Inexpensive, Rapid and Convenient PCR-minigel SSCP Protocol for Polymorphisms and Mutations Analyses of LDL Receptor Gene

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Abstract

Hypercholesterolemia has been recognized as a major risk factor of atherosclerosis and coronary artery disease. The elevation in plasma low density lipoprotein (LDL) cholesterol is frequently due to genetic alteration at the genetic locus specifying the LDL receptors, leading to defective catabolism of LDL. In order to facilitate the molecular diagnosis of LDL receptor disorder, single strand conformation polymorphism (SSCP) analysis of polymerase chain reaction (PCR) amplified genomic DNA fragments has become a simple and sensitive screening method for identification of DNA polymorphisms and mutations in LDL receptor gene prior to DNA sequencing. In addition, SSCP patterns can be detected by silver staining to avoid hazardous radioactive material or other costly nonradioactive detection techniques. However, the original SSCP protocol is generally large-formatted, which is both time and reagents consuming as well as cumbersome. Minigel SSCP protocols have thus been devised but they involve, although commercially available, costly precast gels. We describe here a nonradioactive PCR-minigel SSCP protocol which is sensitive, inexpensive, rapid, reproducible and manually convenient. The results in this study demonstrate that minigel-SSCP (gel size: 10 cm x 7.3 cm x 0.075 cm) can detect conformation polymorphisms in PCR–fragments with a comparative sensitivity to large gel SSCP (gel size: 30 cm x 40 cm x 0.04 cm) as exemplified by the SSCP analyses of exon 13 of the LDL receptor gene.

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For minigel SSCP, the reagents for gel components and silver staining are reduced approximately 9 times and 10 times, respectively. For electrophoresis, electrical power is also reduced 10 times. This improved technique can become routinely used for molecular diagnosis of LDL receptor defect as well as for other genetic disorders.

Key word : Hypercholesterolemia, LDL Receptor Gene, Polymorphism, Mutation, PCR, Minigel SSCP

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Mutation in LDL receptor gene causes primary hypercholesterolemia which is an autosomal codominant disease namely familial hypercholesterolemia (FH)[1]. FH is characterized by an increase of LDL cholesterol in plasma, giving rise to tendon and skin xanthoma, premature atherosclerosis and coronary artery disease (CAD)[2]. FH is one of the most common genetic metabolic disorders. It affects 1 in 500 persons for the heterozygous form and 1 in a million for the homozygous form[3].

Since hypercholesterolemia has been well established as one of the major risk factors of CAD, identification of FH patients has been always attempted. In countries where CAD has been ranked as the first leading cause of death, identification of individuals at risk of developing CAD such as FH patients is an important public health issue[4].

Demonstration of a disease-related mutation in the LDL receptor gene has become more frequently used to diagnose FH subjects. The conventional receptor activity assays are time-consuming, inconvenient and discriminatory power of these assays are insufficient to allow use for the general population[2]. So far DNA-based diagnosis of FH has been exercised in several populations where particular LDL receptor mutations are frequent[2,4,5].

Mutations in the LDL receptor gene are very heterogeneuos at the DNA level. More than 600 different LDL receptor gene mutations have been described and the majority have been associated with a classical FH phenotype[3,6-8]. Although management is based on the phenotype, counseling and future genetic tracing programmes will be helped by knowledge of the genotype so that definitive genetic tests can be offered.

The LDL receptor gene is large (18 exons plus a promoter), i.e. comprising 45 kb for entire gene and 5.3 kb for mRNA[9]. Simple techniques such as PCR and PCR-RFLP can be used to identify any known mutations in a population. However, before these simple techniques can be applied, it is necessary that mutations in the population must be identified and collected as a database and, thus, simple PCR–based assay can be designed and used thereafter. At present, database for LDL receptor gene mutations in a population with a Thai ethnic background is not available. For such a situation with unknown mutations, DNA sequencing is required to identify them. However, DNA sequencing to screen polymorphisms and mutations is time-consuming, labourious and expensive especially for large genes with heterogeneous mutations like the LDL receptor gene in this case. PCR - SSCP is an alternative screening technique prior to DNA sequencing. This technique is simple and convenient for routine identification of unknown DNA polymorphisms or mutations[10]. In this study PCR-SSCP has been used to prescreen mutations, prior to DNA sequencing, in the LDL receptor gene in our patients with primary hypercholesterolemia. In this technique, double - stranded DNA is amplified by PCR, denatured to single-stranded DNA, separated in a nondenaturing polyacrylamide gel and conformational change (due to change in base sequence) is detected as a different pattern of migration on the polyacrylamide gel[11]. However,

the conventional PCR-SSCP analysis involves a lengthy electrophoretic procedure, labour intensive, cumbersome and time consuming. So, we have devised a nonradioactive PCR- minigel SSCP which is economical and convenient. Detection of SSCP pattern is made by silver staining. This paper describes such a SSCP protocol which reduces labour, cost, and time. The application of this technique for the detection of a mobility shift in exon 13 of LDL receptor gene, previously detected by large gel SSCP analysis[12], is described. So far in our laboratory, this minigel SSCP protocol has been used successfully to detect abnormal patterns in any exons of LDL receptor gene.

MATERIAL AND METHOD

PCR

Template DNA from a normal control and from five patients revealing abnormal SSCP patterns of exon 13 in the LDL receptor gene identified earlier by large–formatted SSCP gel in our laboratory were used (unpublished). For large-gel SSCP, Exon 13 was amplified by PCR using primers of which the nucleotide sequences were complementary to 5'and 3'ends of the coding region of exon 13. The sequence of sense primer was 5'-GACAAAGTAT TTTGGACAG-3' and for the antisense primer was 5'-CTCTTGGCTGGGTGAGGTTG-3'[13] A PCR fragment amplified with these primers was 142 bp in size.

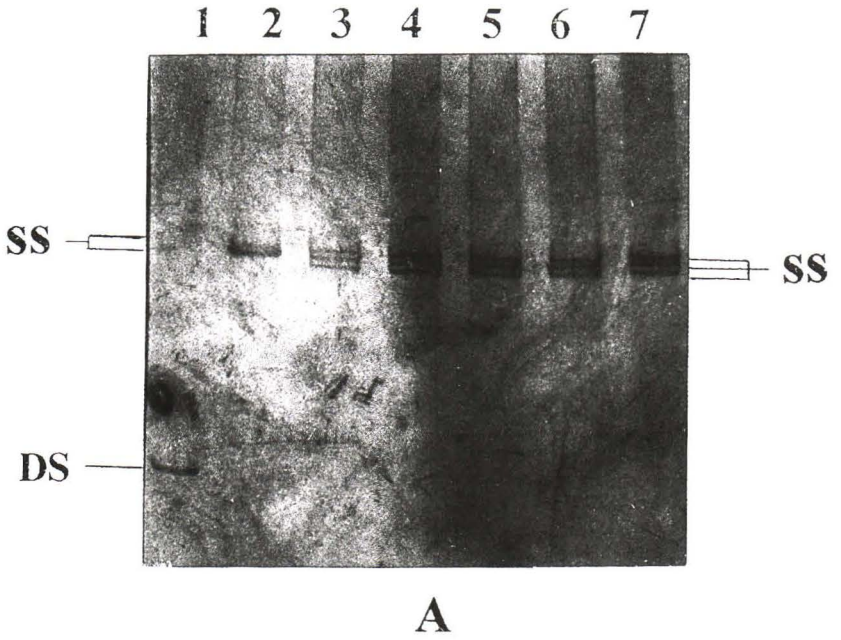
For minigel SSCP analysis, the intron-exon junctions of exon 13 were included in the PCR fragment. The sense and antisense primer were complementary to the 5'end 3' flanking regions of the exon. The nucleotide sequences of sense primer was 5'-GTCATCTTCCTTGCTGCCTGTTTAG-3' and antisense primer was GTTTCCACAAGGAGGTTT CAAGGTT-3'[14]. This PCR product, including splicing sites, was 213 bp in size. Oligonucleotides were synthesized and purified by the Bioservice Unit, Mahidol University, Thailand. The PCR amplification condition for both pairs of primers was the same. Amplifications were performed in a 25 µl reaction containing genomic DNA, 1x PCR buffer, sense and antisense primers at 5 pmol each, deoxynucleotide triphosphate (dNTPs) at 100 µM each and 1 unit of Taq DNA polymerase. Taq DNA polymerase, dNTPs and 10x PCR buffer were commercially supplied (Amersham Pharmacia Biotech, Sweden). The reaction mixtures were denatured at 95°C for 5 min. After the initial denaturation, 35 PCR cycles were performed in a programmable DNA Thermal cycler (Gene Amp PCR system 2400, Perkin-Elmer, USA.) using the temperature profile of 1 min at 95°C, 1 min at 53°C and 1 min at 72°C. The primer extension of the 35th cycle was extended to 5 min.

SSCP analysis

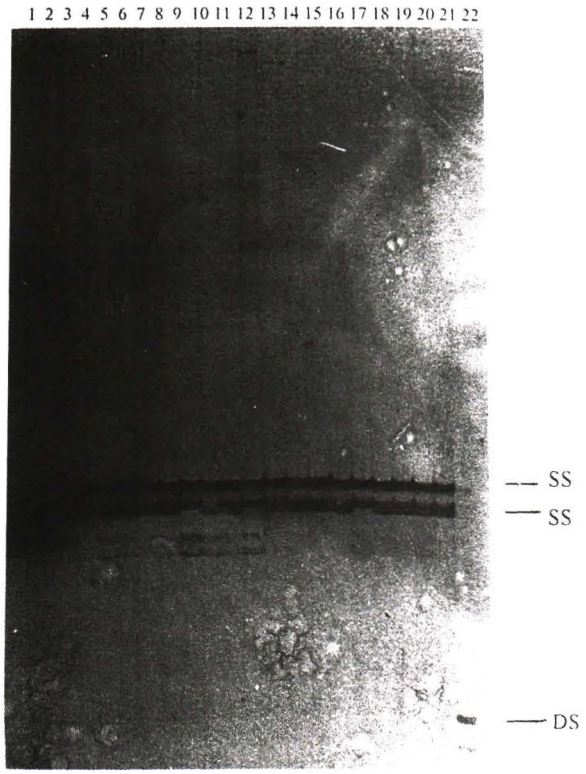
The PCR products were analyzed by SSCP techniques[10,11,15-19]. Both large and small gels SSCP analyses were manually casted. The PCR fragments of 142 bp were analysed by large gel SSCP (gel size: 40 cm x 30 cm x 0.04 cm). One µl of PCR products were mixed with 10 µl of 5x formamide dye mix (Bio101, USA), incubated in a boiling water-bath for 10 min and chilled on ice for 1 min before loading. Electrophoresis was carried out in 6 per cent polyacrylamide gel with 2 per cent crosslink and 0.5 x TBE (1xTBE: 89 mM Tris, 89 mM Boric acid, and 2.5 mM EDTA) without glycerol at 1000 volts for 1.5 hour at 4°C with the S2 DNA sequencing apparatus (BRL, Life Technologies, Inc., USA). The PCR fragments of 213 bp were analysed by minigel SSCP (gel size: 10 cm x 7.3 cm x 0.075 cm). One µl of PCR products were mixed with 10 µl of 5x formamide dye mix and treated in the same manner as described for the 142 bp fragments. Electrophoresis was carried out in 8 per cent polyacrylamide gel with 1.3 per cent crosslink and 0.5x TBE without glycerol at 100 volts for 1.5 hour at 4°C with the Mini-PROTEAN II cell electrophoresis unit (Bio-Rad, USA). Both large gel and minigel SSCP were visualized by silver staining[20-23].

RESULTS

The amplified exon 13 fragments of 142 bp in size were analysed by large gel SSCP. Five samples revealed an identical abnomal SSCP pattern. The same set of DNA samples were then reanalysed using the minigel SSCP protocol. This time, to include intron-exon junction, the DNA samples were amplified with oligonulcotide primers which were complementary to the sequences flanking 5' and 3' ends of exon 13. The amplicons were 213 bp in size. The results of representative SSCP analyses are shown in Fig. 1 (A and B). The mobility shifts in lanes 3, 4, 5, 6 and 7 of minigel SSCP (panel A) corresponded to those in lanes 5, 9, 10, 11 and 12 of large gel SSCP (panel B), respectively. The five abnormal patterns which were detected by the con-



A



B

Fig. 1. **SSCP analysis of LDL receptor gene. Panel A: Lane 1 is double-stranded DNA pattern. Lane 2 is normal SSCP pattern of a normolipidemic subject. Lane 3-7 are abnormal SSCP pattern belonging to subjects with high cholesterol levels. Panel B: Lane 22 is double-stranded DNA pattern. Lane 1-4, 6-8 and 13-21 are normal SSCP pattern of subjects with normolipidemia. Lane 5 and 9-12 are abnormal SSCP pattern belonging to the same hypercholesterolemic subjects presented in lane 3-7 in panel A. Single-stranded and double-stranded DNA are indicated as SS and DS.**

ventional type of analysis were also detected in minigel SSCP.

The comparisons between the large gel and minigel SSCP features are summarized in Table 1. The devised minigel- SSCP protocol in this study allows the analysis of same cases and exons with reduction in labour, cost and time.

DISCUSSION

PCR-SSCP is a simple and convenient method for routine identification of unknown DNA

Table 1. Comparison of large gel and minigel for SSCP analyses of LDL receptor gene.

	Large gel	Minigel
1. Gel casting	- plate sizes are 30x40x0.04 cm - glue is used for binding between glass plates - bind silane (Promaga, USA) is used for binding between gel and one glass plate - one glass plate is coated with SIGMACOAT (Sigma, USA) - amount of gel matrix is ~100 ml/plate	- plate sizes are 10x7.3x0.075 cm - glue is used for binding between glass plates - bind silane is not necessary - SIGMACOAT is not required - amount of gel matrix is 8 ml/plate, i.e., gel matrix is reduced more than 10 times
2. Polymerization	- 2 hours	- 20 minutes
3. Electrophoresis	- pre-run for 30 minutes is required before sample loading - electrophoresis at 1,000 volts for 1.5 hours - high–voltage power supply is required	- pre- run is not required - electrophoresis at 100 volts for 1.5 hours - electrical power is reduced ~10 times.
4. Silver staining	- 1,000 ml of 10% acetic acid , 1,000 ml of 2% $AgNO_3$, 1000 ml of developing solution (30% $NaCO_3$ with 1.5 ml 37% formaldehyde)	- 100 ml of 10 % acetic acid, 100 ml of 2% $AgNO_3$, 100 ml of developing solution (30% $NaCO_3$ with 150 ul 37 % formaldehyde)
5. Advantage & Disadvantage	- expensive - difficult to handle - sensitivity ~ 80%(13,16) - the process takes 5 hours after completion of PCR - post-electrophoresis staining and blotting is inconvenient and cumbersome	- cost of reagents is reduced~9 times for gel components,10 times for silver staining and 10 times for electrical power - convenient to handle - same sensitivity as large gel SSCP, in this study - the process takes 3 hours after completion of PCR - post-electrophoresis staining and blotting is convenient and simple

polymorphisms or mutations(10). In our previous study, PCR following with large formatted SSCP was used to prescreen mutations, prior to DNA sequencing, in LDL receptor gene in patients with primary hypercholesterolemia(12,24). The conventional large-formatted SSCP is labourious, expensive and time consuming. Therefore, the authors have tried a variety of labour saving approaches to achieve convenience as well as to reduce cost and time for such screening. A SSCP method was devised using a minigel, manually casted, to replace the conventional large-gel SSCP. For detection of SSCP patterns, silver staining was used in place of costly autoradiography or other nonradioactive detection techniques. Minigel SSCP has already been applied in molecular genetic studies of several genes such as glucose -6 -phosphate dehydrogenase (G6PD)(25), medium chain acyl-coA dehydrogenase (MCAD)(26), human p53 , human HLA-DQA, human K-ras and rat K-ras(17). PCR-SSCP analysis with silver staining has already been reported as well(20-23). The sensitivity of mini-gel SSCP with silver staining is not inferior to the conventional method using autoradioghaphy and large gel SSCP(26).

In the present study, five DNA samples (from patients) revealing identical mobility shift in amplified exon 13 in large gel SSCP(12) were reanalysed by minigel SSCP. In this analysis, PCR products were denatured by the same protocol as for large–formatted gel SSCP, whereas, eletrophoresis was carried out at a lower voltage (100 volts) in 8 per cent polyacrylamide with 1.3 per cent crosslink instead of high voltage (1000 volts) in 6 per cent polyacrylamide with 2 per cent crosslink in large gel. In the large gel SSCP analysis, the PCR products encompassed only the coding region of exon 13, whereas, in the minigel SSCP the PCR products included intron-exon junctions. The SSCP patterns of both large and minigel SSCP were assessed by

silver staining. The five PCR products which included intron-exon junctions also showed identical mobility shift in minigel SSCP, revealing equivalent sensitivities of both SSCP formats. The reagents, and hence the cost, for gel components and silver staining for minigel SSCP were reduced approximately 9 times and 10 times, respectively. For electrophoresis, electrical power is also reduced 10 times. In addition, the minigel SSCP described here is very convenient to handle, neither special equipment nor skill is required. It takes only 3 hours after completion of PCR.

At present, the minigel SSCP has been applied for screening mutations in other exons of the LDL receptor gene. The results are reproducible and quite satisfactory. However, this mini gel SSCP is being modified further to reduce cost, hands-on time, and also the possibility to do analysis for all 18 exons (plus promoter) of the LDL receptor gene within a few reactions and processing steps so that it can be used for routine screening purposes. Although, simple techniques like PCR and PCR–RFLP can be designed and routinely used to identify an individual at risk for any common or known mutations in the LDL receptor gene, PCR-SSCP is still necessary for screening unknown mutations at this locus. Therefore, economical and rapid SSCP protocol is essential. The authors expect that simple and inexpensive SSCP analysis will eventually accelerate the identification of novel and potentially pathogenic mutations at the LDL receptor locus for a Thai population. Such economic SSCP analysis will certainly be applicable for screening polymorphisms and mutations at any loci implicated in other genetic diseases as well.

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เทคนิค PCR–minigel SSCP แบบประหยัด, รวดเร็วและสะดวกสำหรับตรวจหาความหลากหลายและการกลายพันธุ์ในยีน LDL receptor

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ภาวะโคเลสเตอรอลสูงในเลือดเป็นปัจจัยเสี่ยงที่สำคัญต่อการเกิดภาวะหลอดเลือดแดงแข็งและโรคหลอดเลือดหัวใจอุดตัน ภาวะโคเลสเตอรอลสูงและการเพิ่มขึ้นของระดับแอลดีแอลโคเลสเตอรอลในเลือดส่วนหนึ่งมักเกิดจากความผิดปกติของยีน LDL receptor ซึ่งมีผลทำให้การสลายแอลดีแอลผิดปกติและคั่งค้างอยู่ในกระแสเลือด เทคนิคหนึ่งที่ใช้ตรวจการเปลี่ยนแปลงของยีน LDL receptor คือ PCR–SSCP ซึ่งเริ่มต้นด้วยการเพิ่มจำนวน DNA ในยีน LDL receptor โดย polymerase chain reaction (PCR) ก่อนแล้วจึงใช้เทคนิค single strand conformation polymorphism (SSCP) แยก DNA (ซึ่งในที่นี้คือ DNA ที่ได้ทำ PCR ไว้) ที่มีโครงร่างที่ต่างกัน (เนื่องจากลำดับเบสต่างกัน) ออกจากกัน จากนั้นจึงหาลำดับเบสที่เปลี่ยนแปลงไปโดยการทำ DNA sequencing การดู SSCP pattern นั้นสามารถย้อมดูด้วยเทคนิค silver staining แทนการใช้สารกัมมันตภาพรังสีหรือเทคนิคอื่นที่ไม่ใช้สารกัมมันตภาพรังสีแต่ราคาแพง อย่างไรก็ตาม SSCP ต้นแบบนั้นจะใช้ gel ที่มีขนาดใหญ่ซึ่งเปลืองทั้งเวลา, สารเคมีและยุ่งยาก ดังนั้น จึงได้มีการปรับปรุงเทคนิค minigel – SSCP ขึ้นมาแต่ส่วนใหญ่จะเป็น gel สำเร็จรูปซึ่งมีราคาแพง ในที่นี้เราได้เสนอวิธีทำ PCR – minigel SSCP ที่ไม่ใช้สารกัมมันตภาพรังสี, มีความไว, ประหยัด, ได้ผลเหมือนกันทุกครั้ง และเตรียมทุกอย่างได้สะดวกโดยไม่ต้องใช้ gel สำเร็จรูป เราพบว่าความไวของ minigel SSCP (ขนาด gel : 10 cm x 7.3 cm x 0.075 cm) นั้นเทียบเท่าได้กับ SSCP ที่ใช้ gel ขนาดใหญ่ (ขนาด gel : 30 cm x 40 cm x 0.04 cm) ซึ่งในที่นี้ได้แสดงผลดังกล่าวโดยใช้ exon 13 ของยีน LDL receptor เป็นตัวอย่าง สารเคมีที่ใช้สำหรับเตรียม gel และ ย้อม (เทคนิค silver staining) เพื่อดู SSCP pattern ได้ลดลงประมาณ 9 เท่า และ 10 เท่า ตามลำดับ ขณะนี้เราสามารถใช้เทคนิค minigel SSCP ที่ปรับปรุงนี้สำหรับตรวจกรองหาการเปลี่ยนแปลงของยีน LDL receptor ในผู้ป่วยคนไทยที่มีภาวะโคเลสเตอรอลในเลือดสูงแบบปฐมภูมิ รวมทั้งยังสามารถนำไปใช้กับยีนอื่นได้ด้วย

คำสำคัญ : ภาวะโคเลสเตอรอลสูงในเลือด, ยีน LDL receptor, ความหลากหลาย, การกลายพันธุ์, PCR, minigel SSCP

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