Clinica Chimica Acta 412 (2011) 999–1002

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Multiplexed genotyping of beta globin mutations with MALDI-TOF mass spectrometry ☆

Mee-Lee Looi [a], Mageswary Sivalingam [a], Nor Diana Husin [a], Fara Zela Mohd Radin [a], Raihana Mohamed Isa [a], Syed Zulkifli Syed Zakaria [a], Noor Hamidah Hussin [b], Hamidah Alias [c], Zarina Abd Latiff [c], Hishamshah Ibrahim [d], Rahman Jamal [a,c,*]

[a] UKM Medical Molecular Biology Institute, Universiti Kebangsaan Malaysia, Malaysia
[b] Department of Haematology, Faculty of Medicine, UKM Medical Centre, Universiti Kebangsaan Malaysia, Malaysia
[c] Department of Paediatrics, Faculty of Medicine, UKM Medical Centre, Universiti Kebangsaan Malaysia, Malaysia
[d] Institut of Paediatrics, Hospital Kuala Lumpur, Malaysia

ARTICLE INFO

Article history:
Received 7 January 2011
Accepted 4 February 2011
Available online 17 February 2011

Keywords:
Beta thalassemia
Mass spectrometry
MALDI-TOF
Multiplexed genotyping
Beta globin gene mutation
Molecular testing

ABSTRACT

Background: Beta thalassemia represents a great heterogeneity as over 300 mutations have been identified and each population at-risk has its own spectrum of mutations. Molecular characterization with high accuracy, sensitivity and economics is required for population screening and genetic counseling.
Methods: We used the MALDI-TOF mass spectrometry (MS) platform to develop novel multiplex assays for comprehensive detection of 27 mutations in beta-thalassemia patients. Six multiplex assays were designed to detect 13 common known ß-mutations, namely CD41/42, CD71/72, IVS1-5, IVS1-1, CD26, IVS2-654, CAP + 1, CD19, -28, -29, IVS1-2, InCD (T-G) and CD17; and 14 rare ß-mutations, i.e. InCD (A-C), CD8/9, CD43, -86, CD15, Poly A, Poly T/C, IVS2-1, CD1, CD35/36, CD27/28, CD16, CD37, and 619bpDEL in 165 samples. We compared the efficiencies of genotyping by MS and Amplification Refractory Mutation System (ARMS). Discrepant results were confirmed by sequencing analysis.
Results: A total of 88.7% (260/293 allele) of MS and ARMS results was in agreement. More than fifty percent of the discrepant result was due to the false interpretation of ARMS results. Failed CD19 assay by MS method might be due to the assay design. The MS method detected 5 rare ß-mutations (CD15, CD35/36, CD8/9, Poly A and Poly T/C) presented in 13 alleles, which were not included in the ARMS screening panel.
Conclusion: We revealed that the MS method is a sensitive, high-throughput, highly automated, flexible, and cost-effective alternative to conventional ß-thalassemia genotyping methods.

1. Introduction

Beta (ß) thalassemia is an autosomal recessive disorder which is due to mutations in the ß globin gene on chromosome 11. According to the Malaysian Thalassaemia Registry in the year 2009, there are 4768 thalassemia patients registered in Malaysia [1]. Of these, 59% require regular blood transfusion. About 5% of Malaysians are believed to be carriers meaning about one in 20 Malaysians carry the thalassemia gene [1]. In the year 2004, to reduce the burden of thalassemia in the nation, the Ministry of Health Malaysia approved a comprehensive program which included providing free iron chelation, a population screening program, an education program and the setting up of a thalassemia registry.

ß-thalassemias are heterogeneous at the molecular level and there are more than 300 different mutations which have been identified [2]. Severity of thalassemia depends on the nature of the mutation. The severe form of ß globin gene mutations (ß°) leads to failure of ß globin chain production and hence patients will have the thalassemia major phenotype and present with severe clinical features; mild mutations ($ß^+$) resulting in reduction of ß globin chains and predict for milder clinical features including a much later presentation. As such, the characterization of ß globin mutations in ß-thalassemia is important especially to provide a complete molecular diagnosis which will contribute to a better management of the disease. It will also help during in the screening of the population at-risk and also in prenatal diagnosis.

Each population at-risk is known to have its own spectrum of common mutations [3–5]. Conventionally, thalassemia genotyping is achievable by ARMS (amplification refractory mutation system) [3,5], direct nucleotide sequence analysis [3,6], reverse hybridization assay

☆ Source of support: Ministry of Science, Technology and Innovation, grant number 06-01-02-SF0285.
* Corresponding author at: UKM Medical Molecular Biology Institute, UKM Medical Centre, Jalan Yaacob Latiff, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia. Tel.: +60 3 91717209; fax: +60 3 91717185.
E-mail address: rahmanj@ppukm.ukm.my (R. Jamal).

0009-8981/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2011.02.006

[6] and reverse dot blot [7]. These approaches are relatively time consuming and labor-intensive when it is applied to a large scale characterization of mutation and also in the setting of a population screening program involving multi-ethnic groups. There are also problems with reproducibility and false positive or negative interpretation. It is clear that more efficient and cost effective method that enables high throughput, accurate, rapid, automated and easier interpretation, to aid in massive scale of thalassemia genotyping is required. In the present study, we used the Sequenom MassARRAY matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) platform to develop novel multiplex assays for comprehensive detection of 27 mutations in ß-thalassemia patients. We evaluated its performance by comparing the results from 168 samples with the comparison methods, i.e. ARMS and sequencing.

2. Materials and methods

The study was approved by the Medical Ethics Committee of Universiti Kebangsaan Malaysia (UKM). A total of 165 subjects were recruited from the thalassemia clinic at the UKM Medical Centre after informed consent. There were 154 cases of ß thalassemia and 11 cases of alpha (α) thalassemia. Five milliliters of whole blood was collected from each patient prior to transfusion and placed into an EDTA tube. DNA was extracted from whole blood by salt precipitation method. Briefly, DNA was isolated from white blood cell using proteinase K and sodium dodecyl sulfate at 55 °C. It was then purified using 6 M sodium chloride and ice-cold ethanol. Purified DNA was then solubilized in Tris-EDTA buffer and stored at −80 °C until analysis.

2.1. Genotyping by Mass Spectrometry (MS)

Multiplexed PCR followed by single base extension assays to analyze the β-globin genes was designed using the MassARRAY Assay Designer v3.1 (Sequenom Inc, CA, USA). The assays were designed into 6 multiplexed reactions with 27 assays in total for detection of 13 common ß thalassemia mutations reported in Malaysia [8], namely CD41/42, CD71/72, IVS1-5, IVS1-1, CD26, IVS2-654, CAP+1, CD19, -28, -29, IVS1-2, InCD (T-G) and CD17; 14 rare mutations, namely InCD (A-C), CD8/9, CD43, -86, CD15, Poly A, Poly T/C, IVS2-1, CD1, CD35/36, CD27/28, CD16, CD37, and 619bpDEL.

Multiplexed PCR followed by single base extension was performed according to the Sequenom recommended protocol and the reagents provided (Sequenom Inc, CA, USA). Briefly, each PCR contained 10 ng extracted genomic DNA, 100 nM of each PCR oligo, 1X Sequenom PCR buffer, 5 mM $MgCl_2$, 200 uM each dNTP and 0.1 unit Sequenom PCR enzyme in a 5 uL reaction volume. Amplification for 45 cycles was performed on an AB9700 thermal cycler (Applied Biosystems, CA, USA) with a 5-minute 72 °C final extension. Following PCR, non-incorporated dNTPs were removed by addition of 15% shrimp alkaline phosphatase, and incubated at 37 °C for 40 min followed by 85 °C for 5 min of denaturation.

Typlex homogenous mass extend (Typlex-hME) was performed by adding 2 uL Typlex-hME reaction mix, containing final concentrations of 1XTyplex-hME extend mix, 0.5–1.5uM each extension primer and 0.063 U/uL Typlex-hME Enzyme, to each PCR product. MassEXTEND conditions were 94 °C/2-minute denaturation followed by 40 cycles of 94 °C/5 second denaturation, (52 °C/5 second annealing and 80 °C/5 second extension) X5]. Reaction products were cleaned up by adding 6 mg ion exchange resin (Sequenom, San Diego, California) to the reaction mix.

Ten nL of PCR product was then spotted onto a 384 Spectrochip (Sequenom, San Diego, California) using the Sequenom Nanodispenser (Sequenom, San Diego, California). Mass spectrometry analysis was performed on a matrix assisted laser desorption/ionization time of flight spectrometer (Sequenom, San Diego, California) with data analysis performed by MassArray Typer v4.0 software (Sequenom, San Diego, California).

2.2. Genotyping by ARMS method

The β-globin genes were also analyzed using Amplification Refractory Mutation System polymerase chain reaction (ARMS-PCR) for detection of 13 common ß thalassemia mutations reported in Malaysia [8], namely CD41/42, CD71/72, IVS1-5, IVS1-1, CD26, IVS2-654, CAP+1, CD19, -28, -29, IVS1-2, InCD (T-G) and CD17. Normal and mutant primers paired with common primer as described by Weatherall and Clegg [9] were used. After amplification, 5 μl of product was electrophoresed in a 1.2% agarose gel, stained in ethidium bromide and visualized by UV transilluminator. If there was a discrepancy in the result between MS and ARMs methods, the sample was then subjected to direct DNA sequencing for result confirmation. DNA sequencing was performed by using primers and conditions that were previously described [10].

3. Results

MALDI-TOF MS genotyping was performed in a blinded manner. A total of 95.2% of the cases which were genotyped by ARMS were unambiguously distinguished by MALDI-TOF MS. About 4.0% of alleles failed to be detected by MS partly due to failed CD19 assay. The MALDI-TOF MS also identified 5 rare ß-mutations, i.e. CD15, CD35/36, CD8/9, Poly A and Poly T/C in our sample population. These mutations were not included in our ARMS screening panel, and the results were confirmed by direct sequencing.

In the present study, there was no ß mutation detected in 9 of the 11 alpha thalassemia patients but 2 patients with Hemoglobin H disease were shown to co-inherit the CD26 and IVS1-5 mutations detected by both MS and ARMS methods. Of the 154 ß-thalassemia cases, 4 cases (3 were clinically diagnosed as Hb E beta thalassemia and 1 was Hb Geelong) did not exhibit any mutation by both methods. In total, 293 alleles were characterized. Five cases, which have been phenotyped as thalassemia major (n=3) and Hb E beta thalassemia (n=2) were identified as ß-thalassemia heterozygous with one affected allele.

We also compared the efficiencies of genotyping by MS and ARMS. There were a total of 33 results which differed between the 2 methods (Table 1). Failed CD19 assay constituted 30.3% (10/33) to the discrepancy. All 10 cases were then further validated by direct sequencing. We found 57.6% (19/33) and 12.1% (4/33) of discrepancy were due to false positive and negative results by ARMS and MS respectively. False negative results by MS were observed in CD41/42 assay. After result confirmation was made by sequencing, we identified that MS genotype has 95.2% sensitivity and 100% specificity in detection of 27 beta globin mutations (Table 2).

4. Discussion

ß-thalassemia-causing mutations are diverse and differ between ethnic groups and geographical locations. Efforts have been made to develop a comprehensive screening platform which facilitates the detection of multiple ethnic-specific mutations. As an alternative to the conventional detection methods, real-time polymerase chain reaction using fluorophore-labeled hybridization probes [11], gap-PCR [12], high resolution melting analysis [13] and denaturing high-performance liquid chromatography (dHPLC) [14] assays have been adopted for a multiplex detection approach. Recently, a comprehensive dHPLC assay for the detection of 20 mutations has been developed by Li et al. [15]. They conducted a blinded validation in 27 DNA samples and found dHPLC assay accurately genotyped 100% of samples previously characterized by reversed dot blot and/or direct sequencing. However, it is still relatively not a rapid approach as the dHPLC analysis needs a 9-minute sample-run time to elute the primer