Appl Biochem Biotechnol (2010) 160:927–931
DOI 10.1007/s12010-009-8595-y

Development of a Sensitive Deaminated Single-Strand Conformation Polymorphism (DSSCP)

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Received: 5 November 2008 / Accepted: 24 February 2009 /
Published online: 31 March 2009

Abstract The single-strand conformation polymorphism (SSCP), accompanied by sequencing, is a useful methods for identifying mutations in a DNA fragment. In this study, we have developed a modified SSCP with the aid of sodium bisulfite treatment. The corresponding PCR products for exon 3 of *Hb* gene were sequenced and samples with homozygote and heterozygote single nucleotide substitutions were identified. The PCR products were treated with sodium bisulfite, which deaminates all the cytosine residues. The reaction mixture was then analyzed on non-denaturing polyacrylamide gels. The modified method, which is called deaminated SSCP (DSSCP), was applied successfully in analysis of mutations in the beta-globin gene at positions relevant to codon 6. DSSCP is a very effective and reproducible method providing clear results that are easy to interpret without the involvement of radioactivity.

Keywords Deaminated SSCP · DNA polymorphisms · SSCP · PCR

Introduction

Analysis of DNA polymorphism is important in the study of gene function, genetic diagnosis, and confirmation of the genetic cause of disease. Among the different methods that have been developed for the detection of mutations, polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) has been used extensively. The advantages of this method are its simplicity, sensitivity, and low cost. The principal of SSCP is the altered mobility due to mutation-induced conformational changes in single-strand DNA fragments. Single-strand DNA (ssDNA) in non-denaturing conditions, takes on a three-dimensional structure, due to intra-strand base-pairing that is highly sequence-dependent. Therefore, changes in a single base can cause different secondary conformations, resulting

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in the altered mobility of the fragments when analyzed in a non-denaturing polyacrylamide gel (PAGE).

In practice, the efficiency of SSCP is very variable. In an optimized condition, this method can detect almost 90% of the mutations in DNA fragments of approximately 300 bp or less [1]. In our experience, optimization of conditions for non-radioactive SSCP is laborious and time-consuming. In addition to components of gel and methods of staining, the main obstacle in the establishment of sharp, diagnostically informative, bands is the conditions for the formation of double-strand DNA regions that are required for the detection of mutation-induced conformational changes. In the current study, this difficulty was overcome by sodium bisulfite treatment of PCR products before their analysis on PAGE. Sodium bisulfite treatment chemically converts cytosine residues in DNA to uracil [2]. The consequence of this conversion is that the original DNA strands are no longer complementary. The two DNA strands now represent two non-complementary single-stranded sequences, which are able to form secondary structures with reduced probability of interference by inter-strand hybridizations (Fig. 1). This improved method, called deaminated SSCP (DSSCP), could be applied to PCR products less than 1 kbp.

Materials and Methods

Samples and DNA Isolation

The studied material comprised the blood samples were collected from patients with beta-thalassemia and normal individuals. Genomic DNA was isolated by standard techniques and was used for the PCR reactions [3, 4].

PCR Analysis

PCR amplification of exon 3 of hemoglobin gene (size, 282 bp) was performed in a 25-μl reaction mixture containing 1× PCR buffer, 1.5 mM MgCl2, 160 μM dNTP, 0.5 μM of each primer, 0.5 U Taq DNA polymerase, and approximately 30 ng genomic DNA. PCR amplification was carried out for 30 cycles in a DNA thermal cycler. Each PCR cycle contained denaturation at 94 °C for 40 s, annealing at the 57 °C for 30 s, and extension at

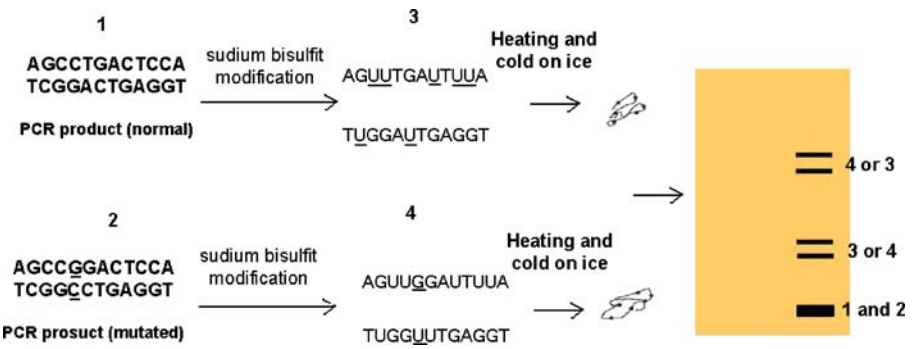

Fig. 1 Principals of DSSCP. Following amplification of template DNA fragment by PCR, the products were denatured and treated with sodium bisulfite, which converts all cytosine residues to uracil. This results in single-stranded DNAs, which are no longer complementary. Three dimensional conformation of each single strand DNA molecule can be revealed by PAGE

72 °C for 30 s. Primers 5′ GATGCTCAAGGCCCTTCATA 3′ (forward) 5′ GAGTCCAAG CTAGGCCCTTT 3′ (reverse) were used.

Sequencing

The corresponding PCR products for exon 3 of *Hb* gene were sequenced by the cycling sequencing method in an ABI automatic sequencer. Each sample was sequenced in forward and reverse directions.

Sodium Bisulfite Treatment of PCR Products

Ten micrograms of PCR products in total volume of 50 μl was denatured by heating at 95 °C for 5 min, 2 μL of NaOH (3 M) was then added and incubated for 10 min at 42 °C, and immediately placed on ice. Bisulfite solution/hydroquinone solution, 0.5 ml, (5 M, pH 5.0) was added to the denatured DNA and incubated at 50 °C for more than 3 h in the dark. DNA purification was performed by using DNA extraction kit (Fermentas, Germany) according to the manufacturer's protocol. Modified DNA was incubated with 5 μl of NaOH (3 M) for 5 min at room temperature. The reaction solution was neutralized by adding 100 μl of ammonium acetate (5 M, NH_4OAc). DNA was precipitated in ethanol for 30 min at −20 °C and high speed centrifugation for 10 min. The precipitated DNA was suspended in 20 μl of TE (Tris–EDTA, pH 8) [5].

DSSCP Analysis

The PCR products of each reaction were treated with sodium bisulfite and then diluted 1:1 with gel loading buffer (20 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol in distilled water). The reaction mixture was denatured for 5 min at 95 °C and then immediately transferred onto ice until gel electrophoresis was carried out. Each denatured single-strand DNA molecule takes on a there dimensional conformation that is dependent on its primary nucleotide sequence. Ten milliliters of the mixture was then separated in a vertical 10% non-denaturing polyacrylamide gel with 5% glycerol [6]. Electrophoresis conditions were as follows: separation distance 15 cm, voltage 190 V, separation time 18 h, temperature 4 °C in 1× TBE (Tris–Boric acid–EDTA) buffer [7]. After electrophoresis, SSCP bands were stained by the fast silver staining method as follows: the gels were removed from glass plates and washed in acetic acid (7.5%) for 10 min. Then the gel was kept at 4 °C in staining solution (1% silver nitrate and 0.6% formaldehyde) for 20 min, washed in distilled water quickly and then soaked in the developing solution (10 ml sodium hydroxide, 37.5%, and 1 ml formaldehyde in 250 ml ddH_2O). When the DNA bands became visible, the gel was washed with water. Analysis of DSSCP gel was performed visually.

Results

PCR products of homozygote DNA templates will produce two distinct SSCP bands. One band corresponds to the upper strand of DNA and the other to the lower strand of the amplified fragment. The detection of more than two SSCP bands in homozygous DNA templates is also possible. These additional bands are, which usually stain more weakly, the result of alternative stable conformations that are the product of both inter- and intra-strand

hybridizations, as well as the primers left over from the PCR reactions. In DSSCP, because of the existence of high quantity of single-stranded DNA and the reduced probability of inter-strand hybridizations, these subsidiary bands were not present (Fig. 2).

In DSSCP, heterozygote samples produce four distinct SSCP bands. Each band corresponds to one strand of the double-stranded DNA molecules. Because of sodium bisulfate treatment, the strands are no longer complementary to each other (Fig. 2). Samples with homozygote mutations, because of the base differences, cause different secondary conformations and thus result in different migration rates of the DNA strands in comparison to the normal controls. Therefore, there are three possible results: two bands in normal condition, two bands with different migration rates compared to normal controls in homozygote mutants, and four bands in heterozygote conditions.

In this study, we used PCR products of exon 3 of human beta-globin for analysis of codon 121 mutations that are associated with hemoglobin D (HbD). As shown in Fig. 2, four bands corresponding to the heterozygote sample with C121S mutation and two bands in the homozygote samples are detected.

Discussion

Single-strand conformational polymorphism is one of the most popular methods for the rapid detection of mutations in short fragments of DNA. However, often several conditions need to be examined in order to optimize the technique for the detection of mutations in a given DNA sequence [8, 9].

In practice, the main obstacle in the application of SSCP is the need to consistently produce the conditions required for obtaining the high resolution bands that are necessary for easy analysis and interpretation of the data. The ideal conditions for production of high throughput high resolution of SSCP gels is the use of DNA strands that are not complementary to each other. In order to accomplish this ideal condition, following amplification, the PCR products could be treated with sodium bisulfite, which converts all cytosine residues to uracil. Therefore, after the bisulfite treatment, the modified single-stranded DNAs would have substantially reduced regions of complementarity. This enabled us to develop a DSSCP method for easy and efficient analysis of single nucleotide

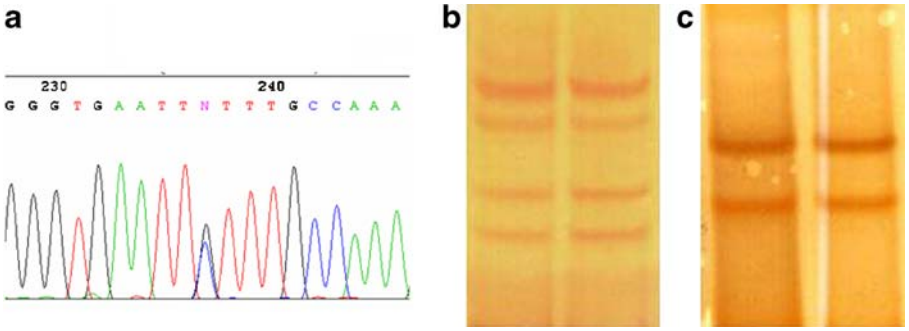

Fig. 2 Examination of codon 121 mutation in beta-globin gene exon 3 with DSSCP. **a** The sequence of a short region of exon 3 of beta globin from an individual whom was heterozygous for the C121S mutation was amplified (positive control). **b** DSSCP analysis of the DNA fragment from exon 3 containing codon 121 mutation. Each band corresponds to the one single strand with special three-dimensional conformation. **c** PCR products were from individuals without mutation in exon 3 of beta globin gene (negative control)

variations in a DNA fragment. The main advantage of this method to the common SSCP is the high resolution of the bands, allowing easy identification of the different single-strand DNA bands following PAGE. Therefore, this method provides a much more robust strategy for high-throughput analysis of mutations in target DNA sequences of up to 1 kb by SSCP.

Acknowledgement This work was supported through a general grant (Pajoohandeh) by the department of research of the University of Isfahan.

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