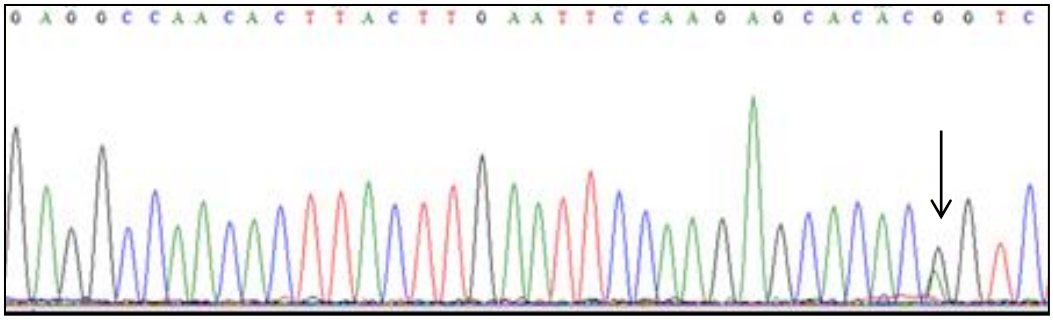

**Figure 2. DNA sequencing result of affected *ApoB* region at exon 26 showing heterozygous mutation R3500Q.**

Meanwhile, at the patient's location c10708, patient XA22 appeared only one peak corresponding to a sequence allele A. Thus patient XA22 carried R3500Q mutation, homozygous (Fig. 3).

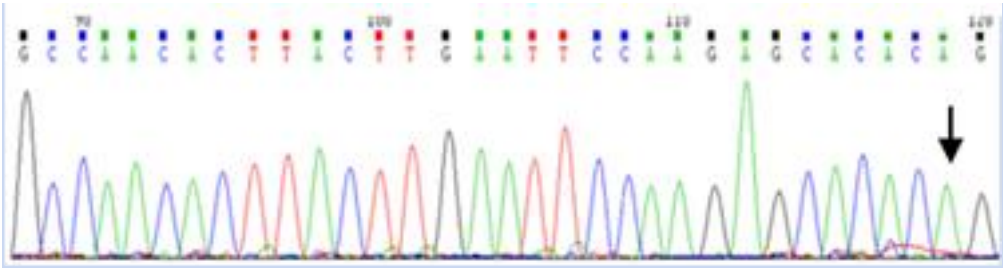

**Figure 3. DNA sequencing result of affected *ApoB* region at exon 26 showing homozygous mutation R3500Q.**

Off total 32 samples enrolled in PCR-Sequencing for detection of R3500Q, 27 patients were shown contain a G→A transition at nucleotide 10,708 and results in the substitution of Arginine by Glutamine at codon 3500 (*ApoB* R3500Q); i.e., five of them were heterozygous for *ApoB* R3500Q, whereas the remained were homozygous (Data not shown). All of the signal of peaks in PCR product sequencing was very good for reading nucleotides, especially at the transition positions (Data not shown). This result was surprising though the sample size was very small, but R3500Q mutation appeared too high, compared to the recorded worldwide, ranging from 0.02% to 57.14%. One possible reason is that the completely subjects were initially chosen as definitive FH patients. In addition, sequencing with a short PCR product as 334 bp can achieve high R3500Q mutation and therefore display better diagnostic performance. *ApoB* R3500Q was demonstrated as changing ApoB protein structure, completely broke the link between LDLR receptor with carrier cholesterol (LDLC) and therefore this is the cause of

familial defective apolipoprotein (FDB), consequently, accumulate of cholesterol in the blood which lead to cardiovascular disease risk (Hevonoja et al, 2000). The Familial defective apolipoprotein B-100 as well as familial hypercholesterolemia is increasing and more diversity in Viernamese population. It means that the risk of serious diseases related to high cholesterol such as heart stroke or other cardiovascular diseases tends to increasingly. Thus, this study will be expanded not only on large samples but also consider to other related genes such as *LDLR* or *PSK9*.

**4. Conclusion**

In summary, we have identified 27 R3500Q mutations in known FDB patients using PCR-Sequencing method. In which, most of patients carried heterozygous mutation R3500Q. PCR-Sequencing method that we have applied in this study proved consistent and so easily identified mutations correctly. With the sequencing cost dropping out, this method will be easy in clinical application for screening of risk FDB, on Vietnamese population in near future.

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