

Research article

## SNP analysis of the inter-alpha-trypsin inhibitor family heavy chain-related protein (IHRP) gene by a fluorescence-adapted SSCP method

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### Abstract

**Background:** Single-nucleotide polymorphisms (SNPs) are considered to be useful polymorphic markers for genetic studies of polygenic traits. Single-stranded conformational polymorphism (SSCP) analysis has been widely applied to detect SNPs, including point mutations in cancer and congenital diseases. In this study, we describe an application of the fluorescent labeling of PCR fragments using a fluorescent-adapted primer for SSCP analysis as a novel method.

**Methods:** Single-nucleotide polymorphisms (SNPs) of the inter-alpha-trypsin inhibitor family heavy chain-related protein (IHRP) gene were analyzed using a fluorescence-adapted SSCP method. The method was constructed from two procedures: 1) a fluorescent labeling reaction of PCR fragments using fluorescence-adapted primers in a single tube, and 2) electrophoresis on a non-denaturing polyacrylamide gel.

**Results:** This method was more economical and convenient than the single-stranded conformational polymorphism (SSCP) methods previously reported in the detection of the labeled fragments obtained. In this study, eight SNPs of the IHRP gene were detected by the fluorescence-adapted SSCP. One of the SNPs was a new SNP resulting in an amino acid substitution, while the other SNPs have already been reported in the public databases. Six SNPs of the IHRP were associated with two haplotypes.

**Conclusions:** The fluorescence-adapted SSCP was useful for detecting and genotyping SNPs.

### Background

Single-nucleotide polymorphisms (SNPs) are considered to be useful polymorphic markers for genetic studies of polygenic traits. A worldwide effort to collect SNPs has re-

sulted in an accumulation of millions of them in the public databases. The SNPs were mainly identified by sequence determination of genomic DNA from restricted samples.

Single-stranded conformational polymorphism (SSCP) analysis has been widely applied to detect SNPs, including point mutations in cancer and congenital diseases [1-4]. This method has been modified to detect signals by use of radio isotope-labeled PCR fragments, detection of PCR fragments by silver staining, PCR using fluorescent labeled primers, and fluorescent internal labeling of PCR fragments [5-10].

Schuelke [11] described the method using fluorescence-labeled PCR fragments for genotyping microsatellites. The procedure used the following three primers: a sequence-specific forward primer with M13 (-21) tail at its 5'-end, a sequence-specific reverse primer, and a universal fluorescent-labeled M13 (-21) primer. PCR and the labeling reactions were performed with a single tube using modified thermo-cycling conditions. In previous studies, this labeling method has been used to analyze polymorphisms of microsatellites from horses and genotyping of many microsatellites has been performed economically [12].

Inter-alpha-trypsin inhibitor family heavy chain-related protein (IHRP) is a novel 120-kDa-glycoprotein in human plasma. IHRP has homology to the heavy chains of the inter-alpha-trypsin inhibitor family (ITI) [13,14]. The IHRP

gene spans 15 kb, is composed of 24 exons ranging from 27 to 207 bp [15] and located to human chromosome 3p14-21 [16]. IHRP binds to actin released from the damaged cells and suppresses its toxic action by preventing the formation of actin fibril. IHRP binds to cell surface actin of polymorphonuclear (PMN) cells and inhibits their phagocytic activities. Therefore, IHRP may act as an anti-inflammatory protein [17,18].

In this study, we describe an application using a fluorescent-adapted primer for PCR and for SSCP analysis. The SNPs of the IHRP gene were then analyzed to identify novel SNPs that might be associated with specific diseases or be available as genetic markers for linkage analysis.

**Methods**

**DNA samples**

Genomic DNA was prepared from whole blood samples collected from 20 unrelated Japanese subjects. All subjects gave their written informed consent to participate in the study and to supply blood samples for DNA analysis. Genomic DNA was extracted using MagExtractor System MFX-2000 (Toyobo, Osaka, Japan) according to the manufacture's protocols.

**Table 1: Primer list for SNP analysis of the IHRP gene**

	Forward primer	Reverse primer
exon 01	ACTAATCCATTCCCCACTTG	TCTCTCATCCCCAGCTC
exon 02	CATTGAAACCCTGCAACCC	GAGCGATGGAGTCATGCAC
exon 03	ACCAGGCTCTCAACAACCTC	CAAGGTGGCCTTTGTCTAG
exon 04	TTGCCTTTGGCCCTTAGTCC	CCATCTGGAGGCAAGATGTG
exon 05	TCTTTGTCCCTGAGCCTCC	CCAGAAATCCGGGCTCATAG
exon 06	AACTTCAGCCCGGATTATTC	GTGGACAGACTTCTAGGCTG
exon 07	GGCCAATTGTAGCTCTGATG	CCACAGCTGATAGCGTGAAG
exon 08	CTATGTTTCCTCTCCCTGAC	AGGGCCACAGAGACACTTA
exon 09	CTTGAGAGTGCGTGTCCTC	TATTTCTGGAACCTCAGAGC
exon 10	TTCTCACCCCTCATCCCAAAC	GCTTTATGACTGCCCACTTC
exon 11	AAGCCCAGCCTTTATGACAC	TTTGAGTCCAGCCCCTCTG
exon 12	ATGGAAGAGCTCAGAAGTGC	CTCCATGCTTAGGCGCTG
exon 13	CTGATGTGCTCCACCTACAG	TGAGACTGCTTTGGTCATC
exon 14	GCAAGATTAACACAAGCGC	CACAGCCTTTGAGGATGTTT
exon 15	CAAGATGCAAACCCAGGTC	CAATCTGTCAATCTCCCCTC
exon 16	GGCCTCAGTTCAGTTGTATC	AATAATGGACCTCCCTCAAG
exon 17	ACCTGAGAGAAGACTGCATC	TCAAGGACCACTCGTGAA
exon 18	TCTGTTTCCGGATGTTCTCTG	ATCGAGACATGTGACAGGTC
exon 19	AACCCAGCCACACCTAGTC	AAGGATGTGAAAGGGGCC
exon 20	TTGGCCCTTTCCACATCC	AGTGGTGTCTGGGTCTCTC
exon 21	GAACTCCAGGGGGTGTGAT	CTGTAGCCTGAAAGGTCAAC
exon 22	GACACTGGGTCTTTCCACAG	AAGCACAGCTGGTTTCTGAG
exon 23	GTCTCTGACCCTTACTGATC	GTCTGCTTGTGCCAAGTCAC
exon 24	TTTGCTGACCACCTGCCTA	GATTTGGCCACATGGAAGTC

### Fluorescence-adapted SSCP

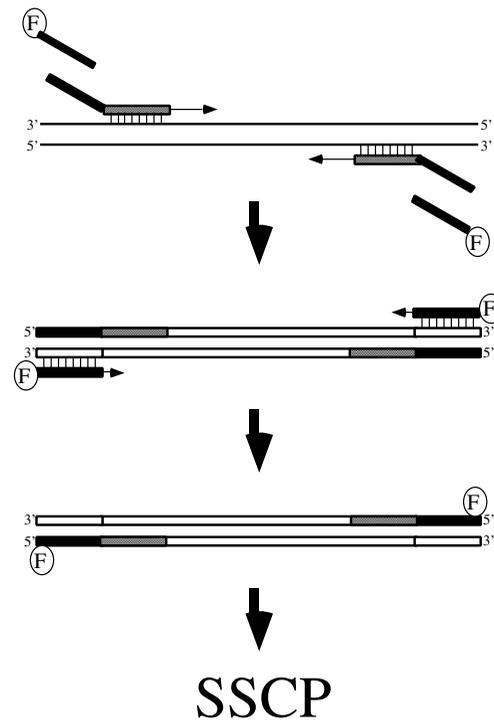
Primers for fluorescence-adapted SSCP were designed using the program DNASIS, Version 3.6 (Hitachi Software, Yokohama, Japan) based on the IHRP gene sequence (DDBJ, EMBL, and GeneBank accession numbers, NM002218), to amplify the fragments that include individual 24 exons and flanking intronic sequences. Primers were about 20 bp in length (Table 1).

An outline of the fluorescence-adapted SSCP is shown in Figure 1. In the fluorescence-adapted SSCP analysis, four primers were prepared as follows: the sequence-specific forward primer conjugated with 5'-TGA CCG GCA GCA AAA TTG-3' tail at its 5' end; the sequence-specific reverse primer conjugated with 5'-TGT AAA ACG ACG GCC AGT-3' tail at its 5' end; the Cy-5 labeled 5'-TGA CCG GCA GCA AAA TTG-3' primer (Amersham Biosciences, NJ, USA); and the Cy-5 labeled 5'-TGT AAA ACG ACG GCC AGT-3' primer (Amersham Biosciences).

The PCR mix contained 50–100 ng of Genomic DNA, 2 pmol of the sequence-specific forward primer conjugated with 5'-TGA CCG GCA GCA AAA TTG-3', 2 pmol of the sequence-specific reverse primer conjugated with 5'-TGT AAA ACG ACG GCC AGT-3', 10 pmol of Cy-5 labeled 5'-TGA CCG GCA GCA AAA TTG-3' primer, 10 pmol of Cy-5 labeled 5'-TGT AAA ACG ACG GCC AGT-3' primer, 200  $\mu$ M of dNTP, 2  $\mu$ L of 10  $\times$  reaction buffer, 1 U of rTaq polymerase (Takara Bio, Shiga, Japan) in a final 20  $\mu$ L reaction volume. The PCR amplification procedure entailed an initial denaturation cycle (94°C, 4 min); 25 cycles of 1 min each at 94°C, 55°C and 72°C, and 10 cycles of 1 min each at 94°C, 50°C and 72°C. This was followed by 7 min at 72°C for final extension in a GeneAmp PCR System 9600 (Applied Biosystems, CA, USA).

### Gel electrophoresis

The fluorescence-labeled DNA fragments were diluted 2–5 times with a loading buffer consisting of formamide and EDTA (100% formamide:50 mM EDTA = 5:1). The DNA samples were heated at 94°C for 5 min and cooled immediately on ice. This solution (1 to 5  $\mu$ L) was applied to a non-denaturing polyacrylamide gel using 0.5  $\times$  MDE gel solution (BioWhittaker Molecular Applications, ME, USA) with 1  $\times$  TBE buffer (100 mM Tris-borate, pH 8.3, 1 mM EDTA). The short gel plate was applied to the Amersham Biosciences Model ALF Express DNA sequencer. The gel temperature was strictly maintained at 15°C. Electrophoresis was performed in 0.5  $\times$  TBE buffer at 30 W for 5 h, and the data were collected and analyzed using Fragment Manager software (Amersham Biosciences).



**Figure 1**

A schematic diagram of fluorescence-adapted SSCP method. F indicates a fluorescent dye (Cy-5). Shaded boxes are specific primer sequences to amplify the IHRP gene, and black boxes are adapted sequences. These PCR reactions are performed in a single tube

### Direct sequencing

DNA sequencing was done with BigDye Terminator Cycle Sequencing FS (Applied Biosystems) and determined with an ABI PRISM 377 sequencer (Applied Biosystems).

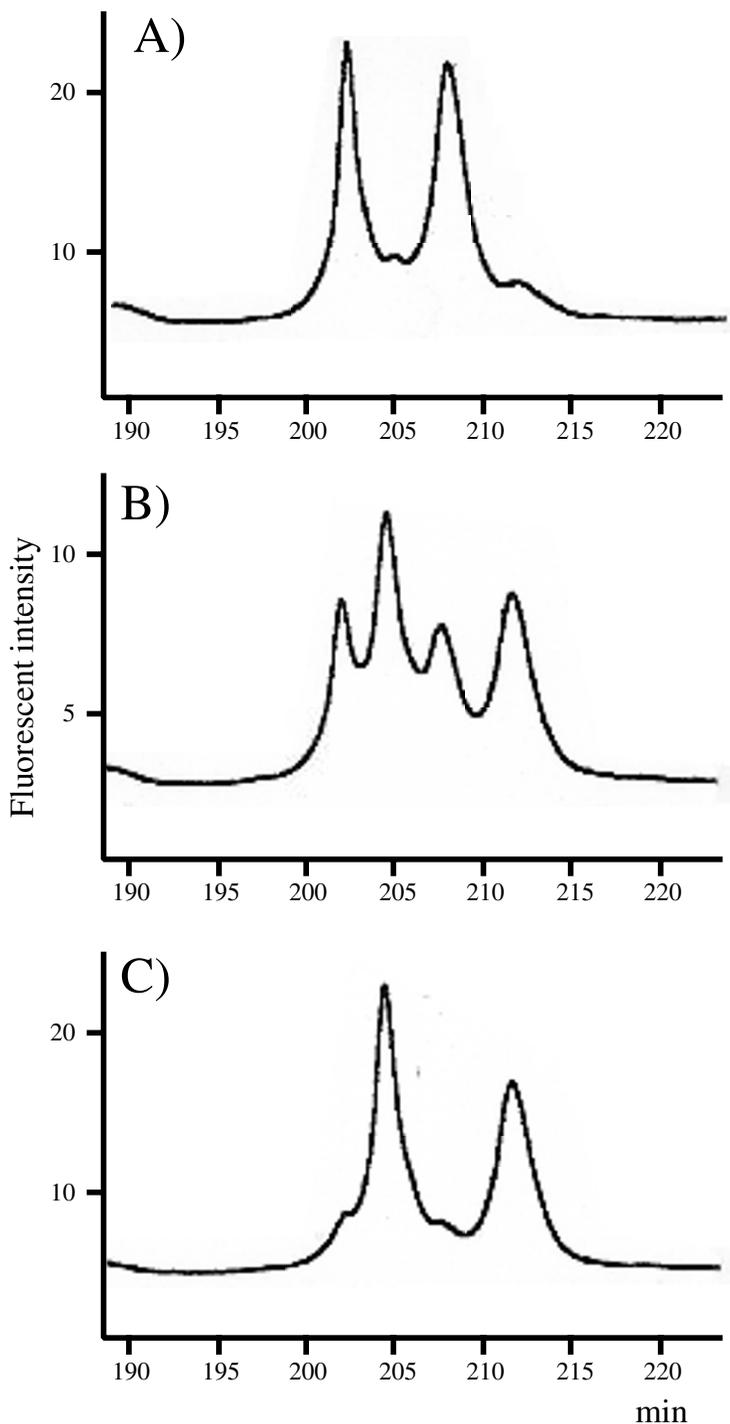
### Determination of allele frequencies and haplotype frequencies

Allele frequencies were determined by direct counting of alleles. Haplotype frequencies in the 40 chromosomes investigated were calculated with Arlequin software (Genetic and Biometry Laboratory, Geneva, Switzerland).

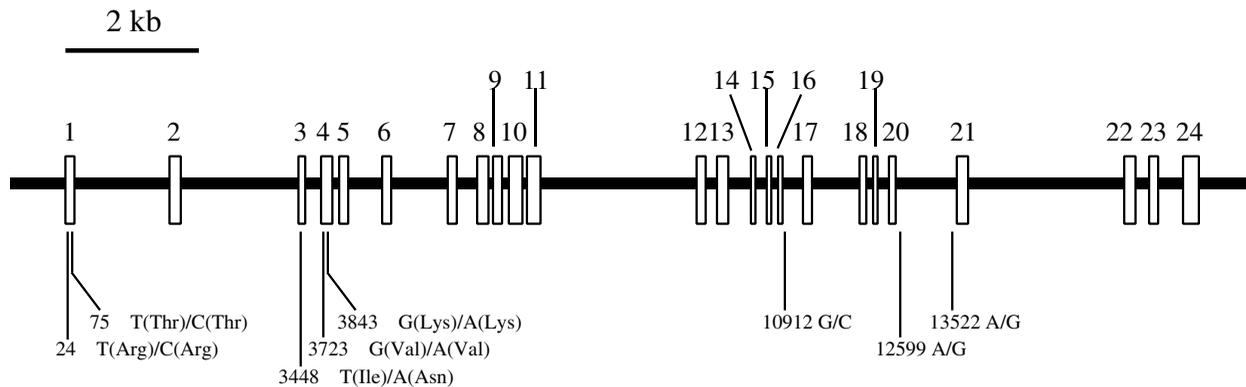
### Results

#### Effect of fluorescence-adapted SSCP

PCR fragments with the primer sets shown in Table 1 were well amplified for SSCP analysis using the fluorescence-adapted SSCP method. Figure 2 shows the migration pattern of the adapter-labeled 322-bp DNA fragments of exon 21 in the IHRP gene on non-denaturing polyacryla-



**Figure 2**  
 Electrophoretic profile on non-denaturing polyacrylamide gel of adapter-labeled 322-bp DNA fragments obtained with exon 21 primer set of the IHRP gene. A) homo-type of main allele, B) hetero-type of main and minor alleles, C) homo-type of minor allele



**Figure 3**  
Relationship between genomic structure and the location of single-nucleotide polymorphisms (SNPs) in the IHRP gene

mid gel. The PCR fragments labeled with fluorescence-adapted primers were well detected on an automatic DNA sequencer. Figures 2A, 2B, and 2C show profiles for the alleles in homozygotes (A, C) and heterozygotes (B). The alleles were clearly sequenced on non-denaturing polyacrylamide gel. The alleles were determined by direct counting.

#### Genotyping

A total of 20 Japanese individuals were genotyped for sequence variations of all 24 exons and the surrounding regions of the IHRP gene. A total of eight SNPs were found in the gene by this method. The locations of these SNPs are shown in Figure 3. Five of the SNPs found in the IHRP gene were located within exons: a T/C at nucleotide (nt) position 24 in exon 1, a T/C at nt position 75 in exon 1, a T/A at nt position 3448 in exon 3, a G/A at nt position 3723 in exon 4, and a G/A at nt position 3843 in exon 4. Three SNPs of the IHRP gene were located within introns: a G/C at nt position 10912 near exon 16, a A/G at nt position 12599 near exon 20, a A/G at nt position 13522 near exon 21. One of the five exon-based SNPs, the T/A at nt position 3448 in exon 3, resulted in an amino acid substitution from Ile to Asn. This is a newly identified SNP, while the remaining seven SNPs have already been reported in the NCBI dbSNP database [<http://www.ncbi.nlm.nih.gov/SNP/>] and the JSNP db SNP database [<http://snp.ims.u-tokyo.ac.jp/>]. Table 2 summarizes the SNPs and their allelic frequencies detected in our samples of 20 Japanese subjects.

#### Allele frequencies

Distributions of genotypes and allele frequencies among all eight polymorphisms are shown in Table 2. The distri-

butions of genotypes and allele frequencies of four SNPs at nt positions 75, 3448, 3723, and 10912 were identical. The distributions of genotypes and allele frequencies of two SNPs at nt positions 12599 and 13522 were also identical. The distributions of genotypes and allele frequencies of these six SNPs were identical except for one sample. Two SNPs at nt positions 24 and 3843 showed rare alleles. Distributions of genotypes of these two SNPs were not identical to each other nor the other six SNPs.

Construction of haplotypes and calculation of their frequencies were carried out using the Arlequin algorithm. The results are summarized in Table 3. The two SNPs at nt positions 24 and 3843 were excluded from this analysis because their minor alleles were rare. Three distinct haplotypes were identified from the 20 Japanese subjects, and two of the haplotypes were more frequent than 30%.

#### Discussion

We previously analyzed microsatellite polymorphisms using fluorescence-adapted labeling methods of PCR fragments [11] in which we designed a novel adapter sequence for the method [12]. In this study, we reported a fluorescence-adapted SSCP method, which was modified from the fluorescence-adapted labeling methods of PCR fragments, using the novel adapter sequence.

SSCP analysis is one of the simplest and most sensitive methods for detection of mutations based on PCR technology [4,19,20]. Since its first report, the SSCP analysis has been widely used to detect mutations in genes responsible for various hereditary diseases and somatic mutations of oncogenes. Generally, fluorescence-labeled primers are available for SSCP analysis, and SNPs are de-

**Table 2: Allele frequencies of eight SNPs of the IHRP gene**

Position* <sup>1</sup>	24	75	3448	3723	3843	10912	12599	13522
NCBI contig position* <sup>2</sup>	505694	505745	509118	509393	509513	516582	518269	519192
exon/intron	exon I	exon I	exon 3	exon 4	exon 4	intron	intron	intron
Allele (frequency %)	T (95)	T (65)	T (65)	G (65)	G (95)	G (65)	A (60)	A (60)
Amino acid	Arg	Thr	Ile	Val	Lys			
	C (5)	C (35)	A (35)	A (35)	A (5)	C (35)	G (40)	G (40)
	Arg	Thr	Asn	Val	Lys			

\*<sup>1</sup>The positions were counted from the ATG initiation codon of the IHRP gene. \*<sup>2</sup>The NCBI contigs were derived from assembled genomic sequence data of locus NT\_005986.

**Table 3: Haplotype frequencies of six SNPs of the IHRP gene**

Position	75	3448	3723	10912	12599	13522	Frequencies
haplotype-1	T	T	G	G	A	A	60%
haplotype-2	C	A	A	C	G	G	30%
haplotype-3	T	T	G	G	G	G	10%

tected with fluorescence-detecting automatic DNA sequencers, such as ALF Express, ABI377 and ABI3100. However, the fluorescence-labeled primers are expensive. In order to detect all SNPs of a gene region, it is necessary to design and synthesize fluorescence-labeled primer sets for about every 400 bp, or the size of an exon. Recently, some SSCP methods using internal fluorescent labeling of PCR fragments were reported [7,8]. Although these methods carried out the labeling reaction using a single tube, the procedures of those methods were complicated because of the requirement to use enzymes and buffers after PCR for the labeling reaction. Our method, the fluorescence-adapted SSCP method, is a simple and efficient method for detection of SNPs because the method only modifies the PCR conditions using fluorescence-adapted primers and the specific primers used with the adapted sequences. Our results were identical to results obtained using fluorescence-labeled primer (data not shown). Only the retention times of detected alleles on non-denaturing polyacrylamide gel between those methods were different. The retention time might be influenced by the adapted sequences. Although our method showed longer retention times compared to SSCP methods using fluorescence-adapted primers, the migration pattern was almost coincident. This phenomenon was observed in analyzing microsatellite polymorphisms [12]. Thus, it is indicated that the adapted sequences were not influenced by SSCP

analysis, nor were non-labeled PCR products. Those results proved that the fluorescence-adapted SSCP method is one of the simplest and most useful methods for SSCP.

In this method, we used two adapted sequences: 5'-TGA CCG GCA GCA AAA TTG-3' from M13 (-21) and 5'-TGT AAA ACG ACG GCC AGT-3', originally designed in a previous study. It is necessary to prepare different thermo-cycling conditions for the cycle sequencing reaction when specific sequence primers are used for SSCP analysis. The adapter sequences are available as primers for direct sequencing to determine nucleotide sequences of SNPs with the same thermo-cycling condition. Although we used one fluorescent dye in this study, (Cy-5, with ALF express DNA sequencer), allele peaks of multi-fluorescent color could be detected by using different fluorescent dye-adapted primers, resulting in determination of nucleotide sequences of alleles for SNPs.

Recently, Inazuka et al. (1996; 1997) [6,8] developed a streamlined and cost-effective mutation/polymorphism detection method (PLACE-SSCP) in which PCR products are post-labeled with fluorescent dyes, and analyzed with an automated capillary electrophoresis system under SSCP conditions. Sasaki et al. (2001) [21] reported a precise estimation of allele frequencies of SNPs by PLACE-SSCP analysis of pooled DNA, and the methods showed

allele frequencies accurately estimated by pooling DNA samples. Peak heights of alleles amplified from the same amount of genomic DNA by the fluorescence-adapted SSCP were coincident, as shown in Figure 1. The results suggested that allele frequencies accurately estimated from pooling DNA samples using fluorescence-labeled primers are identical to allele frequencies estimated from the individual DNAs.

In this study, we detected eight SNPs of the IHRP gene using the method described. Although seven SNPs were reported in the NCBI dbSNP database [http://www.ncbi.nlm.nih.gov/SNP/] and the JSNP db SNP database [http://snp.ims.u-tokyo.ac.jp/], one novel SNP, which had an amino acid substitution, was identified. Those results indicated that our method is as sensitive and accurate as SSCP methods previously reported and is useful for detection and genotyping methods of SNPs.

We detected a novel SNP that showed an amino acid substitution from Ile to Asn. In pig and mouse IHRP genes, this residue is conserved as Ile. The amino acid substitution to Asn might occur in humans or primates. The SNP converted the Sau3AI site from GATC to GAAC: thus the SNP could be detected using PCR-RFLP. Because the SNP has strong linkage disequilibrium with the other five SNPs at nt positions 75, 3723, 10912, 12599, and 13522, the six SNPs were considered to be one locus. Thus, the novel SNP is useful as a genetic marker of the IHRP gene region with PCR-RFLP, because PCR-RFLP is one of the simple methods using conventional restriction enzyme and agarose-electrophoresis.

### Competing interests

None declared.

### Authors' Contributions

TT carried out the molecular genetic studies and drafted the manuscript. NHCM and MT obtained and administered the blood samples. MK helped in setting up the technique. NHCM and MT participated in its design and coordination.

All authors have read and approved the final manuscript.

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