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ORIGINAL ARTICLE

ABSENCE OF APO B R3500Q MUTATION AMONG KELANTANESE MALAYS WITH HYPERLIPIDAEMIA

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Familial defective apolipoprotein B-100 (FDB) is an autosomal dominant genetic disorder associated with hypercholesterolaemia and premature coronary heart disease. FDB is caused by mutations in and around the codon 3500 of the apolipoprotein B (apo B) gene. Apo B R3500Q mutation is the first apo B mutation known to be associated with FDB and it is the most frequently reported apo B mutation in several different populations. The objective of the present study was to determine the association of apo B R3500Q mutation with elevated plasma cholesterol concentration in Kelantanese population in which both hypercholesterolaemia and coronary heart disease are common. Sixty-two Malay subjects with hyperlipidaemia, attending the lipid clinic at Hospital Universiti Sains Malaysia, Kelantan, were selected for this study. The DNA samples were analysed for the presence of apo B R3500Q mutation by polymerase chain reaction-based restriction fragment analysis method using mutagenic primers. This mutation was not detected in the subjects selected for this study. Apo B R3500Q mutation does not appear to be a common cause of hypercholesterolaemia in Kelantanese Malays.

Key words: *Familial defective apolipoprotein B-100, apo B mutations, hypercholesterolaemia*

Introduction

Low-density lipoprotein (LDL) particles are the major cholesterol transport lipoproteins in the plasma. The plasma cholesterol concentration is mainly regulated by the LDL receptor pathway in which LDL receptors mediate uptake and degradation of the LDL particles. Apolipoprotein B-100 (apo B-100) is the sole protein of the LDL particle and it acts as a ligand for the LDL receptor (1).

Familial defective apolipoprotein B-100 (FDB) is an autosomal dominant disorder associated with increased plasma cholesterol concentration (2) and may thus increase the risk of premature coronary heart disease. To date, three apo B mutations, apo B R3500Q, apo B R3531C and apo B R3500W that cause defective apo B, have been reported (3).

Apo B R3500Q mutation is the first reported apo B mutation associated with FDB. This mutation is caused by a G to A transition at nucleotide 10708 in exon 26 of the apo B gene resulting in the substitution of arginine by glutamine at codon 3500 of the apo B and reduced affinity of apo B for the LDL receptor (4). The estimated heterozygous frequency of FDB based on apo B R3500Q mutation in general population is 1 in 500 (5).

Identification of underlying specific mutations in primary hyperlipidaemia cases is required for the detection of carriers in the families as early intervention may prevent the development of premature coronary heart disease. In this study, we determined the association of apo B R3500Q mutation with elevated plasma cholesterol

Figure 1.A. Diagram showing the DNA sequence of part of the apo B gene in normal allele and the position of 5' primer: Msp I *recognition sequence is created in codon 3500 of normal allele when a mismatched base is introduced at nucleotide 10706 using mutagenic 5' PCR primer.*

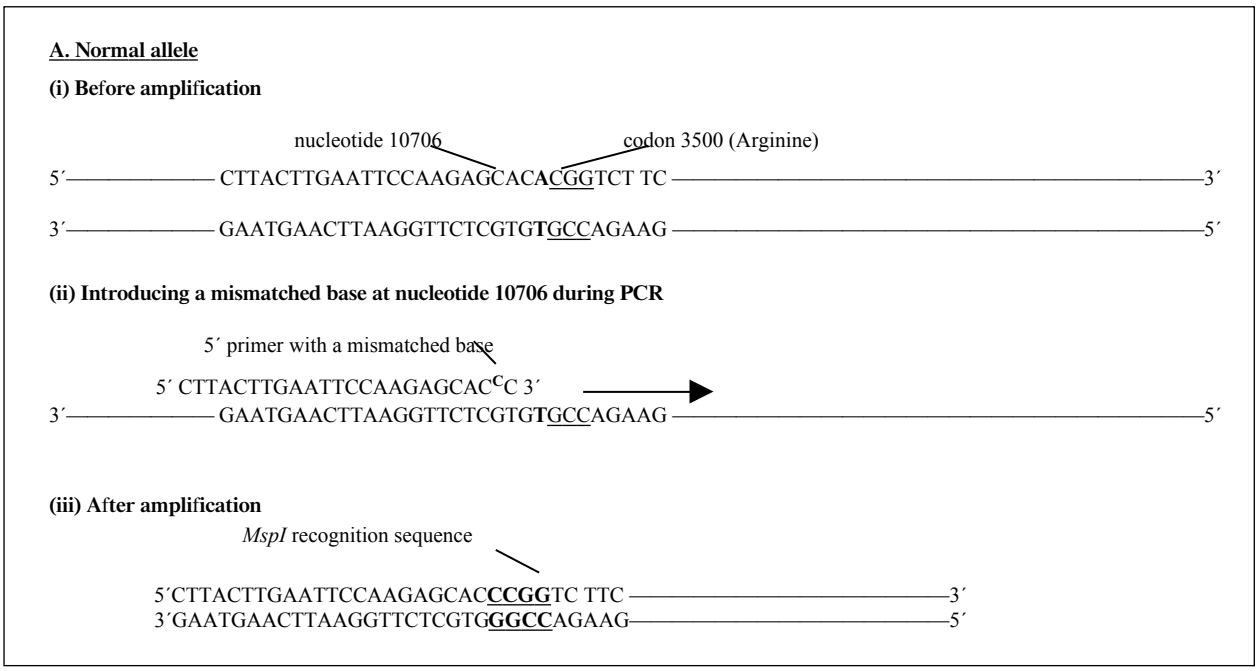
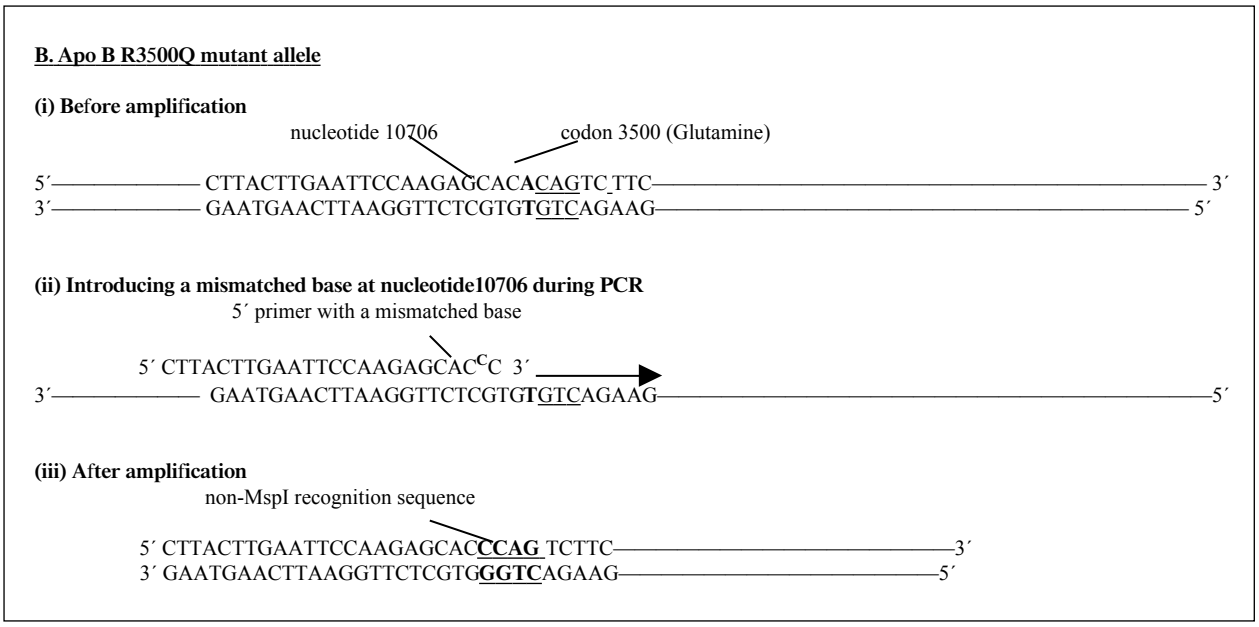


Figure 1.B. Diagram showing the DNA sequence of part of the apo B gene in apo B R3500Q mutant allele and the position of 5' primer: Msp I *recognition sequence is not created in codon 3500 of mutant allele when a mismatched base is introduced at nucleotide 10706 because of G to A transition at nucleotide 10708.*



concentration in Malay subjects attending the lipid clinic at Hospital Universiti Sains Malaysia, Kelantan.

When a point mutation creates or abolishes the restriction enzyme recognition sequence in DNA, it can be detected by polymerase chain reaction (PCR) amplification of the DNA fragments containing target sequence, digestion of the PCR products with restriction enzyme and analysis of the restriction fragments by electrophoresis. If a mutation does not alter any restriction enzyme recognition site, it can be created by PCR, using mutagenic primer with a single-base mismatch, introducing a novel restriction site in the normal allele or in the mutant allele (6). Apo B R3500Q mutation does not create or abolish any restriction enzyme site, however, a recognition sequence for restriction enzyme *Msp I* can be introduced in codon 3500 of the normal allele using mutagenic 5' PCR primer, but not in apo B R3500Q mutant allele (7).

Materials and methods

A total of 62 Malay subjects with hyperlipidaemia, attending the lipid clinic at Hospital Universiti Sains Malaysia, Kelantan, were selected for this study. The study group consisted of 38 males and 24 females, age ranging from 29 to 70 years. Clinical data were obtained from the medical records and subjects were selected on the basis of a plasma total cholesterol pre-treatment concentration of >6.2 mmol/L and individuals with secondary hyperlipidaemia were not included in this study. Their plasma total cholesterol concentrations ranged from 6.31 to 12.35 mmol/L (mean ± SD = 8.02 ± 1.47). Among these subjects, 43 had hypercholesterolaemia alone (plasma triglycerides <2.3 mmol/L) and 19 had mixed hyperlipidaemia (plasma triglycerides ≥2.3 mmol/L). Thirty subjects were classified clinically as familial hypercholesterolaemia (FH) (25 definitive FH and 5 possible FH) based on the criteria of the Simon Broome Register Group (8) and they were not excluded from the study because FDB may present clinically as FH (9).

After an overnight fast (12 hours), 5 ml of venous blood was collected from each subject in a tube containing potassium EDTA as an anticoagulant. Plasma was separated after centrifugation for the determination of lipid profile. For the screening of apo B R3500Q mutation, DNA was extracted from the cellular portion by a simple salting-out procedure (10).

A fragment of 478 bp containing codon 3500 of apo B gene was amplified by PCR using a set of primers (The Bioprocessing Technology Centre, National University of Singapore). These primers correspond to nucleotides 10684-10707 and

Figure 2. Detection of apo B R3500Q mutation according to the length of DNA fragments in the digestion products, bp = base pair.
(A) Schematic representation of the PCR-amplified DNA fragment spanning codon 3500 and 3611 of the apo B gene.
(B) Schematic representation of the expected DNA fragments in Msp I *digestion products according to the presence or absence of* Msp I *cleavage sites at codon 3500 and 3611.*

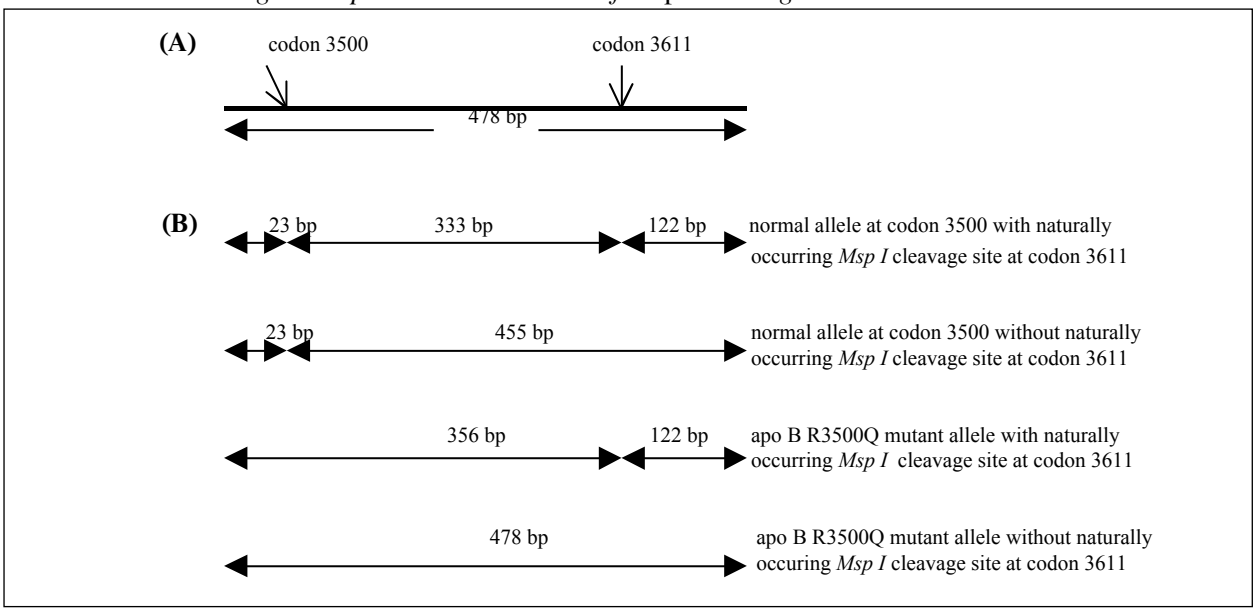
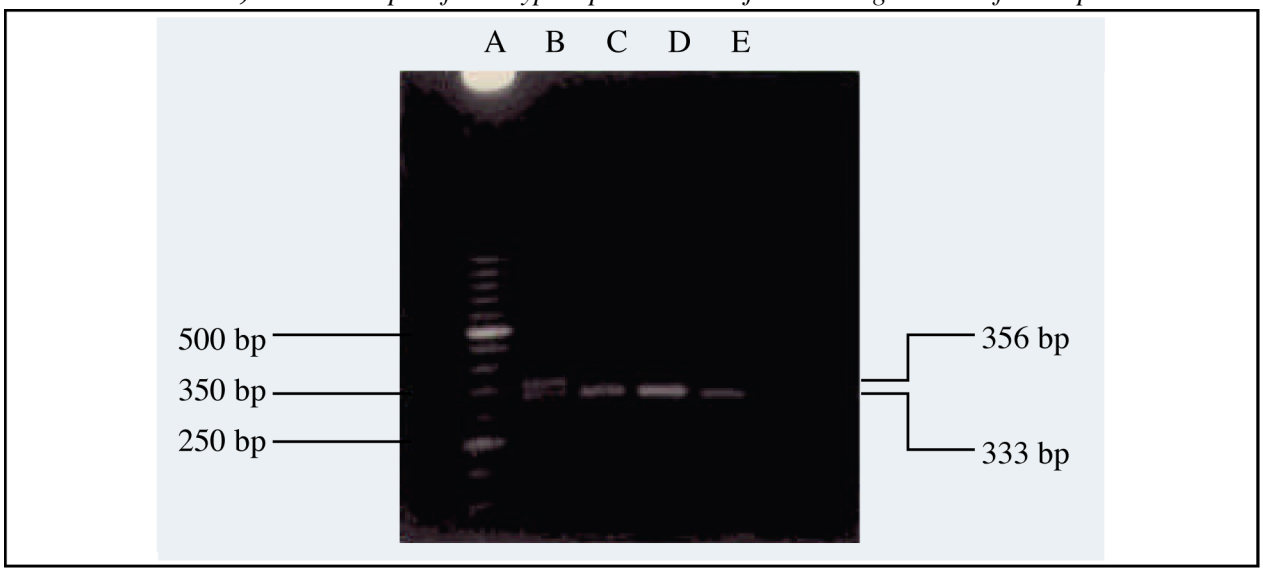


Figure 3. Photograph showing the results of an analysis of Msp I *digestion products by 2% agarose gel electrophoresis, bp = base pair.*
Lane A-50 bp DNA ladder,
Lane B- positive control (heterozygous for apo B R3500Q mutation): 356 bp and 333bp bands obtained,
*Lane **C, D** & **E**-samples from hyperlipidaemic subjects: a single band of 333 bp obtained.*



nucleotides 11138-11161 of the apo B gene respectively. The sequence of the 5' primer was modified, an "A" which is the second last base at its 3' end, was replaced by "C". The terminal 3' end nucleotide of the 5' primer corresponds to the first base of codon 3500.

5'primer -5'CTTACTTGAATTCCAAGAGCAC^{C}C3'
(Superscript letter denotes mismatched base)
3' primer - 5'GGTAGGATGATATTTTTGAGGAAC3'

The mutagenic 5' primer introduced its mismatched base at nucleotide 10706 creating a recognition sequence for *Msp I* (CCGG) in codon 3500 of normal allele (Figure 1.A.). In case of apo B R3500Q mutant allele, the mutagenic 5' primer could not create *Msp I* recognition sequence because of G to A transition at nucleotide 10708 (CCAG) (Figure 1.B.)

The amplified PCR products also spanned codon 3611. Since a naturally occurring *Msp I* recognition site is frequently present in this codon, both normal allele and apo B R3500Q mutant allele may also be cleaved at codon 3611.

PCR mixture contained 300-500 ng genomic DNA, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 2.5 U Taq DNA polymerase and 5 µl PCR buffer (Promega, USA) in a 50 µl reaction volume. PCR was performed in automated thermal cycler (Eppendorf Mastercycler 5330) using the temperature profile of 5 minutes at 96ºC for initial denaturation followed by 35 cycles of denaturation at 96ºC for 2 minutes, annealing at 60ºC for 1 minute and extension at 72ºC for 1 minute. A final extension of 7 minutes was added at the end of the last cycle. In each batch of PCR, a negative control (all reagents except DNA) was included to exclude contamination. The DNA sample from a patient heterozygous for apo B R3500Q mutation was used as a positive control. The PCR products were then digested with *Msp I* (Promega, U.S.A.) at 37ºC for 3 hours. The reaction mixture was prepared by mixing 10 µl PCR product, 10 U restriction enzyme and 2 µl restriction enzyme buffer in a 20 ll volume.

The PCR products and *Msp I* digestion products were analysed by 2 % agarose gel electrophoresis at 90 volts for 1.5 hours. A 50 bp DNA ladder was included in every run of electrophoresis to analyse the size of DNA fragments.

Results

When the DNA samples were analysed, a 478 bp fragment was obtained for all the samples after PCR. The length of DNA fragments in different types of allele after cleavage with *Msp I* depend on the presence of *Msp I* cleavage sites at codon 3500 or

codon 3611 or both codons (Figure 2). The cleavage of a 122 bp fragment from the PCR product after digestion indicates the presence of naturally occurring *Msp I* cleavage site at codon 3611.

When digestion products were analysed by 2% agarose gel, the positive control sample (heterozygous for apo B R3500Q mutation) showed a two-band pattern (356 bp and 333 bp) whereas a single-band of 333 bp was observed in the digestion products of all hyperlipidaemic subjects recruited in this study (Figure 3).

A 356 bp band observed in the positive control was due to the absence of *Msp I* cleavage site

After electrophoresis at 90 volts for 1.5 hour.

The single 333 bp band observed in the digestion products of hyperlipidaemic subjects indicated homozygosity for normal allele at codon 3500 as well as the presence of naturally occurring *Msp I* cleavage site at codon 3611 of both alleles. Apo B R3500Q mutation was not detected in these individuals.

Discussion

Apo B R3500Q mutation is the most commonly reported apo B mutation associated with increased plasma cholesterol concentration (7, 9, 11, 12 and 13). In the previous studies, the frequency of heterozygous FDB with this mutation was estimated to be 1 in 500 and FDB heterozygous were identified in 2-3 % of individuals with diagnosis of FH (9. 11 & 13). In this study, the DNA samples from 62 Malay subjects were analysed to determine the association of apo B R3500Q mutation with increased plasma cholesterol concentration in Kelantanese population. Apo B R3500Q mutation was not detected in the hyperlipidaemic subjects selected for this study. Although this mutation is relatively common in some populations, it does not appear to be a major contributor to elevated plasma cholesterol in the Kelantanese population. Apo B R3500Q mutation was also found to be absent in some populations such as Finnish (14), Japanese (15), South African (16) and French-Canadian (17). Because the number of cases examined in this study is small and hypercholesterolaemic subjects were selected only from the lipid clinic at Hospital Universiti Sains Malaysia, our study group may not represent the whole Kelantanese population. Therefore, further studies involving larger sample sizes are required to confirm the results of this study. On the other hand, screening of mutations other than this apo B R3500Q mutation such as apo B R3531C and apo B R3500W needs to be undertaken since these mutations might be associated with increased plasma cholesterol in the Kelantanese population.

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