APPLICATION OF THE POLYMERASE CHAIN REACTION TO THE DIAGNOSIS OF SICKLE CELL DISEASE IN IRAN

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Background – Sickle cell anemia is one of the most common heritable hematologic diseases affecting humans. Detection of the single base pair mutations at codon 6 of the beta-globin gene is important for the prenatal diagnosis of sickle cell anemia and sickle cell disease. We applied the polymerase chain reaction technology to detect sickle cell patients and heterozygous carriers in a group of patients suspected of sickle cell disease.

Methods – The sample was composed of 45 normal individuals and 45 unrelated sickle cell disease out-patients from Hematology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. All patients were interviewed. Results of their medical histories, physical examination, and the hematologic analysis were recorded. The blood samples were collected in EDTA and genomic DNA was extracted from leucocytes. An amplified 110 base pair fragment of beta-globin gene containing codon 6 was digested with the restriction enzyme MS II, and electrophoresed in 3% agarose.

Results – We have established the technical condition for detection of sickle cell disease using a PCR assay. Thirteen patients having hemoglobin SS (Hb SS) and 32 patients in the heterozygous state (Hb AS) were identified. We confirmed that the normal controls had the Hb AA genotype.

Conclusion – This amplification method is rapid, sensitive, and simple, and has the application which is important for the prenatal diagnosis of sickle cell disease.

Keywords • beta-globin gene • Iran • polymerase chain reaction • sickle cell disease

Introduction

Sickle cell disease (SCD) is a major health problem in many countries with a wide spectrum of clinical severity. The SCD can cause numerous disorders that vary with respect to degree of anemia, frequency of crises, extent of organ injury, and duration of survival. This disease affects over 2 million people in Nigeria with a generally severe clinical course. In some parts of Africa as many as 45% of population have sickle cell trait, approximately 8% of blacks carry the sickle gene, and in the United States, the incidence of sickle cell anemia is around 1 in 625 births. The sickle cell gene has also been reported, though with lower frequency, in southern India, Saudi Arabia, the Mediterranean basin, and the Middle-East. In Iran the frequency of sickle cell gene, though with less severity than thalassemia, has been reported. Iranians and some Indians, had different DNA structure not encountered in Africa. Some genetic factors affecting the clinical severity of SCD, including fetal hemoglobin (Hb-F) level and β-gene haplotypes are associated with the chromosomes. This haplotype is associated with much higher Hb F level (15 – 40%), fewer vasoocclusive sickle crisis, lower complications rate, and mild clinical condition. Iranian patients with SCD, on account of their higher level of Hb F compared to Afro-American patients, present a less severe clinical picture. However, they need sustainable life-long medical attention. Accordingly, screening for the sickle cell gene

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carriers would be of great benefit.\textsuperscript{9, 10}

Diagnosis depends upon documentation of the presence of sickle cell hemoglobin, preferably by electrophoresis.\textsuperscript{11} Rapid methods that are less reliable for the detection of sickle cell hemoglobin include the observation of sickling of red cells containing sickle cell hemoglobin microscopically under a coverslip by suspending the cells in a droplet of a 2\% solution of sodium metabisulfite and solubility tests.\textsuperscript{12} However, such tests do not detect hemoglobin C or $\beta$-thalassemia and do not reliably distinguish between sickle trait and sickle disease and are therefore of limited value. With the refinement and automation of techniques, it has also been possible to detect sickle hemoglobin accurately and economically by high-pressure liquid chromatography and by isoelectric focusing.\textsuperscript{13}

Advances in molecular techniques have allowed elucidation of the molecular basis of some genetic diseases that affect people. Use of the polymerase chain reaction (PCR) to detect the sickle mutation is the method of choice for prenatal diagnosis. This paper describes a technique of DNA amplification \textit{in vitro} and its application on detection of sickle cell (Hb S) gene.

Materials and Methods

Clinical samples

Blood samples from 45 unrelated sickle cell disease patients were obtained from Hematology Research Center of Shiraz University of Medical Sciences between November 2001 and September 2002. SCD was identified by positive sickling test and confirmed by hemoglobin electrophoresis at pH 9.2 on cellulose acetate and application of a 300-volt current for 35 minutes. Forty-five normal individuals with no signs or symptoms of SCD were selected from the laboratory personnel. Patients and normal controls were randomly selected from both males and females.

Hematologic analysis

Complete blood count was carried out using a counter Model LUTON BEDS, (Sysmex, England). Sickle cell hemoglobin was quantitated after elution from a microcolumn of diethylamineethyl cellulose (DE 52) resin as described earlier.\textsuperscript{14} Fetal hemoglobin quantitation was performed by alkaline denaturation procedure.\textsuperscript{15}

DNA extraction

One mL of cold lysis buffer, containing 0.2 g Tris, 21 g sucrose, 0.2 g MgCl$_2$, and 2 mL Triton 100X in 200 mL of distilled water, was added to 500 $\mu$L of EDTA-treated peripheral blood and centrifuged. The precipitate was washed three times and genomic DNA was extracted by adding 100 mL of 50 mM NaOH. Subsequently the tube was heated in a boiling waterbath to solublize the DNA.

Polymerase chain reaction

Target DNA sequence of all samples was amplified with the standard PCR condition,\textsuperscript{16} using the following pair of primers (from TIB, Molbiol, Berlin, Germany) 5'-ACACAACGTGTGTTCCAGTAGC and 5'-CAACTTCATCCACGTTCACC, that primed amplification of an 110 base pair (bp) segment of beta-globin gene containing codon 6. The amplified DNA was digested with the restriction endonuclease MS II, which has a recognition site at codon 6 in normal beta-globin gene.
Application of the PCR to the diagnosis of SCD

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Restriction endonuclease analysis of the amplicon DNA

Thirty μL of the amplified product were treated with 1.5 μL of 10 U/mL of MS II restriction enzyme solution (Boehringer, Mannheim, Germany), and digested at 37°C for 1 hour in SURE/cut buffer A in a total volume of 35 μL. Subsequently, the digestion products were separated according to size on a 2% agarose gel (Pharmacia, Sweden), by application of a 70-volt current for 45 minutes and visualized by ethidium bromide staining under ultraviolet light.

The MS II enzyme has a recognition site at codon 6 in the normal beta-globin gene, and cleaved the normal amplified beta-globin DNA into two fragments, while the fragment amplified from DNA of sickle cell mutation remained uncleaved. The agarose gel electrophoresis of the amplified DNA along with the MS II digested products, detected the homozygous and heterozygous patients and normal controls.

Results

A 110 bp fragment of the beta-globin gene containing codon 6, among normal people and patients was amplified. Figure 1 shows the agarose gel electrophoresis of the amplified DNA along with the digested products with MS II enzyme for two patients and two normal subjects. MS II enzyme has a recognition site at codon 6 in the normal beta-globin gene, and cleaved the normal amplified beta-globin DNA into two fragments of 54 bp and 56 bp which was as an overlap band in agarose gel electrophoresis, while the 110 bp fragment amplified from DNA of sickle cell mutation remained uncleaved owing to a single base substitution (A----T) at codon 6 in the mutation. Line 3 in Figure 1 shows the digested products of the MS II enzyme in homozygous patient, and Line 7 represents the presence of the MS II site in normal individual.

While this site was present in all 90 chromosomes of the normal individuals, it was not found in homozygous state of sickle cell patients. The single band with a molecular weight (MW) of about 50 bp observed in the Line 7, is actually composed of a mixture of two equal size products, due to the fact that the MS II site lies almost exactly in the middle of the amplified DNA. Figure 2 shows the agarose gel electrophoresis of the amplified DNA along with the MS II digested products for three patients in the homozygous state (p) and two heterozygous patients (h). Lines 7 and 11 in Figure 2 represent the heterozygous state with two different bands. One band with a MW of about 110 bp corresponding to the amplified DNA of sickle cell mutation remained uncleaved with MS II digestion, and another band which is a mixture of two equally sized products corresponding to the normal amplified beta-globin gene. This methodology for SCD permitted us to identify 13 patients (14.4 %) with sickle cell anemia (having Hb SS) and 32 patients (35.5%) in the homozygous state (Hb AS) (Table 1). We confirm that the normal controls have the Hb AA genotype.

Discussion

Sickle cell hemoglobin is a mutant hemoglobin in which valine has been substituted for the glutamic acid normally at the sixth amino acid of the β-globin chain. This hemoglobin polymerizes and becomes poorly soluble when oxygen tension is lowered, and red cells that contain this hemoglobin become distorted and rigid.11 The diagnosis of sickle cell anemia rests on the

<table>
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<th>Studied individuals</th>
<th>No. tested</th>
<th>Total (%)</th>
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<tbody>
<tr>
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<td>35.5</td>
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<tr>
<td>Homozygous patients</td>
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<td>14.4</td>
</tr>
<tr>
<td>Normal controls</td>
<td>45</td>
<td>50.0</td>
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Table 1. Identification of the sickle cell gene in patients compared with normal controls.
electrophoretic pattern or chromatographic of hemoglobins in hemolysates prepared from the peripheral blood. However, several relatively hemoglobin variants have an electrophoretic mobility identical to that of Hb S on cellulose acetate.² Although the diagnosis of SCD is straightforward, that of Hb Sβ-thalassemia may sometimes be problematic. In Hb Sβ+ thalassemia, there is a preponderance of Hb S with Hb A comprising 5 – 30% of the total. Hb Sβ0 thalassemia produces an electrophoretic pattern that is visually indistinguishable from that of sickle cell anemia, but a diagnosis can often be made by the presence of elevated Hb A2 level and a decreased MCV. However, detailed family history and DNA-based studies may be necessary to make this distinction.²

In the last 2 decades, the molecular pathology of the β-thalassemia and the mutation phenotype relationships of these disorders have been largely elucidated. This knowledge has been applied to molecular diagnosis, carrier identification, and prenatal diagnosis, and has resulted in dramatic reduction of the incidence of the homozygous state in several at-risk populations.¹⁰ Detection of sickle cell gene by analysis of amplified DNA sequences was done in China⁹ and Venezuela¹⁷ by using the endonuclease MS II. Hatcher et al¹⁸ designed an enzymatic amplification and restriction endonuclease digestion method for detection of Hb S.

In the previous study,³ we reported the frequency of the sickle cell gene in South Iran. In Fars Province, in southern Iran, with a gene frequency of around 0.01, which is one-tenth of β-thalassemia gene frequency, the sickle cell gene is not too prevalent.³ However, infection must have been the most likely selective force operating in the dissemination of the sickle cell gene.¹⁹ So, screening for sickle cell carriers would be of great benefit.¹⁷ In the present study, we have established the technical condition for detection of sickle cell mutation using a PCR assay that eliminates the need for other hematologic analysis. This methodology permitted us to identify the normal controls, the homozygote and heterozygote sickle cell patients. This amplification method is specific and sensitive, thus permitting rapid economical diagnosis of SCD. Also, as the detection of the single base pair mutations at codon six of the beta-globin gene is important for the prenatal diagnosis of sickle cell disease,¹⁸ this technique has research potential that is important for the prenatal diagnosis of sickle cell disease.

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