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Identification of Hb D-Punjab Gene: Application of DNA Amplification in the Study of Abnormal Hemoglobins

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Summary

Hemoglobin D-Punjab (or D-Los Angeles) is a common variant worldwide. It is also the most frequent abnormal hemoglobin in Xinjiang Uygur Autonomous Region of China. A large survey of hemoglobinopathy, including 142,171 people and 21 national/ethnic groups, was carried out in Xinjiang and indicated Hb D-Punjab accounted for 55.6% of the total hemoglobin variants there. Here we describe a simple way—*Eco*RI mapping of the amplified β-globin DNA sampling from dried blood spots on filter paper blotters—of identifying the Hb D-Punjab gene. The primers were designed and synthesized to emzymatically amplify a 144-bp fragment of β-globin gene which included codons β 121 (GAA) and 122 (TTC) representing an *Eco*RI recognition site. The Hb D-Punjab gene could be easily detected by *Eco*RI digestion of the amplified DNA sequence on agarose gel because of a single base change at codon 121. The analysis of amplified DNA sampling from dried blood provides a very useful method for population study of Hb D-Punjab and will be of significance for demonstration of the occurrence of the Hb D-Punjab gene and for understanding of the relations among various nationalities.

Introduction

Hemoglobin D-Punjab or D-Los Angeles (β 121 Glu→Gln) is one of the most commonly encountered abnormal hemoglobins worldwide (Bunn et al. 1977). It is present in a large number of people in Pakistan and Northwest India and at a high frequency in the Punjab (Brittenham 1987). Among Sikhs of the Punjab region, Hb D-Punjab has an incidence of 2%–3%. Many scientists are interested in this abnormal hemoglobin, because Hb D-Punjab not only results in sickle cell/Hb D disease clinically but also has served as a good material for genetic anthropological studies.

Hb D-Punjab is also quite common in China. Over the past 10 years, a large-scale survey for hemoglobinopathies was conducted on the China mainland, and a number of individuals with Hb D-Punjab were found in several national/ethnic groups (Zeng and Huang 1987). During the survey in Xinjiang Uygur Autonomous Region (Li et al. 1986), it was significant to find that Hb D-Punjab was very frequent there and accounted for 55.6% of the total abnormal hemoglobins in Xinjiang. Further investigation revealed that the frequency of this hemoglobin variant in Mongolia was significantly low when compared with that in Uygur, Kazak, and Khalkhas (Li et al. 1986*a;* 1987). This result obviously disproved the hypothesis that the Hb D-Punjab gene originated in Mongolians (Lehman and Huntsman 1974). It would therefore be of great interest and significance to examine and identify Hb D-Punjab in additional populations, for understanding of the origin of this hemoglobin variant.

The Hb D-Punjab mutation can be identified either by protein chemical structural analyses (Baglioni 1962; Zeng and Huang 1984; Li et al. 1986*b*) or by restriction mapping (Zeng et al. 1986). Both methods are labor intensive and time-consuming; furthermore, they require relatively large quantities of blood. The Southern blot procedure needs radioactive isotope for the labeled probes. Therefore, these methods are not suitable for mass screening. The advances in DNA amplifica-

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tion with polymerase chain reaction (PCR) (Saiki et al. 1985) provide a novel technique for the studies of gene mutations. In the present paper, we introduce the application of this technique to develop a new approach to the study of Hb D-Punjab, i.e., nonradioactive restriction mapping of the amplified DNA sampled from dried blood spots on filter-paper blotters. This simple and rapid method greatly facilitates the detection of Hb D-Punjab gene and would provide a useful tool for implementing mass screening of Hb D-Punjab at the molecular level.

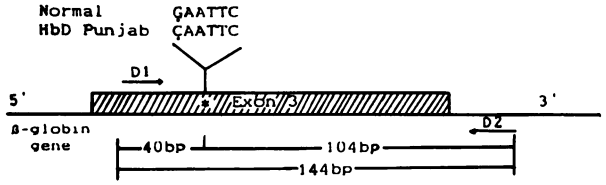

Figure 1 Schematic representation of 144-bp DNA fragment amplified with primers D_1 and D_2. The amplified DNA spans the greater part of exon 3 and the 3′ intronic sequence of β-globin gene. The asterisk (*) indicates the *Eco*RI recognition site.

Material and Methods

Subject

In the survey of hemoglobinopathies in Xinjiang, 85 cases were recognized as Hb D-Punjab with a Glu→Gln substitution at the position of β 121 (GH4) by the chemical structural analyses (Li et al. 1987). Five Hb D-Punjab heterozygous individuals from the 85 cases of Hb D-Punjab carriers, as well as 10 normal individuals, were selected for DNA analysis. They belonged to the Han, Uygur, Mongol, Kazak, and Tibet nationalities, respectively, and lived in different parts of Xinjiang region. Whole-blood samples (50–100 μl) from these individuals were spotted directly or were anticoagulated with EDTA before being spotted on Whatman 3 MM filter papers according to the method we have described elsewhere (Huang et al. 1986; McCabe et al. 1987). Dried blood specimens were mailed to the Laboratory of Medical Genetics, Shanghai Children's Hospital.

DNA Amplification

Dried blood specimens were washed with normal saline two times and then were centrifuged once. The precipitates were lysed in 100 μl of 0.1 M NaCl, 5% SDS solution followed by PCR without prior DNA extraction procedure. The modification of DNA amplification by PCR with *Taq* DNA polymerase (New England Bio-Labs) was performed according to the method described elsewhere (Huang et al. 1988; Saiki et al. 1988). The primers (D_1 and D_2), 5′-AACGTGTGCTGGTCTGTGTGCT-3′ and 5′-AAATTGGACAGCAAGAAAGC-3′, were designed and synthesized by an Applied Biosystems DNA synthesizer and were purified by 20% polyacrylamide/7 M urea gels and C_{18} Sep-PAK column to allow enzymatic amplification of a β-globin DNA sequence from nucleotide 1355 through nucleotide 1498 that included codons 121 (GAA) and 122 (TTC) (fig. 1). The amplified target sequences were detected with ethidium bromide under UV light on 2% agarose-gel electrophoresis.

Restriction Mapping of Amplified DNA

Ten microliters reaction mixture of amplified β-globin DNA was digested with 10 units *Eco*RI (New England Bio-Labs) according to the recommendation of the manufacture and then was analyzed on 3% NuSieve agarose gel (Karlan Chemical Co.).

DNA Oligonucleotide Hybridization

The oligonucleotide probes for detection of normal (N) and β-D Punjab (M) genes were designed and constructed as 5′-GGGTGAATTCTTTGCCAAA-3′ and 5′-TTTGGCAAACAATTCACCC-3′, respectively. One-thirtieth of each reaction mixture of amplified DNA was adjusted to 0.4 N NaOH, 25 mM EDTA in 200-μl volume and was applied to zeta probe nylon filters with a Bio-Dot Spotting Apparatus (Bio-Rad), Oligo-probes were labeled at the 5′ end with [γ-^{32}P] ATP, and dot-blot hybridization was performed with the amplified DNA sequences in zeta probe nylon membranes. After being washed, the membranes were exposed to X-ray films for 4 h at −76C.

Results

The normal and Hb D-Punjab carrier DNA amplified with the primer pair D_1 and D_2 revealed a single band of 144 bp on agarose-gel electrophoresis. The normal amplified DNA gave two fragments, one each of 104 and 40 bp, after digestion by *Eco*RI; however, the amplified DNA from Hb D-Punjab carriers had three visible bands of 144 bp/104 bp + 40 bp when digested with the same restriction enzyme (fig. 2).

Oligonucleotide hybridization experiments showed that the amplified DNA from Hb D carriers could be detected by both the normal and mutant probes; but

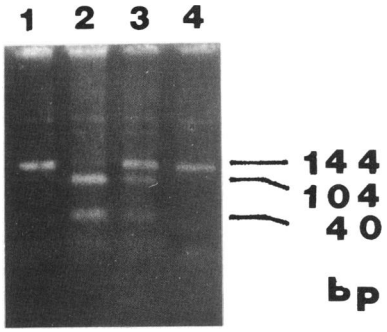

Figure 2 NuSieve agarose gel electrophoresis of amplified DNA after digestion with *Eco*RI (40 MA, 1 h). Lane 1, normal amplified DNA without *Eco*RI digestion; lane 2, normal amplified DNA after *Eco*RI digestion; lane 3, amplified DNA from Hb D-Punjab carriers after *Eco*RI digestion; lane 4, amplified DNA from Hb D-Punjab carriers, without *Eco*RI digestion.

the normal amplified DNA only hybridized with the normal probe (fig. 3).

Discussion

Hemoglobin D-Punjab or D-Los Angeles was first discovered in a family of British and Indian descent in Los Angeles (Itano 1951), and its chemical structure was characterized as Glu→Gln substitution at β 121 in 1962 (Baglioni 1962). This abnormal hemoglobin has been reported in many parts of the world, but mainly in the Indian subcontinent. The origin of this abnormal hemoglobin remains unclear. Recently, a large survey of hemoglobinopathies, including 142,171 people and 21 national/ethnic groups, was carried out in Xinjiang Uygur Autonomous Region of China, and quite a number of Hb D carriers were discovered in this study (Li et al. 1986*b*). This result offers us good opportunities to further investigate the origin, drift, and other interesting aspects of the Hb D-Punjab gene. So far, 85 cases with Hb D have been subjected to the chemical structural analysis (Li et al. 1986*a*). However, there still remain many Hb D cases to be structurally identified. As we know, chemical structural analysis requires substantial effort, and it seems impossible to be used in large quantities of analyses.

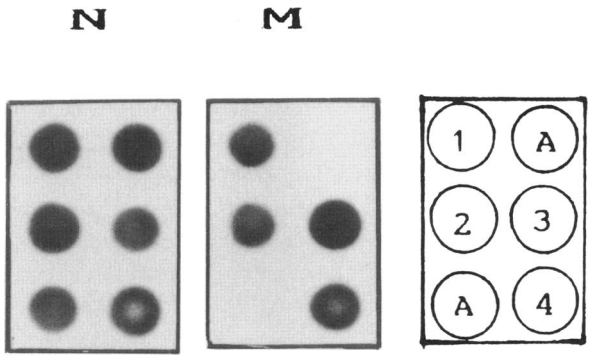

Figure 3 Detection of Hb D-Punjab mutation (β 121 GAA→CAA) by using specific oligonucleotide probes. N = normal probe; M = mutant probe; A = normal amplified DNA; 1–4 = amplified DNA from four cases of Hb D-Punjab carriers.

The present paper describes a simple way to identify the Hb D-Punjab mutation. We used a pair of primers to direct amplification of a 144-bp fragment that flanked most of exon 3 and a portion of the 3′ intron in the β-globin gene; this region included codons 121 (GAA) and 122 (TTC). The latter sequence (5′-GAATTC-3′) was an *Eco*RI recognition site, so the normal amplified DNA could be digested by this enzyme and cleaved into two fragments of 104 and 40 bp that could be visualized on gel electrophoresis. Hb D-Punjab results from a single base substitution at codon 121 (GAA→CAA) of the β-globin gene; thus, the *Eco*RI site was disrupted and the intact 144 bp of amplified DNA remained. Therefore the amplified DNA from Hb D-Punjab carriers revealed three fragments, one each of 144, 104, and 40 bp (fig. 2).

In the analysis of the Hb D-Punjab mutation by the method of restriction mapping of the amplified DNA, a minor band of 144 bp would appear sometimes in the amplified DNA samples from normal individuals, owing to incomplete digestion. However, this 144-bp band is quite faint when compared with the major bands of 104 and 40 bp. On the contrary, the 144-bp band is much more intense than the bands of 104 and 40 bp in the amplified DNA from heterozygotes for Hb D-Punjab (fig. 2). Therefore, it can easily distinguish a normal individual from a Hb D-Punjab carrier by using this method.

It also should be considered that two other hemoglobin variants, Hb Beograd (β 121 Glu→Val) and Hb O-Arab (β 121 Glu→Lys), also can be detected by *Eco*RI digestion, because of the base changes at the same codon as in Hb D-Punjab (Forget 1979; Phillips et al. 1979). For further characterization of the Hb D mutation, a pair of oligonucleotides specific for the normal and mutant β-globin sequences were hybridized with the amplified DNA (fig. 3). It not only distinguished the normal sequences from the abnormal, but this approach could also be used to distinguish Hb D-Punjab from Hb O-Arab or Hb Beograd, and an Hb D homozygote

from Hb D/β-thalassemia disease. Our results indicated that the cases we analyzed in Xinjiang belonged to the C→G mutation at codon 121 of the abnormal β-globin gene. But we suggest that, if this variant is very frequent in the studied regions, it is unnecessary to routinely use the oligo-hybridization procedure in every case of Hb D-Punjab, especially for the population-screening purpose.

Sampling from dried blood specimens would ease the sample transport. Our experience suggested that it was very useful for sample collection from rural areas and for individuals who were culturally and geographically isolated. DNA amplification with PCR from dried blood samples, without prior DNA extraction, offered a rapid and simple method for detection of Hb D carriers in the general population, and it will be of great significance for understanding the origin of Hb D-Punjab and the drift of this mutant gene, as well as the relations among various nationalities.

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