Detection of Common Beta-thalassemia Mutations by Reverse Dot Blot Analysis

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Abstract: Beta (β)-thalassemia is the most common genetic disease of anemia caused by mutations on beta globin gene. In Vietnam, there is a high frequency of β-thalassemia carriers with a prevalence ranging from 1.5 to 25.0 % in the different ethnic groups. The most common mutations are the nonsense in codon (CD) 17 (A>T), codon 26 (G>A) and the frameshift at codons 41/42 (-TTCT). The polymerase chain reaction-amplification refractory mutation system (PCR-ARMS) is challenged by using a great number of primer sets for detecting normal and mutated alleles. Reverse dot-blot hybridization using oligonucleotide probes can simultaneously detect allelic specific mutations on a membrane. In this study, we designed oligonucleotide probes specific to the three most common mutations in CD17, CD26 and CD41/42, and used them to optimize hybridization conditions at 68°C in 6xSSC hybridization buffer, 2XSSC washing buffer for detecting homozygous or heterozygous alleles of beta globin gene in fifteen individuals of 5 families. Our results were consistent with those detected by PCR-AMRS method, indicating that oligonucleotide probes created by this study was specific, and that the reverse dot-blot hybridization using these oligo probes was convenient for analysis of beta thalassemia disease in Vietnam.

Keywords: Beta globin gene, beta thalassemia disease, polymerase chain reaction-amplification refractory mutation system (PCR-ARMS), reverse dot-blot analysis.

1. Introduction

Thalassemias, the commonest monogenic disorders among the people living in Southeast Asia, result from mutations on genes encoding globin proteins [1]. Mutations on alpha (α)- and beta (β) globin genes lead to defective synthesis of the globin chains of adult hemoglobin.
were observed to alpha thalassemia whereas the most common point mutations were found in beta thalassemia. At molecular level, beta-thalassemia represents a great heterogeneity as more than 200 mutations have been identified for the beta-globin gene responsible for this disease [3]. Based on the reduced (β+) or absent (β0) synthesis of the beta globin chains, clinical consequences increase from mild to severe anemia that require regular blood transfusion [3]. The major methods for mutation detection of beta thalassemia are PCR-based techniques such as amplification refractory mutation system (ARMS) and allele specific oligonucleotide probes (ASO) or reverse dot blot assay (RDB) [3]. The ARMS is a simple method in which two primers identical in sequence except for 3’ terminal nucleotide; one complementary to normal DNA and the other to mutant DNA at 3’ terminal nucleotide. The ASO is method in which probe pairs are bound to nylon membrane in the form of dots or slots then amplified labelled DNA is hybridized to membrane; this method can detect multiple mutations at once [4].

In Vietnam, the carrier rate for β thalassemia varies from 1.5 % to 25 % depending on the ethnic groups of the population [5]. The ARMS method has been routinely applied for detection of mutations in small samples of β thalassemia [6,7]. Molecular analysis of β thalassemia by using the RDB method was reported a long time ago [8]. In this study, we designed oligonucleotide probes that were specific to three common mutations (CD17, CD26, CD41/42) in beta thalassemia and applied them to RDB for analysis of this disease in North Vietnam.

2. Materials and methods

Tissue samples: Fifteen anticoagulant blood samples from 5 families each included father, mother and children who suffered from β thalassemia disease were obtained from Military Medical University in the period from August, 2013 to September, 2015. Heterozygous state of three codon CD17, CD26 and CD41/42 of fifteen samples were confirmed by ARMS analysis.

DNA Isolation: DNA was isolated from blood samples by using Mini DNA Extraction Kit (Qiagen). The concentration of DNA was quantified by determination of OD260 and the quality of DNA was estimated on electrophoresis agarose gel.

Oligonucleotides and primers: Oligonucleotides for RDB and primer set for biotin labeled PCR products were designed from the nucleotide sequence of the beta globin gene (GeneBank version: U01317.1). They were designed on basis of FastPCR programme and supplied from IDT (USA). The oligonucleotides were modified by adding NH2 group at 5’ end. The NH2 modified oligonucleotides, primer sequences and PCR conditions were presented in Table 1.

<table>
<thead>
<tr>
<th>Oligos/Primer Sequences</th>
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<tr>
<td>Oligos/Primer Sequences</td>
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<tr>
<td>CD17 N NH2-C12-GTGGGGCAAGGTGAACGTG</td>
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<tr>
<td>CD17 M NH2-C12-GTGGGGCTAGGTGAACGTG</td>
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<td>CD26 N NH2-C12-CAGGGCCATACCAACCA</td>
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<td>CD26 M NH2-C12-TTCGGTGTAAGGCCCT</td>
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<tr>
<td>CD41 N NH2-C12-CCCAGAGGTTCTTGTCC</td>
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<tr>
<td>CD41 M NH2-C12-CCCAGAGGTTGAGCCTT</td>
<td></td>
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<tr>
<td>ASO-F TCCTGAGGAGAAGTCTGCCGTT</td>
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<tr>
<td>ASO-R GTTGGCCTAAACGCATCAGGAGT</td>
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Dot blot analysis: Biodine C membrane was activated in 10% EDC for 10 min; it was rinsed briefly in water and air-dried for 30 min at room temperature. NH2-oligonucleotides were
diluted with 0.5 M NaHCO₃/Na₂CO₃ buffer, pH 8.4 for application into the membrane. Dot blot analysis were carried out at two temperatures of 55 °C or 65 °C. The membranes were prehybridized for 20 min and then hybridized for 40 min. The solutions for prehybridization and hybridization were 2 x SSC, 5 x Denhart and 0.1 % SDS for procedure at 55 °C and 6 x SSC, 5 x Denhart and 0.1 % SDS for procedure at 65 °C. The membranes were washed twice with 5 x SSC at room temperature for 5 min, and blocked with 1 x TBS solution containing 0.1 M Tris–HCl pH 7.6 and 0.15 M NaCl. Color spots were detected by enzymatic color reaction using streptavidin alkaline phosphatase (Promega).

3. Results

Genomic DNA was extracted from blood samples of five family each included three member (father, mother and children suspected in beta thalassemia) by using Mini DNA Extraction Kit (Qiagen) and quality of DNA was checked on 1 % electrophoresis agarose gel. Figure 1 indicated that genomic DNA has good quality and was suitable for further analysis.

Genomic DNA was used as template for preparation of biotin labeled PCR products. The PCR products were checked on electrophoresis gel (Fig. 2). The results revealed that fragment labeled with biotin was larger than the non labeled one, indicating that PCR products were labeled successfully.

The biotin labeled PCR products were hybridized to membrane on which a serial dulution of the 5’ NH₂-oligonucleotides were deposited. The solutions of 2 x SSC or 6 x SSC were used in all hybridization procedure (prehybridization, hybridization); however, hybridization temperature was varied from 55 °C to 65 °C corresponding to 2 x SSC and 6 x SSC solutions, respectively.

The results shown in Fig. 3 indicated that color signals distinguished specific alleles were obversed when 0.5 μM of oligonucleotides were deposited on the membrane and optimized hybridization occurred in the 6 x SSC solution at the temperature of 65 °C.
Subsequently, biotin labeled PCR products amplified from DNA that was extracted from individuals of each family were applied to dot blot assay with the optimized conditions. Fig. 4A showed the membrane that have been hybridized with the biotinylated DNA amplified from individuals with different mutations in the \( \beta \) globin gene.

![Dot blot analysis](image)

Figure 4. (A) Reverse dot-blot analysis for a family (Bo: father, Me: mother and Con: children). M and N refer to the mutant and normal oligonucleotides corresponding to CD17, CD26 and CD41/42. P+: biotin labeled oligonucleotides. P-: non-labeled oligonucleotides designed from the first intron sequences. (B) ARMS analysis for detection of the mutations at CD17 and CD41/42 from the family. (-) Negative control without DNA template. IC: Internal Control. Heterozygous state was observed to CD17 (father and children) and CD41/42 (mother and children). The children was homozygous \( \beta \) thalassemia (consisted two mutations).

Dot blot analysis performed to fifteen individuals revealed heterozygous state of single or double mutations in the each family. The parents of 5 families were all mutation carriers (one mutation at heterozygous state) and their children received two mutations at different codons (homozygous beta thalassemia). These results were consistent with those obtained either by using Strip Assay (data not shown) or by ARMS analysis (Fig. 4B).

4. Discussion and conclusion

The common mutations on the \( \beta \) globin, which caused beta thalassemia disease are point mutations [3]. Different approaches were performed to screening and determination of mutation carrier and heterozygous/homozygous states of \( \beta \) thalassemia [4]. The ARMS and RDB assays were common methods due to their sensitivity and specificity [4]. In addition, these methods allowed to detect simultaneously multi mutations at once. In the ARMS, mutations were confirmed based on the presence/absence of PCR products on the electrophoresis gel while in the RDB, based on the presence/absence of colour signals visible on membrane. Therefore, developing the RDB method gives much advantageous such as simplifying procedure and time-saving. Indeed, RDB was developed in prenatal diagnosis of \( \beta \) thalassemia [9,10] or molecular epidemiological characterization of thalassemia [11]. Detecting \( \beta \) thalassemia in Vietnamese population by using the ARMS method has been numerously reported [6,7]; however, by using the RDB method has been scarce. Our study created oligonucleotides for RDB assay. Oligo concentrations deposited on membrane were evaluated and hybridization procedure were optimized, that allowed to dishtinguish normal from mutated alleles of the \( \beta \) globin gene. The RDB assay using these oligos was performed for detection of three mutations CD17, CD26 and CD41/42 in 5 families each has a children suspected \( \beta \) thalassemia. Our result revealed heterozygous and homozygous states of \( \beta \) thalassemia in each individual, that indicating the parent were mutation carriers.
(heterozygous) and the children were homozygous β thalassemia. Our result was confirmed by ARMS and consistent with Strip Assay (data not shown).

5. Conclusion

We have created oligonucleotides and applied them to the RDB assay for successful detection of three mutations CD17, CD26 and CD41/42. Developing the RDB for detection of more than three mutations at once is needed in further studies, that will support government plan for controlling inherited thalassemia in resource-limited settings.

Acknowledgements

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References

Sàng lọc đót biễn phó biên gây bệnh beta thalassemia bằng kỹ thuật lai điểm ngược (Reverse dot blot)

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Tóm tắt: Beta thalassemia là bệnh thiếu máu di truyền rất phổ biến gây ra do các đót biến trên gen beta globin. Ở Việt Nam, tỷ lệ người lành mang gen bệnh dao động từ 1.5 đến 25 % tùy thuộc vào các dân tộc khác nhau. Đót biến gây bệnh beta thalassemia phổ biến nhất ở Việt Nam gồm các đót biến với nghịch đảo di truyền CD17 (A>T), mã CD26 (G>A) và đót biến lệch không đóc ở hai mã di truyền CD41/42 ((-TTCT)). Phân ứng PCR-ARMS (tam dịch là kỹ thuật PCR đặc hiệu allen) cho phép phát hiện trang thái đóng hợp/di hợp allen nhưng có những điều yếu cầu sử dụng nhiều cấp môi. Kỹ thuật RDB (Reverse Dot Blot-tam dịch là lai điểm ngược) cho phép phân biệt đóng hợp/di hợp của nhiều đót biến trong cùng một thí nghiệm. Trong nghiên cứu này, chúng tôi đã thiết kế các oligonucleotide đặc hiệu cho 3 đót biễn CD17, CD26 và CD41/42 xảy ra phổ biến ở Việt Nam và sử dụng các oligo này cho kỹ thuật RDB để tối ưu điều kiện lai và rित trong 6xSSC và nhiệt độ lai 65 °C. Kết quả RDB phân tích 3 loại đót biến này cho 15 thành viên của 3 gia đình cho kết quả hoàn toàn phù hợp với ARMS, do đó khẳng định chúng tôi đã thành công khi đưa kỹ thuật RDB vào việc phân tích đót biễn gây bệnh beta thalassemia ở Việt Nam.

Từ khóa: Bệnh thiếu máu thalassemia, gen beta globin, đót biến điểm, PCR-ARMS, lai điểm ngược.