Homozygous DNA Variants in Exon 9 of the LDL Receptor Gene in a Thai Patient with Primary Hypercholesterolemia Phenotype

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Abstract

Mutation in low density lipoprotein (LDL) receptor gene causes an inherited primary hypercholesterolemia namely familial hypercholesterolemia (FH). In this study, 46 Thai patients with primary hypercholesterolemia were screened for mutations in exon 9 of the LDL receptor gene by polymerase chain reaction - restriction fragment length polymorphism (PCR - RFLP). The analysed fragment was 224 bp in length. According to the published cDNA sequence, exon 9 of the LDL receptor gene contains several hypermutable CpG dinucleotides. Three of these sites are Hpa II recognition sites. PCR product of exon 9 obtained from amplification of wild-type DNA sample would yield four fragments after Hpa II digestion. The expected sizes of these restriction fragments were 15, 30, 40 and 139 bp. All normocholesterolemic subjects (n = 33) showed normal RFLP. However, in one patient (72 year old female), abnormal RFLP from Hpa II digestion of the amplified exon 9 was observed, i.e., a fragment of 70 bp and another one smaller than 139 bp. Such RFLP reflects that exon 9 of both alleles of the LDL receptor gene in this patient lost one and gained one Hpa II site. It is interesting that this patient, eventhough harbouring two mutations on both alleles of the LDL receptor gene (presumably homozygous genotype of FH), apparently revealed lipid levels of heterozygous phenotype of FH without symptoms of coronary artery disease. It has yet to be proved whether these genetic variations are disease-related mutations or presumably common DNA polymorphisms.

Key word : Hypercholesterolemia, CpG Dinucleotides, Gene Mutations, PCR-RFLP, LDL Receptor

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Hypercholesterolemia has been recognized as a major risk factor of atherosclerosis and coronary artery disease (CAD)[1]. This lipid disorder can be the consequence of both genetic and environmental factors. However, contribution from genetic factors has been observed in several populations. Both mutations and common DNA polymorphisms in genes involving lipid metabolism can affect lipid phenotypes[2]. Mutations in LDL receptor and apolipoprotein (apo) B-100 genes cause primary hypercholesterolemia namely familial hypercholesterolemia (FH) and familial defective apolipoprotein (apo) B-100 (FDB), respectively[3,4]. Some common polymorphisms at the LDL receptor locus have also been found to affect cholesterol phenotypes in normal populations[5-8].

In this study, the authors focused attention on the LDL receptor gene. It has been observed that CpG dinucleotides appear to be hypermutable irrespective of methylation-mediated deamination and mutations at this site have been preferentially found[9]. According to the published cDNA sequence [10], CpG dinucleotides are found all along the LDL receptor nucleotide sequence. Analysis of published mutations in South African Indians has shown that more than 50 per cent of the different LDL receptor mutations occur at CpG hotspots[11]. In exon 9, three of its CpG sites are recognized by Hpa II (5'CCGG3'). It was thus decided to exploit these Hpa II recognition sites for mutation screening in this exon by PCR-RFLP. A similar strategy, making use of CpG dinucleotides, was previously described by Kotze et al[11]. The authors collected DNA samples from Thai patients with primary hypercholesterolemia and also from normolipidemic subjects for this investigation. These DNA samples (from 46 patients and 33 normal individuals) were amplified by the polymerase chain reaction (PCR) and digested with Hpa II. The RFLPs were then analysed on polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. By this simple strategy, the authors documented one patient who had abnormal RFLP revealing one gain and one loss of the Hpa II site in both alleles of the amplified exon 9 of the LDL receptor gene.

MATERIAL AND METHOD

Subjects

Forty-six patients and thirty-three normolipidemic subjects were recruited on the basis of plasma cholesterol levels. The subjects whose plasma total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C), corrected for sex and age, exceeding the 90th percentile were selected as hypercholesterolemic subjects. The cut off at the 90th percentile of lipid levels was made accordingly as defined by the NIH (The National Institute of Health) [12]. Informed consent was obtained from each participant. Patients with secondary hypercholesterolemia were excluded from this study. None possessed the apo B 3500 mutation[13], as determined by previously described methods[14,15].

Biochemical analysis

Triglyceride (TG), total cholesterol and high-density lipoprotein (HDL) cholesterol were analysed by enzymatic methods, and LDL-C was calculated using Friedewald's formula as previously described[12].

Amplification of DNA

Leukocyte DNA was extracted from the fasting EDTA blood samples by Guanidine-HCl method as previously described[16]. DNA samples were amplified by PCR in a DNA thermal cycler (Gene Amp PCR System 2400, Perkin Elmer Cetus, USA). The oligonucleotide primers used for amplification of exon 9 were SP70 (5'-CCTGACCTCG CTCCCC GGACCCCCA-3') and SP71 (5'-GGCTG CAGGCAGGGGCGACGCTCAC-3'), as described by Leitersdorf et al[17]. The resulting fragment after amplification was 224 bp in length. The amplification reaction (25 µl) contained an appropriate amount of genomic DNA, 1xPCR buffer (supplied by the manufacturer as 10xPCR buffer), 0.2 mM each dNTP (dGTP, dATP, dTTP, and dTTP), 10 pmol of each oligonucleotide primer, and 0.5 unit of Taq DNA polymerase. Taq DNA polymerase and dNTPs were commercially supplied by Amersham Phamacia Biotech, (Sweden). The PCR was subjected to 36 cycles at 95°C 1 min, 72°C 1 min, with denaturation at 95°C for 3 min in the initial cycle and extension at 72°C for 5 min in the final cycle.

RFLP analysis

The amplified products were digested with Hpa II by the protocol provided by the manufacturer (Amersham Phamacia Biotech, Sweden). The digestion reactions contained an excess amount of the enzyme and the reactions were incubated overnight to obtain complete digestion. The products of digestion were analysed by 8 per cent polyacrylamide gel

electrophoresis, stained with ethidium bromide and visualized under UV light. All analyses were done in duplicate.

RESULTS

The amplified exon 9 of all the DNA samples from both normal and hypercholesterolemic subjects used in this study yielded 224 bp fragment. According to the published cDNA sequences[10], exon 9 of the LDL receptor gene contains three Hpa II sites. Digestion of the amplified exon 9 with Hpa II would yield four fragments of 15, 30, 40 and 139 bp in size for wild-type DNA sample. All wild- type DNA samples from normolipidemic subjects revealed wild-type restriction pattern. DNA samples from primary hypercholesterolemic subjects also showed the restriction pattern of the wild-type DNA, except one DNA sample from a female patient which revealed different RFLP. The result of Hpa II digestion, presenting the abnormal RFLP pattern, is shown in Fig. 1. The abnormal RFLP pattern revealed a DNA fragment smaller than 139 bp and another fragment 70 bp in size. Such a restriction pattern indicated that both alleles of exon 9 in the LDL receptor gene gained one and lost one of the Hpa II site in this particular patient. This patient was a 72 year-old woman who had approximately a two fold increase in plasma LDL-C and TC levels (on average) which can be attributable to heterozygous condition of FH. She was 68 years old at the time of her first diagnosis of hypercholesterolemia (plasma TC = 379 mg/dl, LDL-C = 318 mg/dl). In addition, she apparently revealed no xanthomas or symptoms of coronary artery disease.

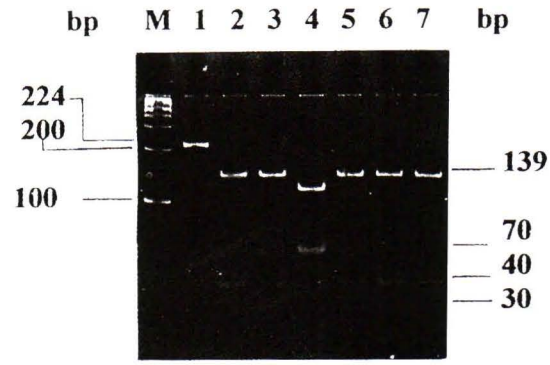

Fig. 1. Screening for mutations at CpG dinucleotides in the amplified exon 9 of the LDL receptor gene using PCR-RFLP technique. There are three CpG sites in exon 9 which are recognized by Hpa II (5' CCGG 3'). Hpa II digested PCR products of wild-type DNA were 139, 40, 30 and 15 (unobservable) bp in size (lane 2, 3, 5, 6 and 7). Lane 4 was the homozygote for HpaII digestion. The homozygote was a 72 -year- old woman whose plasma total cholesterol and LDL cholesterol were 379 mg/dl. and 318 mg/dl. respectively. Lane M was 100 bp ladder markers. Lane 1 was PCR product without Hpa II digestion.

DISCUSSION

A defect in function of the LDL receptor, causing primary hypercholesterolemia namely FH, is caused by mutation in the LDL receptor gene. FH is clinically characterized by markedly elevated plasma LDL-C, tendon xanthomas and premature CAD[18]. The disorder is inherited in an autosomal codominant manner. There is a gene dosage effect in this disorder. Heterozygous patients usually have approximately a two fold increase in plasma LDL and TC levels and CAD is generally developed at the age of 40 and 50 years in males and females, respectively[19,20]. Homozygotes or compound heterozygotes who have two defective copies of the LDL receptor gene generally have a greater than 5-fold increase in plasma LDL and suffer from fatal CAD at a young age[19].

In this investigation, the authors presumably found a homozygote who harboured two mutations in both alleles in exon 9 of the LDL receptor gene. The common mutation R3500Q in apo B-100 gene was not observed in this patient. Based on such a homozygous genotype, conventionally, this hypercholesterolemic patient should be homozygous FH. However, in this patient, her cholesterol levels before any medication, on average, were similar to those found in FH heterozygotes and she apparently has had no symptoms of CAD. The result from this study demonstrated a variation in clinical expression of certain mutations in LDL receptor gene. Exon 9 encodes a part of the epidermal growth factor (EGF) precursor homology domain.

A function of this domain is to recycle the receptor protein[21]. The mutations observed in exon 9, in this study, may disturb the recycling process. However, other functions, encoded by other parts of the LDL receptor gene, including gene expression may not be disturbed. Therefore, the LDL receptor, in sufficient number, may still partially perform its function, i.e., endocytosis of LDL particles. Hence, the heterozygous condition is apparent in this patient.

On the other hand, the observation from this study might support the view that cholesterol lowering mechanism possibly exists and that this mechanism can be activated, even in homozygotes as in this case, perhaps by mutations in known or hitherto unknown genes[22]. The heterozygous phenotype in lipid levels and the delay in CAD manifestation in this patient may reflect that gene-gene or gene-environment interaction has played a role here. Such a variation in clinical phenotypes was also previously reviewed[23]. An intrafamilial variation of clinical expression of FH occurring due to a mutation, D206E, was reported by Kotze et al[24]. The index case was a 54-year-old man who suffered angina pectoris at the age of 30 years, but his 84-year-old father, who also carried the mutation, was healthy and unaffected. In a Chinese population, heterozygotes exhibited a mild phenotype although they carried serious mutations in the LDL receptor gene[22,25]. Obligate heterozygotes are not usually identified as hypercholesterolemic, at least in relation to the Western reference value. On the other hand, Chinese heterozygotes living in Canada have a phenotype similar to that of other FH patients in Western societies[22].

These observations including the result of this study might reflect the impact of nongenetic factors such as dietary habits[22,26] and/or life style. However, before any further conclusion can be made, more studies are required. These studies should include characterization of the DNA sequences at the sites of mutations and the possible impact of such sequence variations on protein structure and function. Other gene loci involving lipid metabolism should be examined. Analyses of other exons, introns and the promotor region at the LDL receptor locus itself should also be performed. In addition, a family study is also inevitable. Such studies would provide information whether these Hpa II mutations are disease-causing mutations or not.

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การกลายพันธุ์ชนิด homozygote ใน exon 9 ของยีน LDL receptor ในผู้ป่วยคนไทยที่มีภาวะโคเลสเตอรอลในเลือดสูงแบบปฐมภูมิ

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การกลายพันธุ์ในยีน LDL receptor ก่อให้เกิดภาวะโคเลสเตอรอลในเลือดสูงแบบปฐมภูมิซึ่งเป็นโรคที่ถ่ายทอดได้ทางพันธุกรรมเรียกว่า familial hypercholesterolemia (FH) การศึกษานี้เป็นการค้นหาการกลายพันธุ์ใน exon 9 ของยีน LDL receptor ในผู้ป่วยคนไทยที่มีภาวะโคเลสเตอรอลในเลือดสูงแบบปฐมภูมิ (n = 46) โดยใช้เทคนิค PCR–RFLP ขนาดของ exon 9 ที่ได้จากการทำ PCR คือ 224 bp จากข้อมูลลำดับเบสของ cDNA ในยีน LDL receptor ที่ได้ตีพิมพ์ไว้ แสดงให้เห็นว่า exon 9 มี CpG อยู่หลายตำแหน่ง CpG เป็น dinucleotides ที่กลายพันธุ์ง่าย ใน exon 9 มี CpG อยู่ 3 ตำแหน่งที่ตัดได้ด้วยเอ็นซัยม์ HpaII exon 9 (ที่ได้จากการทำ PCR) ที่มีลำดับเบสปกติเมื่อตัดด้วยเอ็นซัยม์ HpaII จะได้ DNA ที่มีขนาด 15, 30, 40 และ 139 bp ในคนปกติ (n = 33) RFLP ที่ได้เป็นปกติทุกราย อย่างไรก็ตามมีผู้ป่วยหญิงหนึ่งราย (อายุ 72 ปี) ที่มี RFLP ผิดปกติ กล่าวคือ มีชิ้น DNA ที่มีขนาด 70 bp และ อีกชิ้นมีขนาดเล็กกว่า 139 bp ผลที่ได้นี้แสดงว่ามีตำแหน่งที่ตัดได้ด้วย HpaII หายไปหนึ่งตำแหน่งและเกิดขึ้นใหม่ หนึ่งตำแหน่งใน exon 9 ของทั้ง 2 alleles ในยีน LDL receptor ของผู้ป่วยรายนี้ สิ่งที่น่าสนใจคือผู้ป่วยรายนี้ มีการกลายพันธุ์ 2 ตำแหน่ง ของทั้ง 2 alleles ในยีน LDL receptor ซึ่งควรเป็นลักษณะของ homozygous FH แต่ปรากฏว่าผู้ป่วยรายนี้ มีระดับโคเลสเตอรอลสูงเทียบเท่ากับ heterozygous FH และยังไม่ปรากฏอาการของโรคหัวใจ อย่างไรก็ตาม การที่จะสรุปว่าการกลายพันธุ์ที่พบนี้จะเป็นการกลายพันธุ์ที่ก่อโรคหรือเป็นเพียง polymorphisms ยังต้องศึกษาเพิ่มเติมต่อไปอีก

คำสำคัญ : ภาวะโคเลสเตอรอลในเลือดสูง, CpG dinucleotide, การกลายพันธุ์, PCR–RFLP, ยีน LDL receptor

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