Beta Globin Frameworks in Thalassemia Major Patients from North Iran

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Abstract

Objective: Four combinations of five neutral sequence changes at rs713040, rs10768683, rs7480526, rs7946748, and rs1609812 occurring in the human beta globin gene defined as frameworks have been reported in beta globin gene. Here we report for the frequency of these frameworks in thalassemia major patients of North Iran.

Methods: Beta globin gene frameworks of 46 thalassemia major patients of North Iran were determined using Denaturing Gradient Gel Electrophoresis.

Findings: All these frameworks called framework 1, 2, 3, 3a were present at the frequency of 23.9%, 45.7%, 6.5% and 23.9% respectively.

Conclusion: These frameworks may be used for tracking mutant alleles in prenatal diagnosis programs.

Introduction

Techniques that allow the detection of single base changes in genomic DNA have had a major impact on our understanding of human genetic diseases, both by identifying specific mutations that result in disease[1,2] and by identifying DNA polymorphisms that are used as genetic markers in linkage studies[3-5]. Detection of beta globin gene lesions in thalassemic patients and carriers is based on screening for the mutations most often found in the relevant ethnic group. But in circumstances in which an unknown mutation is encountered, another difficulty arises when at-risk couples present at a late stage of pregnancy with an unusual beta globin mutation asking for prenatal diagnosis. Since the nature of the parental beta thalassemia alleles must be determined before fetal sampling, it is fundamental to find either parental specific mutations or DNA polymorphisms in beta globin gene cluster for linkage studies. In some instances base changes have been identified as restriction fragment length polymorphisms (RFLPs) near or within beta globin gene[6,7]. RFLPs are detected by determining the presence or absence of a restriction enzyme cleavage site within a DNA fragment. Few intragenic polymorphic sites detectable by restriction enzymes were reported for beta globin gene. This concerns rs713040 or codon2 (C-T) recognized by HgiAI, rs10768683 or IVS2-16 (C-G) recognized by AvaII and rs1609812

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or IVS2-666 recognized by SspI[8,9]. As there are hot spots for recombination upstream of beta globin gene[10,11] and few RFLPs within beta globin gene, it is wiser to study either polymorphism in both sides of beta globin gene or look for polymorphisms within beta globin gene for prenatal diagnosis. Moreover in many instances RFLPs are not informative due to the low degree of polymorphism and each restriction site's study necessitates a separate PCR reaction. The combined PCR and Denaturing Gradient Gel Electrophoresis (DGGE) can be used to detect the presence of uncommon mutations or natural polymorphisms within beta globin genes[12,13]. This method permits the separation of strands of small DNA fragments (200-700bp) according to the nature of their sequence. It involves the electrophoresis of double stranded DNA fragments through an acrylamide gel containing a linear gradient of DNA denaturants, such as formamide and urea. Using this method, 4 different beta globin gene frameworks called FW1, FW2, FW3 and FW3a representing combinations of 5 natural sequence changes at exon one (one site) and intron two (4 sites) of beta globin can be distinguished upon a single PCR reaction[12]. These five neutral sequence changes occur in the human beta globin gene in codon 2, IVS 2 (nt 16, 74, 81 and 666). The five neutral sequence changes at codon 2 C/T, IVS 2 nt 16 C/G, nt 74 G/T, nt 81 C/T, nt 666 T/C occurring in the human beta globin gene define four frameworks called: 1, 2, 3 and 3a (Table 1) that differ from each other by at least one nucleotide[12]. To detect these polymorphisms, we analyzed the DGGE pattern of the fragment corresponding to nucleotides +188 to +621 of beta globin and containing IVS2 (nt 16, 74, 81) and report the frequency of these frameworks among 92 beta globin alleles belonging to 46 thalassemia major patients for the first time in northern Iran.

Subjects and Methods

Blood samples were collected from 46 unrelated regularly transfused thalassemic children of Mazandaran Province, Iran. For each sample, DNA was obtained from 0.5 ml blood after alkaline lysis of white blood cells[14]. All patients were compound heterozygote with one or both mutations being unknown or present at a frequency lower than 2% in the population studied. Mutations studied are those reported by Akhavan-Niaki et al[15].

DNA amplification: PCR was performed using a Techne progene thermal cycler (UK). The primer sequences and PCR conditions are as described in Ghanem et al[12], for fragment G amplification. A 474 bp DNA fragment corresponding to nucleotides +188 to +621 of beta globin gene was amplified using Forward 5'CTGGGCATGTGGAGAC-AGAG3' primer containing an additional 5'GC rich oligomer which creates a high temperature melting domain: 5'GCGGGCGGGGCGGGGCGCGGCG-GGCGGGGC called GC clamp and a Reverse 5'CACTGATGCAATCATTCGTC3' primer (Bioneer Company, South Korea). Fig. 1 shows the position of the amplified fragment and the 5 single nucleotide polymorphisms.

DGGE was accomplished according to Ghanem et al[12]: 10 μl of PCR product was subjected to electrophoresis at 160 V for 5 hours in a 6.5% polyacrylamide gel containing a linearly increasing denaturant gradient of 10% - 60% in a water bath at 60℃. After migration the gel was stained by Ethidium bromide and the position of different bands visualized under 312 nm UV wavelength. As DGGE is based on the principle that two double-stranded DNA fragments of the same size, but differing in sequence, melt at different points in a denaturing gradient and can be distinguished by differential migration, a
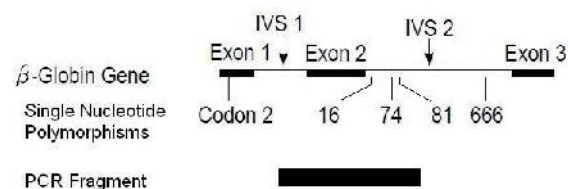

Fig 1. Beta globin gene representation: A portion of beta globin gene spanning from nucleotide +188 to +621 and covering exon1 and 2 was amplified using PCR. The position of the 5 single nucleotide polymorphisms (one in Exon 1 and four in Intron 2) is also presented. Three polymorphic nucleotides IVS2(nt 16,74,81) are present in the amplicon, and their association defines the beta globin frameworks.

Table 1: Nucleotide combinations defining the beta globin gene frameworks (Ghanem et al[12])

Framework	Codon 2	IVS2-16	IVS2-74	IVS2-81	IVS2-666
Framework1	C	C	G	C	T
Framework 2	C	C	T	C	T
Framework 3	T	G	T	T	C
Framework 3a	T	G	T	C	C

uniform temperature with a linear denaturant gradient formed by urea and formamide creates the denaturing environment in DGGE.

Findings

DGGE analysis of amplicons belonging to 46 thalassemia major patients revealed that framework 2 is the most abundant among mutant alleles analyzed and represents 45.7% of the cases. Frameworks 1 and 3a represent each 23.9% of the cases and framework 3 is the less frequent, namely 6.5%. Table 2 shows the frequency of each framework in the population studied and Fig 2 shows DGGE patterns for individuals with different combinations of these frameworks. As expected, in individuals homozygous for a particular framework, heteroduplex cannot form upon renaturation of the amplicon's strand and only homoduplexes corresponding to complete matching between the two complementary strands of amplicons are possible and consequently a single band is visible upon electrophoresis in a gradient gel and under denaturing conditions (Fig. 2, lane 6). In heterozygous individuals, 4 bands are detectable under similar conditions: 2 bands migrating faster and representing homoduplexes corresponding to each framework and 2 other bands which migrate slowly comparing to homoduplexes due to mismatches between complementary DNA strands belonging to different frameworks. Each framework combination has its particular pattern of heteroduplexes and homoduplexes migration as nuclotidic sequences and consequently DNA migration pattern under denaturing gradient gel electrophoresis varies in each framework. The higher the number of G and C in the polymorphic

sites of the amplican, the faster is the migration of homoduplexes. Thus, amplicons corresponding to framework 1 which contains IVS2 nucleotide polymorphisms C, G and C respectively at positions 16, 74 and 81 migrate faster than amplicons corresponding to framework 3a with nucleotides G, T and C and framework 2 with nucleotides C, T and C at those polymorphic positions. Amplicons corresponding to framework 3 with nucleotides G, T and T at the above mentioned positions are the slowest (Fig. 2).

Table 2: Frequency of beta globin gene frameworks in 92 mutant beta globin alleles in northern Iran

Framework	Number (%)
Framework1	22 (23.9)
Framework 2	42 (45.7)
Framework 3	6 (6.5)
Framework3a	22 (23.9)
Total	92 (100)

Discussion

Although extensive studies were performed for molecular characterization of beta globin mutations in Northern Iran[15-17], but as in any prenatal diagnosis program, new or undefined mutations may be encountered. Thus other methods are needed to detect the mutant alleles. Different restriction site polymorphisms have been described near beta globin gene[6,7], but few of them are intragenic markers[8,9]. Here we report for the first time polymorphisms for five intragenic markers in North Iran using DGGE analysis. The combination of these 5 polymorphisms defines 4 frameworks. In this study, we have determined the frequency of these
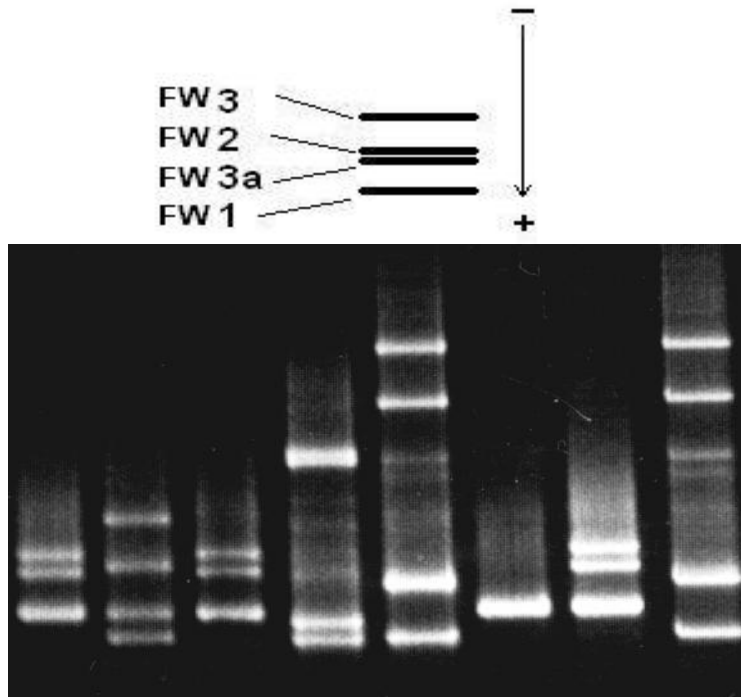
Fig. 2: A: Schematic representation of relative migration pattern expected for different frameworks' homoduplex after Denaturing Gradient Gel Electrophoresis. B: Pattern of different combinations of beta globin gene frameworks using DGGE analysis. Lanes 7 and 8 controls with 2/3a and 1/3 frameworks respectively. Lanes 1, 3: 2/3a – Lane 2: 1/2 – Lane 4: 1/3a – Lane 5: 1/3 – Lane 6: 2/2. In each lane, lower bands migrate faster and thus correspond to homoduplexes while upper bands correspond to heteroduplexes.

4 different frameworks among mutant beta globin alleles present in Northern Iran. Our data show that all 4 frameworks exist in the population studied. Their frequency vary between 45.7% for the most abundant (framework 2) to 6.5% for the less abundant (framework 3).

DGGE analysis of beta globin gene was also performed in other populations. In India where framework 3a is the most prevalent with a frequency of 66.5% followed by framework 1 with a frequency of 25.2%, frameworks 2 and 3 represent 5.8% and 2.5% respectively of the 206 mutant alleles studied[18]. Another study accomplished in North Thailand showed that the frequency of FW1 and FW3a was 73.3% and 26.7% respectively in 30 mutant alleles studied[19]. Similar frequencies were also reported in a limited number of mutant alleles analyzed in Korean population[20]. The difference in framework distribution and their frequencies among population studied reflect the differences in both mutation spectrum and the degree of heterogeneity within a population. Indeed framework 2 was the most prevalent in North Iran with a frequency of 45.7% while it was absent in East Asians and rather rare in Indian population studied. As IVSII-1(G-A) mutation is the most prevalent mutation in North Iran with a frequency of around 60%[15,16] and compound heterozygotes presenting IVSII-1(G-A) alleles included in this study were also possessing the framework 2 (Data not presented), IVSII-1(G-A) may be associated to framework 2. This could explain the difference in framework distribution and frequencies observed in Iranian and Indian or East Asian populations where IVSII-1(G-A) was not reported.

Although direct mutation detection is routinely performed either by using ARMS PCR or Reverse Dot Blot analysis or sequencing analysis in molecular diagnosis of thalassemia, this indirect method which necessitates segregation analysis

may be used either in cases where unknown mutations are encountered or as a complementary method to confirm the genotype. Many diagnostic laboratories apply traditional haplotype analysis by co-segregation studies within a family either in order to detect an unknown mutation for carrier screening or prenatal diagnosis in families with a previous history of disease or as a complementary analysis to direct mutation methods in prenatal diagnosis to confirm the genotype of the fetus. However, most polymorphic sites studied routinely using restriction enzymes are either located outside the gene which may be subject to errors in genotype determination at beta globin gene locus in rare cases of recombination due to the presence of a hotspot for recombination located upstream of beta globin gene[10,11] or may be located within the beta globin gene with a low degree of polymorphism and therefore non-informative in many families[9]. The frameworks characterized in the present study represent a combination of 5 single nucleotide polymorphisms all located within the beta globin gene which abolishes errors due to recombination. Moreover a single PCR reaction allows the simultaneous evaluation of the genotype in all 5 intragenic polymorphic sites determining each specific framework, while in segregation analysis using RFLP method, genotyping of each polymorphic site necessitates a separate PCR reaction followed by enzymatic digestion. Regarding their abundance these polymorphisms could be studied either as frameworks or as single polymorphic sites using restriction endonuclease analysis: Alw44 for codon 2[8], AvaII for IVS2-16[8], MaeIII for IVS2-81[12] and SspI for IVS2-666[9] as intragenic markers for detection of beta thalassemia by linkage analysis. Polymorphism at IVS2 nt666 was studied separately in North Iran as the T nucleotide at this position is recognized by SspI[8]. Our previous findings show that IVS2 nt 666(T) is present in about 85.7% of mutant alleles[8]. This nucleotide is present in frameworks 1 and 2 which together represent about 70% of studied mutant alleles. As Table 1 indicates the C allele at codon 2 is in the same linkage phase than IVS2 nt 16(C) and IVS2 nt 666(T). The two former polymorphisms can also be distinguished by restriction analysis using Alw44 and AvaII respectively. Thus as Alw44 is commercially less expensive than AvaII, we recommend the use of the former enzyme in segregation analysis using RFLP.

Conclusion

All 4 defined frameworks were present in beta thalassemia chromosomes at a frequency higher than 5% and consequently may be useful for tracking mutant alleles in prenatal diagnosis programs*.*

Acknowledgment

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