Applications of Molecular Genetics to the Diagnosis and Prevention of Thalassemia

Samuel S. CHONG, PhD

Department of Pediatrics, Yong Loo Lin School of Medicine, National University of SINGAPORE

Preimplantation Genetic Diagnosis Centre, Khoo Teck Puat – National University Children’s Medical Institute, National University Health System, SINGAPORE

Molecular Diagnosis Centre, Department of Laboratory Medicine, National University Hospital, National University Health System, SINGAPORE
Alpha- and Beta-Thalassemia

- Autosomal recessive disorders of hemoglobin (Hb) synthesis.
- Among the most common monogenic diseases globally.
- ~14,000 births of lethal (Hb Bart’s hydrops fetalis syndrome) and severe (Hb H disease) α-thalassemia annually.
  - Within Asia, the -α^{3.7} and -α^{4.2} α+ thalassemia single α-globin gene deletions, as well as the --SEA, --FIL, --THAI and --SA α0-thalassemia double α-globin gene deletions are prevalent.
- ~23,000 births of severe β-thalassemia major (Cooley’s anemia) annually.
  - Predominantly caused by different combinations of β+ and β0 point mutations within the β-globin gene.
- No effective cure for α- or β-thalassemia.
- Prevention through prenatal diagnosis, however, involves termination of affected pregnancies.
Genetic Basis of $\alpha$-/β- Thalassemia

- **$\alpha$-thalassemia**
  - Due to defects within the chromosome 16p13.3 $\alpha$-globin gene cluster.
  - Most commonly caused by deletions including $HBA2$ ($\alpha2$ globin) and $HBA1$ ($\alpha1$ globin), and sometimes $HBZ$ ($\zeta$ globin) gene.
  - Point mutations are less common.

- **$\beta$-thalassemia**
  - Due to defects within the chromosome 11p15.5 $\beta$-globin gene cluster.
  - Most commonly caused by point mutations within the $HBB$ ($\beta$ globin) gene.
  - Deletions are less common.
PCR Sequencing of *HBA1*, *HBA2* and *HBB* Genes

**HBA1** amplicon: 1380 bp

**HBA2** amplicon: 1303 bp

**HBB** amplicon: ~1700 bp

Exon 1

- CCAAT

Exon 2

- ATAAA

Exon 3

- Cap

~800 bp

~600 bp
Common $\alpha$-Globin Point Mutations

Wildtype \textit{HBA2} sequence

\[
\begin{array}{c}
\text{GCTGACC} \text{CTCCAAATTACCGT} \text{TAAAAGC} \text{TGAG} \text{CC} \text{TCC} \\
\text{390} \quad \text{400} \quad \text{410} \quad \text{420}
\end{array}
\]

Constant Spring mutation: TAA$\rightarrow$CAA

\[
\begin{array}{c}
\text{GCTGACC} \text{CTCCAAATTACCGTCAAGC} \text{TGAG} \text{CC} \text{TCC} \\
\text{390} \quad \text{400} \quad \text{410} \\
\end{array}
\]

Pakse mutation: TAA$\rightarrow$TAT

\[
\begin{array}{c}
\text{GCTGACC} \text{CTCCAAATTACCGTTATGC} \text{TGAG} \text{CC} \text{TCC} \\
\text{390} \quad \text{400} \quad \text{410} \quad \text{420}
\end{array}
\]
IVS II,654 C→T Point Mutation

C/T

G/A
β-Thalassemia Mutations in Singapore

- IVS2 nt 654 (C/T) (22.3%)
- HbE (20.5%)
- -28 (A/G) (6.6%)
- Cd17 (A/T) (4.9%)
- IVS1 nt 5 (G-C) (6.4%)
- 42 mutations (9.1%)
- IVS1 nt 1 (G-T) (1.3%)
- Cds71/72 (+A) (1.2%)
- Unknown (0.6%)
- Cds41/42 (-TCTT) (27.3%)

N=2409
IVS II, 654(C→T) Minisequencing Assay

Wild-type allele (C)

654-F

5’

TTAAGG C/T AAT AGC

654-R

3’

Mutant allele (T)

654-F

5’

Wild-type allele (C)

3’

AAT TCC G TTA TCG

Heterozygous

Homozygous Wildtype

Homozygous Mutant
HBB Multiplex Minisequencing

-29-F  Cd41/42-F  Cd71/72-F  InII 654-F

-28-R  Cd17-R  Cd26-R  Inl 5-R

aa | A   | G   | g   | C   | G   | C   | c   | g

Normal

Cd41/42 / InII 654

C→G

-29 / β

A→G

C→T
$HBA2$ Multiplex Minisequencing

\[\begin{array}{cccccccc}
C_{d0}-F & C_{d30}-F & S_{D}-F & Q_{S}-F & C_{S}-F & C_{d59}-R \\
T & G & C & T & T & A & T \\
A & C & T & T & A & A & A \\
\end{array}\]

\(\alpha^{\alpha}/\alpha^{\alpha}\)

\(\alpha^{CS\alpha}/--SEA\)

\(\alpha^{Ps\alpha}/\alpha^{\alpha}\)

\(T\rightarrow C\)

\(T\rightarrow A\)
### Alpha Globin Gene Deletions

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal</strong></td>
<td>αα/αα</td>
</tr>
<tr>
<td><strong>Heterozygous α-thalassemia 2</strong></td>
<td>-α/αα</td>
</tr>
<tr>
<td>“silent carrier”</td>
<td></td>
</tr>
<tr>
<td><strong>Heterozygous α-thalassemia 1</strong></td>
<td>--/αα</td>
</tr>
<tr>
<td>“α-thal trait”</td>
<td></td>
</tr>
<tr>
<td><strong>Homozygous α-thalassemia 2</strong></td>
<td>-α/-α</td>
</tr>
<tr>
<td>“α-thal trait”</td>
<td></td>
</tr>
<tr>
<td><strong>Hb H disease (β₄)</strong></td>
<td>--/-α</td>
</tr>
<tr>
<td><strong>Bart’s hydrops fetalis (γ₄)</strong></td>
<td>--/--</td>
</tr>
</tbody>
</table>
-Thalassemia Mutations in Singapore

N=3056

-SEA (70.6%)
-Thai (0.9%)
-Fil (0.4%)
-α3.7 (19.1%)
-α4.2 (4.3%)
-αCSα (1.5%)
-αQSα (1.1%)
-αCd30 (0.1%)
-αCd59 (0.6%)
-Other (1.2%)
-Unknown (0.6%)
Detecting Deletions by Gap PCR

Forward primer

Reverse primer

5’ 0.5kb 1 kb 0.5kb 3’

20 kb Deletion

PCR

1.5 kb fragment is amplified; “21 kb” fragment unamplifiable

PCR

1 kb fragment is amplified
Multiplex Gap-PCR for Common $\alpha$-Thalassemia Deletions
**α-Thalassemia Multiplex Gap-PCR Results**

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Lanes</th>
</tr>
</thead>
<tbody>
<tr>
<td>αα/αα</td>
<td>M</td>
</tr>
<tr>
<td>αα/α3.7</td>
<td>αα</td>
</tr>
<tr>
<td>α3.7/α3.7</td>
<td>α3.7</td>
</tr>
<tr>
<td>αα/α4.2</td>
<td>α4.2</td>
</tr>
<tr>
<td>α3.7/α-SEA</td>
<td>α-SEA</td>
</tr>
<tr>
<td>αα/α-THAI</td>
<td>α-THAI</td>
</tr>
<tr>
<td>αα/α-20.5</td>
<td>α-20.5</td>
</tr>
<tr>
<td>αα/FIL</td>
<td>α/FIL</td>
</tr>
<tr>
<td>αα/−MED</td>
<td>α−MED</td>
</tr>
<tr>
<td>αα/−FIL</td>
<td>α−FIL</td>
</tr>
<tr>
<td>αα/−SEA</td>
<td>α−SEA</td>
</tr>
<tr>
<td>αα/−THAI</td>
<td>α−THAI</td>
</tr>
<tr>
<td>αα/−MED</td>
<td>α−MED</td>
</tr>
<tr>
<td>αα/−FIL</td>
<td>α−FIL</td>
</tr>
</tbody>
</table>

**Molecular Markers:**

- 3 kb
- 2.5 kb
- 2 kb
- 1.5 kb
- 1 kb
- 0.75 kb
- 0.5 kb

LIS1 markers:

- α3.7
- α4.2
- α-3.7
- α-4.2
- α-20.5
- α−MED
- α−FIL

Reference:

Preimplantation Genetic Diagnosis

- Genetic testing on blastomere(s)/trophectoderm of morula/blastocyst stage embryos derived from *in vitro* fertilization (IVF).
- Transfer disease-free (normal/unaffected) embryo(s) for implantation.
- Detect *inherited monogenic disorders* & chromosomal abnormalities.
- Unlike prenatal diagnosis, affected pregnancies are avoided altogether - avoids pregnancy terminations.
Thalassemia PGD

- Thalassemia couples may be carriers of different mutations/deletions.
  - PGD for α-thalassemia using gap-PCR entails significant assay customization.
  - PGD for β-thalassemia also requires customization.
- Most PGD assays now also include analysis of linked microsatellite markers
  - To track transmission of normal and mutant alleles.
  - To detect external DNA contamination, to prevent misdiagnosis.
  - To monitor allele drop-out (ADO), to prevent misdiagnosis.
- Informative markers are required – entails significant assay customization.
α-thalassemia PGD – Simultaneous Deletion and Linkage Analysis
Hb Bart’s and Hb H Disease

α-globin cluster

Hb Bart’s
(-α^3.7/---SEA)

Hb H
(-α^3.7/---SEA)

Hb H
(-α^4.2/---SEA)

Hb HCS
(α^CSα/---SEA)
Observed heterozygositites of α-thalassemia microsatellite markers
PGD Assay for Hb Bart’s & Hb H Disease

Blastomere / cell → Heat → Lysis Buffer

Neutralizing Buffer → Multiplex PCR → GeneScan™ Analysis

Mutation Detection

16PTEL06 16PTEL05 Y1

Diagnostic Report

Haplotype Analysis

155 169 179 227 256 281 380 382 393 429 484 485

16PTEL03 D16S525 HBA572 HBA876 D16S521 HBA950 HBA370
Hb Bart’s PGD

Wife: αα/--SEA

Husband: αα/--SEA

Hb Bart’s embryo: --SEA/--SEA

Unaffected embryo: αα/αα

Carrier embryo: αα/--SEA
β-thalassemia PGD - Combined Mutation Detection and Linkage Analysis

The diagram illustrates the genomic region associated with β-thalassemia, focusing on the location of various genes and mutations. The key genes and regions highlighted include:

- **HBE1**, **HBG2**, **HBG1**, **HBBP1**, **HBD**, and **HBB**
- **Exon 1**, **Exon 2**, and **Exon 3**
- **IVSI 1**, **IVSI 2**, **IVSII 1**, **IVSII 654**, and **IVSII 745**
- **Poly(A)**

The mutations and markers are denoted by specific coordinates and are crucial for understanding the genetic architecture and potential linkage analysis in β-thalassemia PGD (Prenatal Genetic Diagnosis).
Observed heterozygosities of β-thalassemia microsatellite markers

<table>
<thead>
<tr>
<th>Markers</th>
<th>CH</th>
<th>ML</th>
<th>IN</th>
<th>CA</th>
<th>AA</th>
<th>Combined average</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBB4506 D11S988</td>
<td>0.89</td>
<td>0.88</td>
<td>0.89</td>
<td>0.85</td>
<td>0.90</td>
<td>0.89</td>
</tr>
<tr>
<td>HBB4677</td>
<td>0.89</td>
<td>0.88</td>
<td>0.89</td>
<td>0.90</td>
<td>0.90</td>
<td>0.89</td>
</tr>
<tr>
<td>D11S2362</td>
<td>0.80</td>
<td>0.83</td>
<td>0.88</td>
<td>0.85</td>
<td>0.85</td>
<td>0.85</td>
</tr>
<tr>
<td>HBB5089 D11S1243</td>
<td>0.80</td>
<td>0.82</td>
<td>0.80</td>
<td>0.80</td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td>HBB5138 HBB5178</td>
<td>0.75</td>
<td>0.76</td>
<td>0.76</td>
<td>0.78</td>
<td>0.76</td>
<td>0.78</td>
</tr>
<tr>
<td>HBB5205</td>
<td>0.69</td>
<td>0.77</td>
<td>0.77</td>
<td>0.78</td>
<td>0.78</td>
<td>0.78</td>
</tr>
<tr>
<td>D11S1760 HBB5576</td>
<td>0.74</td>
<td>0.77</td>
<td>0.77</td>
<td>0.78</td>
<td>0.78</td>
<td>0.78</td>
</tr>
<tr>
<td>HBB5655 HBB5820</td>
<td>0.68</td>
<td>0.73</td>
<td>0.73</td>
<td>0.74</td>
<td>0.74</td>
<td>0.74</td>
</tr>
<tr>
<td>HBB5859 D11S1338</td>
<td>0.65</td>
<td>0.71</td>
<td>0.71</td>
<td>0.72</td>
<td>0.72</td>
<td>0.72</td>
</tr>
</tbody>
</table>
PGD Assay for β-Thalassemia

Blastomere / cell → Lysis Buffer → Heat → Neutralizing Buffer → Multiplex PCR → Aliquots

SBE GeneScan™

Minisequencing

STR GeneScan™

Diagnostic Report

Mutation Detection + Haplotyping Analysis
Direct single-cell multiplex PCR or after whole genome amplification

Multiplex PCR from 10 ng gDNA

Multiplex PCR from Whole Genome Amplified Single Cell Aliquot

Direct Single Cell Multiplex PCR

D11S2362  D11S1338  HBB4677  D11S1243  HBB5820
D11S988   HBB5178   HBB4506   HBB5576   HBB5859
HBB5178   HBB5089   HBB5655   HBB5576   HBB5205
HBB5859   HBB5138   HBB5205
Multiplex PCR of HBB exons & markers
Minisequencing assays for PGD of β-thalassemia mutations

- **IVS II,654 (C→T)**
  - Normal: C
  - Carrier: C, T
  - Affected: T

- **Cd 41/42 (ΔTTCT)**
  - Normal: C
  - Carrier: G, C
  - Affected: G

- **Cd 26 (C→T)**
  - Normal: C
  - Carrier: T
  - Affected: T

- **IVS I,1 (C→A)**
  - Normal: C
  - Carrier: A
  - Affected: A

- **-28 TATA (T→C)**
  - Normal: T
  - Carrier: T
  - Affected: T

- **IVS I,5 (G→C) AS**
  - Normal: C
  - Carrier: G, C
  - Affected: G
**β-Thalassemia PGD**

Husband’s genotype: β / -28TATA G→A

Wife’s genotype: β / IVSII,654 C→T

<table>
<thead>
<tr>
<th></th>
<th>PGD Cycle 1</th>
<th>PGD Cycle 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocytes retrieved</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>Oocytes fertilised after ICSI</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Embryos biopsied</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Embryos diagnosed as:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (β / β)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Carrier (β / -28)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Affected (IVSII,654 / -28)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Embryos without a diagnosis</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Embryos transferred</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Genotypes of transferred embryos</td>
<td>β / β</td>
<td>β / β</td>
</tr>
<tr>
<td></td>
<td>β / β</td>
<td>β / -28</td>
</tr>
<tr>
<td>Outcome</td>
<td>No pregnancy</td>
<td>Male singleton; Term delivery (β / -28)</td>
</tr>
</tbody>
</table>

**IVS II,654 site**

- β / β
- β / IVS II,654
- β / -28TATA

**-28TATA site**

- IVS II,654 / -28TATA

**Husband’s genotype:**

β / -28TATA G→A

**Wife’s genotype:**

β / IVSII,654 C→T

**Outcome:**

No pregnancy

Male singleton;
Term delivery (β / -28)
The Future of Thalassemia Diagnostics

- Next Generation Sequencing
  - For post-natal diagnosis
  - For carrier screening
  - For prenatal diagnosis (invasive and non-invasive)
  - For preimplantation genetic diagnosis
- α-thalassemia deletions
  - Quantitative methods – no information on type of deletion
  - Qualitative methods – requires deep sequencing (shotgun) or targeted sequencing (gap-PCR)