

Research Article

## Identification of Human Leukocyte Antigen (HLA) Patterns in Beta-Thalassemia Patients and their Relevance to the Mutational Spectrum of the Human Beta-Globin Gene (HBB)

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### ABSTRACT

**Aims:** HLA genotyping is beneficial in both clinical and research settings in order to understand the mechanism of associated diseases as well as organ transplantation. Beta-Thalassemia is endemic in Saudi Arabia. Studies showed more than 200 mutations affecting the HBB gene thus causing the disease with the IVS-1-5 mutation is considered the most common mutation in KSA. HLA matching in beta-thalassemia patients can support therapeutic interventions through hematopoietic stem cell transplantation (HSCT) as it is estimated that only 30% of patients can find an HLA-identical donor within their families. **Methods:** We have investigated the genetic polymorphism of 16 Short Tandem Repeat (STR) loci (D6S291, TAP1, D6S2880, D6S1014, D6S2973, MICA, MOGc, MIB, MIB outer, D6S2674, D6S2959, D6S2908, Ring3CA, D6S2812, D6S248 and D6S2707) in 15 transfusion-dependent beta thalassemia patients' and 15 healthy volunteers using a fluorescent-labelled singleplex-PCR typing method. **Results:** The result showed wide variation in alleles and high level of genetic polymorphisms. In addition, we found a high level of homozygosity in patients samples at 3 STR loci; Ring3CA, D6S2812 and D6S248 where this homozygosity is significantly associated with the IVS-I-5 mutation (p-value <0.05, <0.001 and <0.001 respectively). **Conclusion:** We conclude that HLA matching using the fluorescent-labelled singleplex-PCR typing method provides a useful technique supporting HSCT as well as raising questions about the potential association between homozygosity at certain STR markers and particular HBB mutations.

### Keywords:

### INTRODUCTION

The most polymorphic gene in human is Human leukocyte antigen (HLA)<sup>1</sup>. It has an important role in organs transplantation and immune response and also have a relation to several diseases<sup>2</sup>. The variation of alleles has previously been identified by serological assays. At present, molecular typing techniques have developed to be high accuracy and potential to identify allelic variation. Genotyping of HLA loci is significant in research to understanding the mechanism of HLA associated diseases, in Discovering new informative markers have relation between HLA and specific disease help to using as clinical tool for risk evaluation for disease with well-defined associations.

Thalassemia is a group of inherited hematologic disorders caused by deficient in synthesis of one or more globin polypeptide that lead to an imbalance of alpha and beta globin chain which lead to deficient production of normal hemoglobin. Thalassemia patients cannot synthesis

enough hemoglobin, that causing severe anemia<sup>3</sup>. In Saudi Arabia thalassemia consider high prevalence, especially in the Eastern Province of Saudi Arabia, has highest frequencies of both Alpha Thalassemia and beta Thalassemia<sup>4,5</sup>. Beta thalassemia is a group of inherited blood disorders result by reduction or absence of beta globin chain synthesis leading to decrease of hemoglobin in red blood cells which decreases the production of red blood cell therefore, lead to anemia<sup>6</sup>.

Molecular studies showed more than 200 affecting the HBB gene thus causing the disease with the IVS-1-5 mutation, substitution of G by C at position 5 of the IVS-I of the human beta globin gene leading to alternative cryptic splice which leads to severe beta thalassemia. This mutation is considered the most common mutation in the middle east as well as in KSA<sup>7,8</sup>.

Currently, only effective treatment for thalassemia patients is bone marrow transplantation. Patients without risk factor such as liver fibrosis, hepatomegaly, and severity of iron

accumulation have a disease-free survival over 90% when stem cell transplant from HLA-identical sibling. In fact, approximately less than 30% of patients can find HLA-identical from the same family<sup>6</sup>.

Due to limitation of finding matched sibling donor and high prevalence of beta-thalassemia in Saudi Arabia as well as the result of development in molecular techniques, it became necessary to starting the applications of HLA molecular typing techniques to contribute to the global information on the level of therapeutic strategies for hematological malignancy. We utilized the molecular typing technique for the analysis of specific loci in the HLA region by using 16 fluorescent-labelled STR marker in beta-thalassemia patients who carrying IVS-I-5 (G>C) mutation and healthy volunteer to determine the efficiency of utilizing molecular technique in HLA typing and HLA-associated disease.

## METHODS AND MATERIALS

Fifteen blood samples were collected from transfusion-dependent beta-thalassemia patients' at King Abdulaziz University Hospital (KAUH). Those patients carrying IVS-I-5 (G>C) mutation which was identified by Alwazani *et al.*<sup>9</sup>. Meanwhile, Fifteen control samples obtained from saliva from healthy volunteers. Ethical approval obtained from the local authority for samples collection. DNA of the positive samples was extracted from whole blood using the QIAamp DNA Maxi Kit (Qiagen) according to the manufacturer's protocol (Qiagen, USA). DNA of the control samples was extracted from saliva using Oragene<sup>TM</sup>/Saliva kit based on the manufacturer's instructions. each sample was tested with 16 fluorescent-labelled primers using singleplex polymerase chain reaction. Therefore, the amplified products were loaded on the genetic analyzer to detect the size of fragments and Reading by using GENE MAPPER SOFTWARE V5.

*Amplification of the HBB gene, sequencing and reading*  
HBB gene was amplified from Extracted DNA using 16 fluorescent-labelled primers by using commercially PCR kit (QiaTaq) according to the manufacturer's instructions. Briefly, extracted DNA from each clinical sample was used as a template for screening using singleplex PCR Master Mix kit (QiaTaq) in a final reaction volume of 25  $\mu$ l. The PCR conditions were set for initial denaturation at 95°C for 3 min. this was followed by 35 cycles of amplification consisted of denaturation for 30 sec at 96°C, annealing for 45 sec at 60°C and extension for 1 min at 72°C. Finally, the final extension for 7 min at 72°C. Sequencing of the PCR product were prepared and loaded on the ABI Prism 3130 genetic analyzer for DNA fragments analysis following Standard Operating Procedure SOPs (maintenance and use of ABI Prism 3130). Briefly, 12.3 $\mu$ l of Master Mix consist of 12 $\mu$ l of HiDi formamide and 0.3 of appropriate size standard (LIZ500) were prepared for each sample. Then, 1 $\mu$ l of the PCR product was added to each well of the plate for each sample. Therefore, the plate was covered with septa and denatured for 5 min at 95°C. Finally, the plate was loaded on the ABI Prism 3130 genetic analyzer and the run started. Reading of the fragment size result was performed

by using GENE MAPPER SOFTWARE V5. The display was showed the peaks. The peak represents specific loci act as fingerprinting when tested with STR primer that is lead to identifying the inherited alleles from the parents. When the display showed one peak that's considered homozygous allele which means inherit the same allele from father and mother. Conversely, if showed two peaks that refer to heterozygosity state which mean two different alleles inherited from the parents. The peak of interest was magnified and recorded the result of size for all primers for each sample.

## RESULT

Genetic analysis in the target area of HLA complex showed wide variation in alleles according to the fragment size results and highly level of genetic polymorphism. In addition, widely diversity in the fragment size for each locus in all samples. The fragment size results of the analysis of HLA loci in patients and control samples is showed in (figure1). However, Among the 16 STR loci, the homozygosity in fifteen patient samples at three STR loci (Ring3CA, D6S2812 and D6S248) was 8 samples (53.3% of the total) at Ring3CA, 8 samples (53.3% of the total) at D6S2812 and 4samples (27 % of the total) at D6S248. Conversely, the homozygosity in fifteen control samples at same loci was 4 samples (27 % of the total) at Ring3CA, 2 samples (13.3% of the total) at D6S2812 and no homozygosity (0 % of the total) at D6S248. The homozygosity at the Ring3CA, D6S248 and D6S248 loci are high significantly associated with beta-thalassemia IVS-I-5 (G>C) as indicated by p-value (Chi-square test) showed in (tables 1). The molecular weight of each fragment was shown by peaks. The peak represents specific loci act as fingerprinting when tested with STR primer that is lead to identifying the inherited alleles from the parents. When the display showed one peak that's considered homozygous allele which means inherit the same allele from father and mother. Conversely, If showed two peaks that refer to heterozygosity status which means two different alleles inherited from the parents as shown in (Figure 2)

## DISCUSSION

Several advantages for establishing HLA molecular typing techniques, one of them is hematopoietic stem cell transplantation from an HLA-matched unrelated donors. The Italian Group by Giorgio *et al.*<sup>10</sup> using molecular typing method to both HLA class I and II loci for Bone Marrow Transplant to 68 thalassemia patients from unrelated donors. The Overall survival in the cohort was 79.3% and disease-free survival with transfusion independence was 65.8%. They concluded that the outcomes of patients receiving a BMT from unrelated donors which selected by using molecular typing technique for HLA loci and well matched at the molecular level are favorable and comparable to what observed when a transplant from an HLA-identical family donors. Similarly, a study published from the group in France, strongly suggests that transplantation from HLA-matched unrelated donors, well matched at the molecular level can

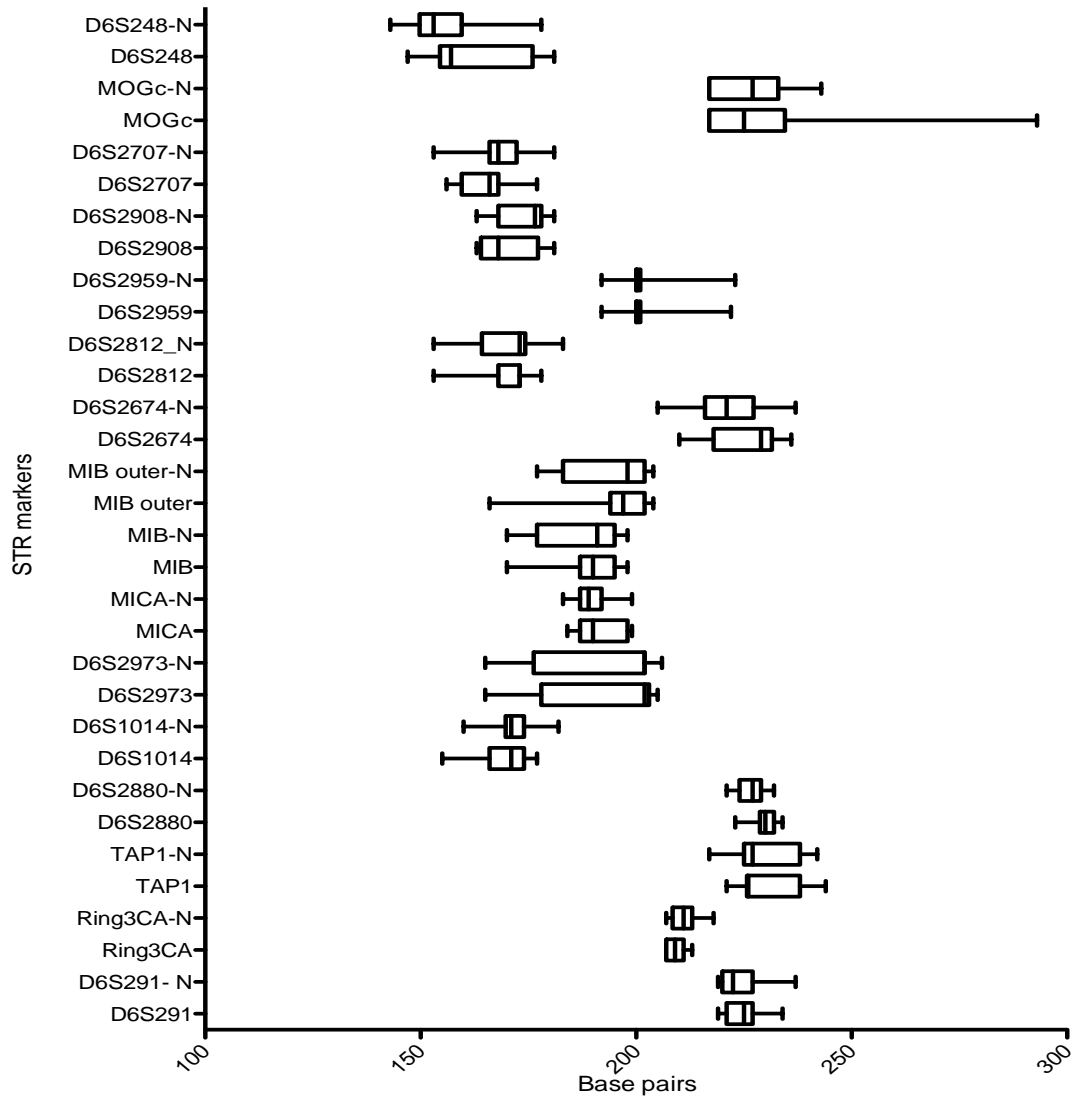


Figure 1: Graphical representation of the fragment sizes results. “N” indicates the fragment sizes in the control group.

Table 1: Homozygosity and p-value of significant STR loci.

group	NO. of tested sample	Number of homozygous sample at loci		
		Ring3CA	D6S2812	D6S248
patient	15	8 of 15 (53.3%)	8 of 15 (53.3%)	4 of 15 (26.6%)
control	15	4 of 15 (26.6%)	2 of 15 (13.3%)	0 of 15 (0%)
p-value		0.05<	0.001<	0.001<

offer a possibility of cure similar to that of HLA-identical donors<sup>11</sup>. Interestingly, using molecular typing technique led to identified a new HLA-C alleles and filled the gap of the most serological typing, that supported by a study conducted of two cohorts of healthy Saudis. They compared a molecular and serological typing of HLA-C, the result showed that the serological typing identified HLA-C\*01-\*07 with different frequencies and failed to identify 23.5 % of the HLA-C alleles and defined as blank. On the other hand, performing sequence-specific primers (PCR-SSP) molecular typing identified HLA-C\*01-\*08, C\*12, C\*14, C\*15, C\*16, C\*17 and C\*18 comparing to C\*01-\*07 which identity by serological typing<sup>12</sup>.

In the traditional Saudi community, consanguineous marriage is common. High consanguinity has an advantage in terms of finding tissue donors. extended family searches by using molecular typing techniques in this population have a high probability of finding HLA-matched non-sibling related family donor. In a recent study, Hussein *et al* in King Hussein Cancer Center (KHCC) in Jordan were able to identify suitable non-sibling donor using extended family typing for 28 ( 8.2%) patients of the total 341 patients with thalassemia major and they did not observe increased risk of Graft-versus-host disease (GvHD) in the patients. They reported that all treated patients are alive and disease free<sup>13</sup>.

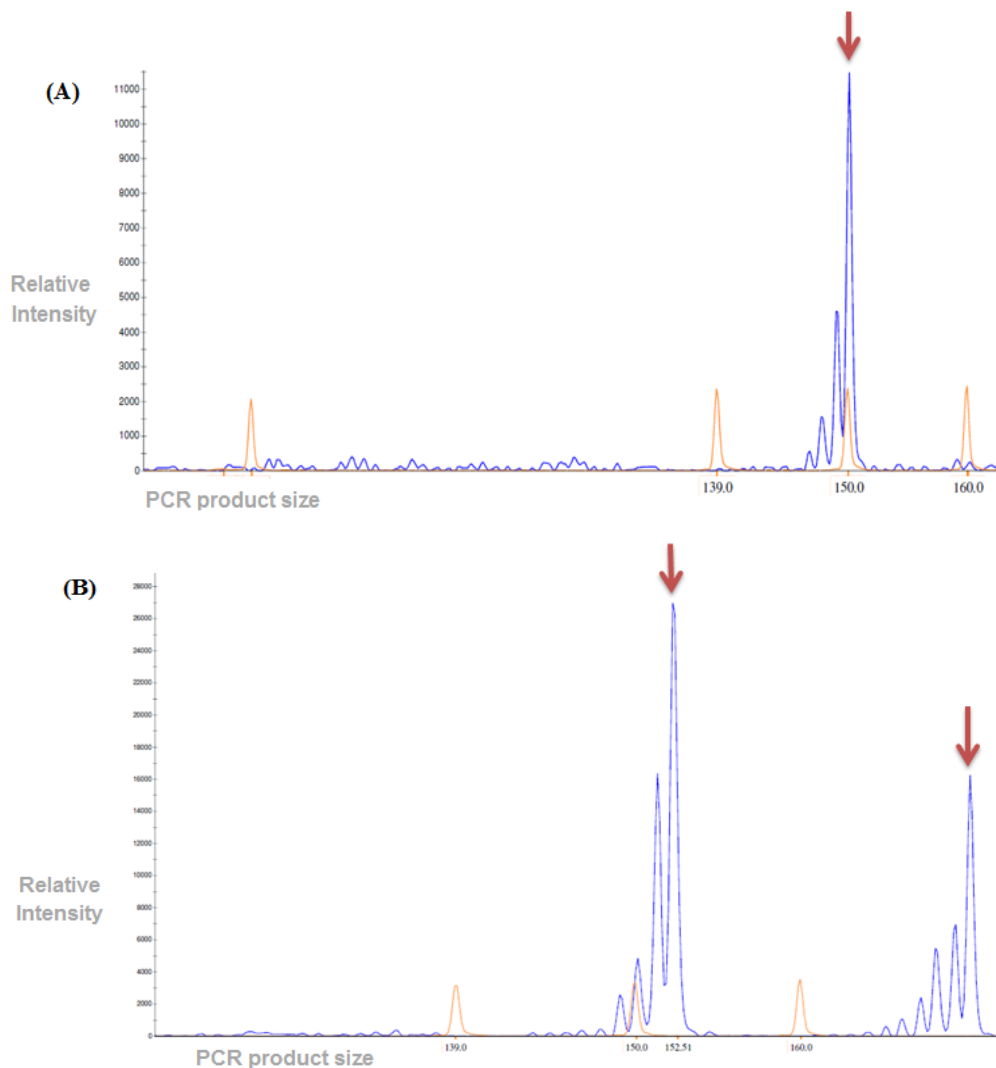


Figure 2: Homozygosity status of the D6S248 STR locus in a patient sample with IVS-I-5 (G>C) shows one peak in (A). On the contrary, heterozygosity status in control sample is shown as two peaks as displayed in (B). The major peaks are illustrated by an arrow.

Work in this study evaluated the efficiency of utilizing the molecular typing technique for the analysis of specific loci in the HLA region by using 16 fluorescent-labelled singleplex-PCR STR marker in beta-thalassemia patients samples who carrying IVS-I-5 (G>C) mutation and healthy volunteer samples collected from KAUH. The results show wide variation in the amplified fragments' sizes indicating a high level of genetic polymorphism. Wherefore, the presence of high genetic polymorphisms, heterozygosity and broad distribution of alleles in the results support the extensive polymorphisms of HLA in nature. This could give good support for using the molecular typing technique as an effective tool in HLA typing and genetic study. Interestingly, Among the 16 STR loci, the homozygosity in fifteen patient samples at three STR loci (Ring3CA, D6S2812 and D6S248) was 8 samples (53.3% of the total) at Ring3CA, 8 samples (53.3% of the total) at D6S2812 and 4 samples (27 % of the total) at D6S248. Conversely, the homozygosity in fifteen control samples at the same loci was 4 samples (27 % of the total) at Ring3CA, 2 samples (13.3% of the total) at D6S2812 and no

homozygosity (0 % of the total) at D6S248. The presence of homozygosity at the three loci in patients group more than in healthy individuals could be indicate a common founder for the IVS-1-5 mutation.

During last decades, several studies have identified a long list of human diseases are associated with HLA complex, i.e. a particular HLA allele occur more frequently through patients compared to healthy people. For example, class I HLA alleles (HLA-B\*27:02 and HLA-B\*27:05) have been identified in more than 90% of Caucasian patients with Ankylosing Spondylitis(AS)<sup>14</sup>. Narcolepsy is a brain disorder characterized by irresistible sleep attacks last for 3 to 5 minutes during the day, carrying the HLA class II (DQB1\*06:02) allele in Caucasian patients with Narcolepsy approaching to 90%<sup>15</sup>. Ehrlich *et al.* They concluded that 90% of patients with type I Diabetes Mellitus, carrying HLA-DRB1\*03/DQB1\*02:01, or HLADRB1\*04/DQB1\*03:02 gene haplotypes, compared to only 40% of controls<sup>16</sup>. In Australians, a study on the influence of HLA-DRB1 to Multiple Sclerosis (MS). They found a highly significant association between HLA-

DRB1\*03 and risk of MS. Moreover, They observed Individuals homozygous for either HLA-DRB1\*15 or HLA-DRB1\*03 were considerably more at risk of MS than heterozygotes and non-carriers<sup>17</sup>. In 2002 Bao R *et al.* suggest that HLA-DQB1\*06 allele is associated with pathogenesis of the major beta-thalassemia in Guangdong area<sup>18</sup>.

In conclusion, HLA matching by utilizing fluorescent tag primer and amplification of the STR probes is a useful technique, relatively easy to perform, cost-effective, no complex equipment required and can be applied in high throughput manner. Excess of homozygosity in the HLA complex is a known factor associated with diseases, we have identified homozygosity at three STR marker associated with beta-thalassemia IVS-I-5 (G>C) mutation.

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