DEVELOPMENT OF SINGLE-STRAND CONFORMATIONAL POLYMORPHISM TO SCREEN FOR MUTATIONS IN HOTSPOT REGIONS OF BETA-GLOBIN GENE OF BETA-THALASSEMIA PATIENTS OF SRI LANKA

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Abstract. Beta-thalassemia is prevalent in Sri Lanka and imposes a heavy economic and social burden in the country due to the patients' life-long need for regular blood transfusion and treatment with iron chelation therapy. Thus, there is a need to develop a rapid, reliable and effective population-based presymptomatic and prenatal screening method for beta-thalassemia. Single-strand conformational polymorphism (SSCP) technique was developed as an adjunct for the previously developed allele-specific PCR (ASP) technique to screen the presence of mutations in beta-globin gene. A hotspot region of beta-globin gene containing 98% of known beta-thalassemia mutations was amplified from 24 clinically diagnosed beta-thalassemia patients and two normal individuals. Two overlapping amplicons of 238 bp and 268 bp were subjected to SSCP analysis. The SSCP banding patterns of these two fragments from beta-thalassemia patients were different from the corresponding regions of normal individuals. Sequence analysis of these regions revealed the presence of 4 mutations in the form of deletion and substitution that have not been reported previously from Sri Lanka. Therefore, the SSCP protocol developed in this study together with ASP should provide an appropriate screening approach for presymptomatic and parental diagnosis of beta-thalassemia in the Sri Lankan population.

Keywords: single-strand conformational polymorphism, beta-globin, beta-thalassemia, allele specific priming technique, mutations, Sri Lanka

INTRODUCTION

Beta-thalassemia is one of the most common autosomal recessive single gene disorders characterized by deficiency in synthesis of beta-globin chains (Weatherall and Clegg, 1981). The severity of the disease depends on the type of mutations and in severe forms patients require regular blood transfusions and continuous iron chelation therapy (Winichagoon *et al*, 2000). Over 600 point mutations have been reported in beta-globin gene, of which more than 200 are associated with beta-thalassemia (Hung *et al*, 2008). However, mutations involving large deletions and insertions in beta-globin gene have been

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reported in beta-thalassemia patients (Frischknecht and Dutly, 2007; Huang *et al,* 2008; Lou *et al,* 2010) as well as mutations in beta-globin gene promoter regions. Sri Lankan patients are reported to have 14 mutations with IVS-I-5 (G→C) and IVS-I-1 (G→A) accounting for 83% of detected mutations (Old *et al*, 2001).

Beta-thalassemia mutations are characterized according to the extent of defect in formation of beta-globin chains with β^o refering to the mutations that prevent the synthesis of β-globin chains while β^+ to mutations that allow some synthesis of β-globin chains to occur (Muncie and Campbell, 2009). In either situation, there is a relative excess of α-globin chains, which do not form homotetramers and instead, they bind to red blood cell membrane causing reduced red cell lifespan. Therefore, mutation analysis of beta-globin gene is useful for the prediction of clinical phenotype of beta-thalassemia, presymptomatic diagnosis of family members at-risk, prenatal diagnosis (Maggio *et al*, 1993; Petrou and Mondell 1995; Old *et al*, 2001) and also for prevention and control programs. However, due to the marked heterogeneity in mutations, screening of mutations in beta-thalassemia is time consuming and expensive.

Various techniques such as PCR mediated restriction fragment length polymorphism (Rahiminejad *et al*, 2011; Sivalingam *et al*, 2012), reverse dot blot analysis with allele specific oligonucleotide probes (Saiki *et al*, 1989), allele specific priming technique (ASPT) (Newton *et al*, 1989), and DNA sequencing have been developed to detect mutations in beta-globin gene. In addition, single strand conformational polymorphism (SSCP) (Yip *et al*, 2004; Kakavas *et al*, 2006, 2008) and denaturing gradient gel electrophoresis (Gorakshakar *et al*, 2004) assays have also been developed to analyze unknown mutations in beta-globin gene. Furthermore, systems based on a microelectronic chip (Foglieni *et al*, 2004), denaturing high-performance liquid chromatography (Colosimo *et al*, 2002) and real time PCR-based techniques (Vrettou *et al*, 2003; Feriotto *et al*, 2004; Hung *et al*, 2010) have also been used in mutational screening of known beta-globin alleles.

Beta-thalassemia is found very frequently in north and east divisions of Sri Lanka. Therefore, the development of a screening method, which is affordable to most of the heath care laboratories in Sri Lanka and suitable for the Sri Lankan population, is required. The main objective of this study is to develop a sensitive and effective screening method, namely SSCP, that indicates the presence or absence of mutations in the hotspot region of beta-globin gene in Sri Lankan beta-thalassemia patients and ASP PCR that is tailored to analyze known mutations in beta-globin gene of beta-thalassemia patient (Old *et al*, 2001) has also been used in this study as an adjunct to SSCP technique.

MATERIALS AND METHODS

Sample collections

EDTA blood samples of 24 beta-thalassemia patients attending for blood transfusion at health care sectors of Sri Lanka and also blood samples of two normal individuals were collected and genomic DNA was extracted (Miller *et al*, 1988). The procedures followed were in accordance with current ethical standards.

ASP PCR

THALF/THALIVS1-1, THALF/THAL1-5, THALB1/THAL8/9, THALF/THAL41/42, THALB1/THAL 16C and THAL F/ THAL 15G primer pairs were

employed to analyze IVS1-1, IVS 1-5, CODONS 8/9, 41/42, 16 and 15 mutations of beta-globin genes, respectively, and THALF/THALIVS1-1N, THALF/ THAL1-5N, THALB1/THAL8/9N, THALF/THAL41/42N, THALB1/THAL 16CN and THAL F/ THAL 15GN primer pairs were employed to analyze IVS1-1, IVS 1-5, CODONS 8/9, 41/42, 16 and 15 wild type alleles of beta-globin genes, respectively (Table 1). Primers were custom synthesized (Sigma-Genosys). The PCR conditions were optimized with regards to DNA and primer concentrations, $MgCl_2$ and dNTP until most of the nonspecific bands disappeared and the general optimized conditions for all ASP were 50 µl PCR mix containing 3.5 mM $MgCl_2$, 1x Colorless GoTaq® Reaction Buffer, 0.2 mM dNTPs, DNA (300 ng), 0.2 µM of each primers and 1.25 U GoTaq® DNA Polymerase (Promega, Madison, WI). The thermocycling parameters were initial heating at 94ºC for 5 minutes, and 35 cycles of 94ºC for 1 minute, 55ºC for 1 minute and 72ºC for 1 minute.

PCR amplification of hotspot region of beta-thalassemia DNA

The over lapping segments of ~268 bp and ~236 bp of the hotspot region (~ 481 bp) of the beta-globin gene were amplified using primer combinations of THAL15G/ THAL15G/N and THAL 41/42/ THAL 41/42N and of THAL16/ THAL16C and THALB1, respectively. Genomic DNA was extracted from the whole blood of beta-thalassemia patients and normal persons (Miller *et al*, 1988). The PCR conditions were optimized with regards to DNA and primer concentrations, $MgCl_2$ and dNTP until a single band is produced, and the optimized conditions were 25 µl PCR mix containing 1x Pfu buffer, 0.2 mM dNTPs, DNA (300 ng), 0.2 µM of each primers and 0.5 U *Pfu* DNA polymerase (Promega). The thermocycling parameters were initial heating at 94ºC for 5 minutes, and 35 cycles of 94ºC for 1 minute, 56ºC for 1 minute and 72ºC for 2 minutes.

SSCP

PCR amplified overlapping segments (~268 bp and ~236 bp) of hotspot region were denatured by incubating at 94ºC with an equal volume of formamide dye for 5 minutes and rapid cooling on ice. The denatured DNA (~80-100 ng) samples were separated in 10% acrylamide gel-electrophoresis at 70 V for 10 minutes and then at 100 V for 4.5 hours at 4ºC in 0.5x Tris-borate butter. Banding profiles were visualized by silver staining. The reproducibility of SSCP banding patterns was checked by repeating SSCP experiments on three separate occasions.

Sequencing and analysis

Amplicons of hotspot region of beta-globin gene of selected beta-thalassemia patients were sequenced (Univesity of Colombo, Sri Lanka) using Thermo Sequenase TM CY5 Dye Terminator kit and ALF express TM DNA Sequencer (Amershan-Pharmacia, Uppsala, Sweden). Alignments of nucleotide sequences ware carried out using *ClustalW* of the BioEdit software program (Hall, 1999) and sequence analyses were carried out both automatically and manually.

RESULTS

ASP PCR

Beta-thalassemia is largely caused by the mutations in beta-globin gene. Therefore, initially, ASP PCR assays were developed to identify the six known mutations in beta-globin genes of subset of beta-thalassemia patients in Sri Lanka. In this technique, a panel of primers tailored to analyze the known mutations in beta-globin genes of Sri Lankan beta-thalas-

Table 1
Sequences and amplicon sizes of allele specific oligonucleotide primers and their corresponding alleles used for common mutation analysis and primers used for SSCP analyses of region 1 (238 bp) and 2 (268 bp) of hotspot region of β-globin genes of Sri Lankan beta-thalassemia patients.

Mutation	Primer	Sequence of primer (5′→3′)	Size of PCR product (bp)
IVS-1-1,G→A	THAL IVS 11[a]	TTAAACCTGTCTTGTAACCTTGATACCGAT	122
	THAL IVS 11N[a]	TTAAACCTGTCTTGTAACCTTGATACCAAC	
IVS-1-5,G→C	THAL IVS 15[a]	CTCCTTAAACCTGTCTTGTAACCTTGTTGG	126
	THAL IVS 15N[a]	CTCCTTAAACCTGTCTTGTAACCTTGATAC	
Codon 8/9,+G	THAL 8/9[b]	CCTTGCCCCACAGGGCAGTAACGGCACACC	211
	THAL 8/9N[b]	CCTTGCCCCACAGGGCAGTAACGGCACACT	
Codon 41/42 N,	THAL 41/42[a]	GAGTGGACAGATCCCCAAAGGACTCAACCT	285
-TCTT	THAL 41/42 N[a]	GAGTGGACAGATCCCCAAAGGACTCAAAGA	
Codon 16-C	THAL 16 C[b]	TCACCACCAACTTCATCCACGTTCACGTTC	236
	THAL 16 CN[b]	TCACCACCAACTTCATCCACGTTCACCTTG	
Codon 15,G→A	THAL 15 G	TGAGGAGAAGTCTGCCGTTACTGCCCAGTA	268
	THAL 15 GN	TGAGGAGAAGTCTGCCGTTACTGCCCTGTG	

In allele specific PCR, primer combinations were primer[a] and THALF (ATGGTGCACCTGACTCCT-GAGG), primer[b] and THALB1 (AGAAGAGCCAAGGACAGG), and THAL 15 GN and THAL 41/42 or THAL 15 G and THAL 41/42 N. For SSCP analyses the primer combinations were THAL B1 and THAL 16 C/ THAL 16CN for region 1 and THAL 15 G/ THAL 15 GN and THAL 41/42/ THAL 41/42N. N, normal primer designed to analyse wild type allele.

semia patients and their corresponding normal primers were used in screening of samples (Table 1 and Fig 1). DNA samples analyzed in the study revealed that some of patients have both the normal as well as mutant alleles, indicating that the majority of them are compound heterozygotes (Table 2). The majority of patients have IVS 1-1, IVS 1-5, and codon 15 mutations, while mutations in codons 41/42 and 8/9 were present in a few patients and codon 16 was not seen in the patients analyzed (Table 2).

SSCP

As ASP PCR can only screen the known mutations and new and rare mutation can be missed in such analysis and further, degradation of 3′ ends of primers can result in false positive results, SSCP technique was developed. Good clarity of the SSCP banding patterns are obtained only when the DNA fragments to be analyzed are 150-300 bp long, and thus the hotspot 481 bp region (Old *et al*, 2001) was divided into two overlapping regions of 238 bp (containing a part of the promoter region, 5′ untranslated sequence and exon 1 of beta-globin gene, region 1) and 268 bp (intron 1, 70 bp of exon 2 of beta-globin gene, region 2) by using primer combinations (Table 1) and the PCR protocols were optimized to result in single amplicons in PCR (Fig 2). The latter PCR products were then analyzed using optimized SSCP techniques. In order to achieve a good clarity,

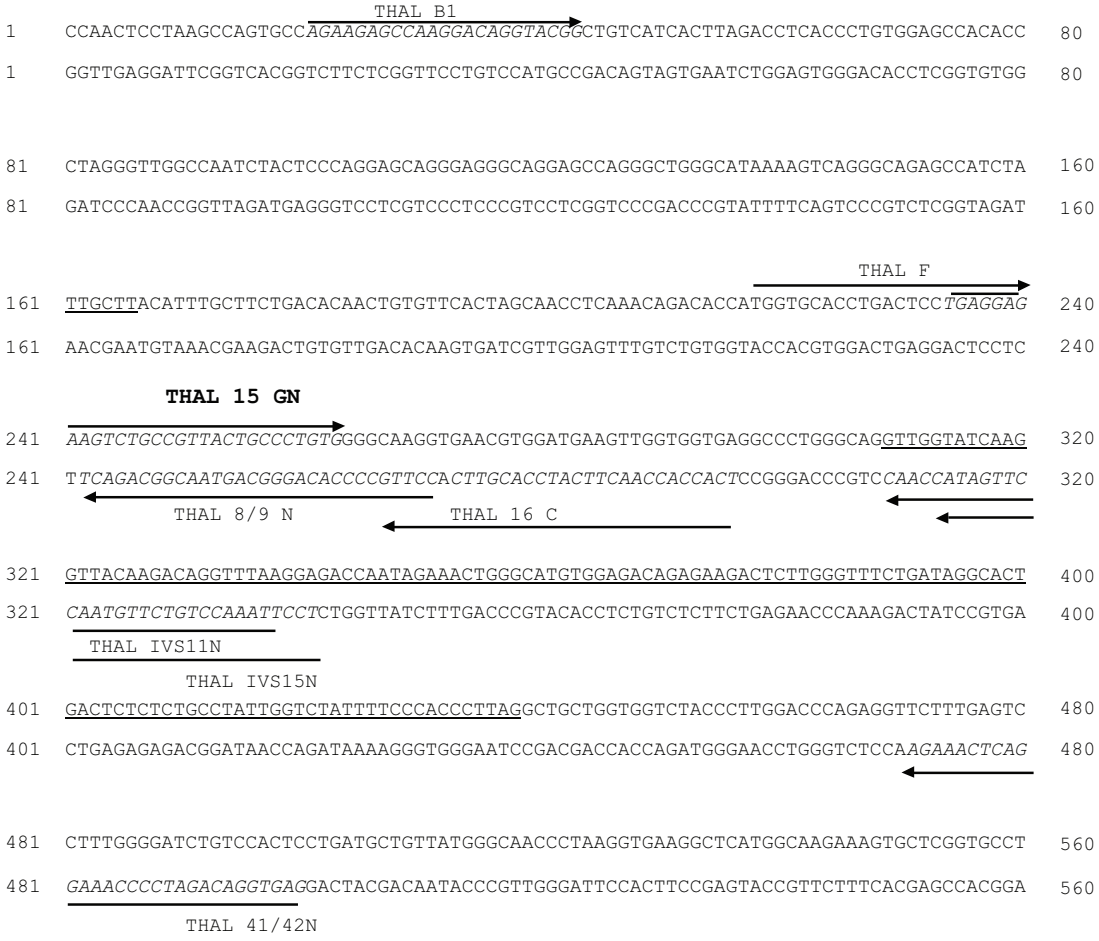

Fig 1–Human beta-globin gene containing the promoter region, exon 1, intron 1 and S' region of exon 2. Underlined arrows indicate the location of normal primer (designed to analyse wild-type allele) annealing regions of beta-globin gene used in this study for ASP and SSCP assays.

SSCP was carried out at low temperature (4ºC) using approximately 80-100 ng of PCR amplicons.

SSCP banding profile analyses revealed differences in banding patterns in either one or both hot spot regions of some samples when compared to SSCP banding profiles of corresponding regions of normal DNA, and this difference is more prominent in region 1 compared to region 2, indicating that region 1 is more prone for the acquisition of mutations than region 2 (Fig 3a). SSCP banding patterns of region 2 showed banding pattern differences in TDNA 7, TDNA 12, TDNA 13, TDNA 15, TDNA 17 and TDNA 20, in which one band was revealed at the top-most level of the banding profile compared to the normal DNA that revealed two bands (Fig 3b). In order to determine whether the differences in banding profiles are associated with mutations, a representative of each unique banding patterns, namely, TDNA1, TDNA 5, TDNA 10, and TDNA 13 (regions 1), and TDNA 17 and THAL DNA (region 2) were subjected to DNA sequencing.

Table 2
Results of allele specific PCR assay.

T DNA	Mutation					
	IVS -1-1 (G-A)	IVS-1-5 (G-C)	CODON 8/9+G	CODON 15 (G-A)	CODON 16-C	CODON 41/42 -TCTT
T DNA 1	X	P[a]	X	X	X	X
T DNA 2	X	P	X	X	X	X
T DNA 3	X	P[a]	X	P	X	X
T DNA 4	X	P	X	X	X	X
T DNA 5	X	P[a]	X	X	X	X
T DNA 6	P	X	X	X	X	X
T DNA 7	P[a]	X	X	X	X	P
T DNA 8	X	P[a]	X	P	X	X
T DNA 9	P	X	X	X	X	X
T DNA 11	X	X	X	P	X	P
T DNA 12	X	P	X	X	X	X

Mutations and DNA samples tested using primer combinations (Table 1) are given. Box with "P" indicates the presence of the investigated allele, "X" indicates absence of the investigated allele and [a]indicates homozygous allele.

Sequence analysis

Analyses of the hotspot regions sequences of selected TDNA of beta-thalassemia patients that elicited different banding patterns in SSCP compared to the corresponding regions of normal DNA revealed deletions and mutations in nucleotide sequences, which result in frame shifts and changes in amino acid sequences (Table 3). Mutations in the promoter regions of TDNA 10, TDNA 13 and THAL and misense mutations in codon 23 of TDNA 1 are new mutations (Table 3) in Sri Lankan beta-thalassemia patients. Homozygous mutations were detected in codon 23 of TDNA 1, codon 15 of TDNA 5, IVS1-5 of TDNA 1 and 5 and codon 41 of TDNA 17.

DISCUSSION

Beta- thalassemia poses a public health problem and is found in high frequency in north and east divisions of Sri Lanka. This highlights the urgent need for the development of a rapid, reliable and effective population-based screening method. Mutation analysis of the gene encoding the beta-globin is useful for prediction of clinical phenotypes, presymptomatic diagnosis and parental diagnosis.

ASP PCR used in this study can detect known mutations in Sri Lankan beta- thalassemia patients by comparing the results using a normal set of primers and set of mutant primers. However, this technique sometimes fails due to degradation of primer 3' ends. In this study it was found that IVS-1-1, IVS-1-5 and CODON 41/42 are the most commonly occurring mutations confirming previous findings (Old *et al*, 2001). As primers are designed to study specific mutations, ASP PCR can detect only known mutations. However, the advantage of this method is that it is

Table 3
Analysis of the sequences of hotspot regions from selected TDNA of beta-thalassemia patients that revealed different banding patterns in SSCP compared to the corresponding regions of normal DNA.

Name	Length of sequence (bp)	5′ Flanking region	Exon 1	Intron 1	Exon 2
TDNA1	453	Not detected	CD 23 GAA E CCA P	IVS1-5 (G>C)	Not detected
TDNA5	441	P -56-54 (GGG)-(TAA)[a]	CD 15 CTG L CTA Q	IVS1-5 (G>C)	Not detected
TDNA10	448	P-11(A)-(C)[a], 5UTR -28del (T),	Not detected	Not detected	Not detected
TDNA13	439	P – 55 del(G), -11-12 (AC)-(CG)[a]	Not detected	Not detected	Not detected
TDNA17	451	Not detected	Not detected	IVS1-5 (G>C)[a]	CD 41 AGG R del(TCTT)
THAL	468	P -55 (G)-(T)[a], -41 del (G)	Not detected	Not detected	Not detected

P, promoter region and 5′UTR, 5′ untranslated region of beta globin gene; c, codon; del, deletion; [a] , heterozygous allele

a rapid and simple method.

SSCP assay was developed as an adjunct to ASP PCR. Two overlapping SSCP assays covered the hotspot region of beta-globin gene and produced atypical banding patterns for some of the DNA (TDNA 1, TDNA 5) tested compared to corresponding banding profiles of normal individuals. DNA sequencing of the regions that contain atypical SSCP banding profiles and comparison with nucleotide sequence of the corresponding regions of normal DNA (Accession No: GU324922) revealed the presence of mutations in these regions, showing the reliability of SSCP assay developed in this study to detect mutations in beta-thalassemia patients. Hence, combining the two techniques (SSCP and ASP) would be an ideal screening protocol for presymptomatic and parental diagnosis of beta-thalassemia in Sri Lanka, where sequencing facilities are limited.

Sequencing of hot spot region of beta-globin gene of beta-thalessemia patients indicated the presence of new mutations in the promoter region and 5′ UTR from deletions missense mutations in exon 1 from substitutions and frameshift mutations and in exon 2 from deletions, in addition to already known mutations in intron 1, indicating that the underlying genetic defects in beta-globin genes of Sri Lankan beta-thalassemia patients may be somewhat more complex than what is known already, warranting further studies.

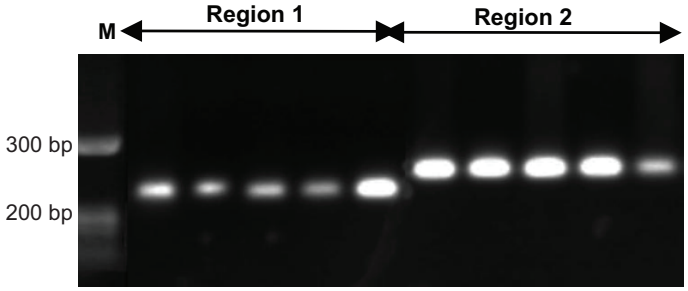

Fig 2–PCR amplification of region 1 (238 bp) and 2 (268 bp) of the hot spot region of human beta-globin gene of beta-thalassemia pateints used for SSCP analysis. Primer combinations of Thal B1 and Thal 16C/ Thal 16CN and Thal 15G/ Thal 15GN and Thal 41/42/ Thal 41/42N, respectively were employed using optimized PCR conditions. M, 100 bp ladder marker.

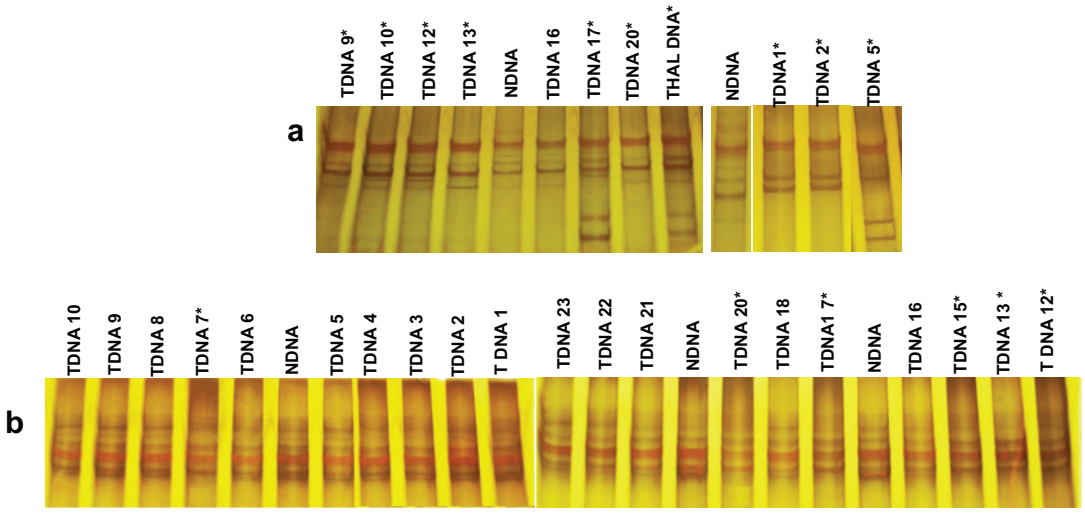

Fig 3–SSCP banding profiles of PCR products obtained from amplifying (a) region 1 (238 bp) and (b) region 2 (268 bp) of hot spot region of beta-globin gene from beta-thalassemia patients. Primers Thal B1 and Thal 16/ Thal 16 CN and Thal 15 G/Thal 15 GN and Thal 42/42 N/Thal 41/42 were used for (a) and (b), respectively. N DNA, DNA of healthy individuals. * indicates SSCP banding profiles different from profile of normal individuals.

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