Thalassemia Prevention: Screening and Prenatal Diagnostic Approaches

Distance Learning Course
From Research to practice: Training course in Sexual and Reproductive Health Research Community Genetics

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This presentation includes:
- Introduction
- Thalassaemia control programs
- Strategy for the prevention of the disease
- Prenatal diagnostic approaches
Haemoglobinopathies

- Structure of globin chain
- Rate of synthesis of globin chains (Thalassaemias)
- Hereditary Persistence of Fetal Haemoglobin (HPFH)
Haemoglobinopathies

Thalassaemias (350)

\[ \text{α-thalassaemia} \]

\[ \text{β-thalassaemia} \]

Abnormal Haemoglobins (887)

\[ \text{Hb S} \]

\[ \text{Hb D} \]

\[ \text{Hb E} \]
Thalassaemia

- Reduction or absence of one of the globin polypeptides making up haemoglobin
- Haemoglobin is a tetramer composed of 2 α-type globin chains and 2 β-type globin chains
Human Haemoglobin Inheritance and Globin Genes

- Thalassaemias are hereditary blood disorders caused by a reduced synthesis of one or more of the globin chains.
Haematopoiesis

Embryonic haemoglobins
- Gower-I: $\zeta_2\epsilon_2$
- Gower-II: $\alpha_2\epsilon_2$
- Portland: $\gamma_2\zeta_2$

Foetal Haemoglobin
- Hb-F: $\alpha_2\gamma_2$

Adult haemoglobin
- Hb-A: $\alpha_2\beta_2$
- Hb-A$_2$: $\alpha_2\delta_2$
β-thalassaemia

>200 β-globin gene mutations

Common β-globin gene mutations
β-thalassaemia

- Globin chain imbalance
- Accumulation of excess α-globin chains in erythroid precursors (ineffective erythropoiesis) and RBC (haemolytic anaemia)
Common Deletional and non-Deletional α-Thalassaemia Mutations

Non deletional α-thal mutations

α2 IVSI Donor site GA[GGTGA]GG → GAGG....(5nt deletion)

α2 Poly(A) signal   AATAAA → AATGAA     (PA-2)
World Distribution of Haemoglobinopathies

- One of the most common inherited blood disorder in the world
- 250 million people (4.5%) are carriers of a potentially pathologic gene
- 300,000 infants are born with a major haemoglobinopathy
Thalassaemia

- Severe anaemia
- Regular blood transfusion
- Iron chelation therapy
- Bone marrow transplantation (BMT)
- Gene therapy
- Drug therapy
Thalassaemia control programs

- National program - effective strategy
- Infrastructure
- Patient Treatment
- Prevention of the disease
Thalassaemia control programs
National Program – Effective Strategy

- Help from WHO and TIF and experts in the field
- Extend of the problem
- Community priorities
- Economic situation
- Distribution
- Ethical (therapeutic abortion option)
- National financial support of the program
Thalassaemia control programs
Infrastructure – Thalassaemia Center

- Clinics
- Haematology Lab
- Molecular Biology Lab
Thalassaemia control programs
Prevention

- Public education
- Carrier screening
- Genetic counseling
- Prenatal Diagnosis
Prevention Programs

- Euro Mediterranean countries (Italy, Greece, Cyprus)
- Middle East countries (Iran 1997)
- SE-Asia countries (Asian Network for the control of thalassaemia was established on 2004)
Prevention Programs
Public education

- Schools
- Leaflets
- Media
- Conferences/Seminars
- Professionals
- To inform NOT to stigmatize
Prevention Programs
Carrier Screening

- Population screening
- High risk groups
- Pregnant women
Carrier Detection

- Haematology
- Hbs electrophoresis
- Biosynthesis
- Family study
- Molecular diagnosis

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<td>&gt;3.5</td>
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<tr>
<td>Hb A+A2</td>
<td>A+(F)+A2</td>
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Thalassaemia Carrier Screening Flow Chart

MCH (pg) >27
HbA2(%) <3.3
Hb A+A2

<27
>3.5
A+(F)+A2

<27
<3.5
A+A2

IRON STUDIES
α-GLOBIN GENE
BY PCR
α-THAL
NORMAL α-GENES
GLOBIN CHAIN
SYNTHESIS AND/OR
δ-GENE ANALYSIS
δ+β-THAL,
γδβ THAL
OTHER NORMAL
HbA2 β-THAL

<27
<3
A+F+A2

HbF

α / β RATIO ANALYSIS

δβ-THAL
HPFH

SCREENING FOR COMMON
β-THAL MUTATIONS
UNDEFINED
β-THAL MUTATION
DGGE
DIRECT SEQUENCING
NORMAL

β-THAL

MCH (pg) >27
HbA2(%) <3.3
Hb A+A2
Prevention Programs
Genetic counseling

- Risks
- Clinical features
- Patient treatment
- Options
- Procedures to follow
Prevention Programs
Prenatal Diagnosis

- Blood samples from family members
- CVS biopsy/Amniocentesis
- Molecular analysis (ARMS, Sequencing etc)
- Diagnosis
Prenatal Diagnosis
Cyprus example

- Amniocentesis (2\textsuperscript{nd} Trimester)
- CVS (1\textsuperscript{st} Trimester)
- PGD (Pre Implantation)

- Non invasive prenatal diagnosis
  (EC FP6 Network of Excellence “SAFE”)
Prenatal Diagnosis for haemoglobinopathies

- β-thalassaemia/Hb variants
- Hydrops Fetalis (α-thalassaemia)
- Severe haemoglobin H disease
Steps followed up for prenatal diagnosis by CVS:

- Thalassaemia trait testing
- Card and Premarital certificate
- Genetic counseling
- Pregnancy
- Blood samples and family tree
- DNA extraction
- DNA analysis of family members
- Ultrasound
- CVS biopsy at 11th week of gestation
- CVS cleaning under microscope
- DNA extraction

Diagnosis Thalassaemia center CING Molecular Biology Lab Obstetrician CING Molecular Biology Lab

MATERNAL TISSUE

CHORIONIC VILLI

BLOOD CLOT (Maternal origin)
Steps followed up for prenatal diagnosis by CVS - Cyprus experience

- Thalassaemia trait testing
- Card and Premarital certificate
- Genetic counseling
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- DNA extraction
- DNA analysis of family members

- Ultrasound
- CVS biopsy at 11th week of gestation

- CVS cleaning under microscope
- DNA extraction
- Molecular analysis
- Diagnosis
Typical couple at risk for β-thalassaemia

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Typical couple at risk for β-thalassaemia
Typical couple at risk for β-thalassaemia

- **N/IVS-I-110**
- **N/IVS-I-110**
- **N/IVS-I-110**
- **N/IVS-I-110**

**CVS**

- **β-thal/Father**
- **β-thal/Mother**

β-thal major
Atypical couple

One parent is a typical β-thalassaemia carrier while the other partner has abnormal haematological indices and normal HbA$_2$

- α-thalassaemia
- δ and β thal comp. heter.
- Silent β-thalassaemia
- α, β and δ thal comp. heter.
- β-thal with low HbA$_2$
- γδβ-thal
Preimplantation Genetic Diagnosis
Preimplantation Genetic Diagnosis

- Preimplantation Genetic Diagnosis (PGD) uses in vitro fertilisation (IVF) to create embryos
- Tests one or two cells from each embryo for a specific genetic abnormality
- Identifies unaffected embryos for transfer to the uterus
- The approach through PGD assists couples at risk of an inherited disorder to avoid the birth of an affected child.
STAGES

- Counseling
- Induction of ovulation
- Oocyte collection
- Fertilization by ICSI
- Embryo biopsy
- Genetic diagnosis
- Implantation of 1-2 suitable embryos
- Confirmation of pregnancy
- Prenatal diagnosis (ESHRE guidelines)
Disorders tested by PGD

- FISH
  - Chromosomal Disorders

- PCR-based
  - Single gene defects
    - Thalassaemia
    - Cystic Fibrosis
    - Haemophilia
    - Muscular dystrophies
    etc
PGD approaches

- Polar body analysis
- Blastomere biopsy analysis
Induction of ovulation

- In order to obtain a large number of oocytes, the patients undergo controlled ovarian stimulation (COH), with the use of FSH.
- Ultrasound-guided trans-vaginal oocyte retrieval
Fertilization

- Intracytoplasmic sperm injection (ICSI)
- Pronuclear formation (+ 2nd polar body)
- Pronuclear fusion
- Zygote
**In vitro maturation**

Immature oocyte → mature oocyte

**In vitro fertilisation**

Immature oocyte + spermatozoon → zygote

**PGD**

1-cell → 2-cell → 4-cell → 8-cell

**In vitro production**

- Inner cell mass
- Blastocoel
- Trophoderm
- Expanded blastocyst
- Early/Expanding blastocyst
- Morula
- Compacting
Micromanipulator
Second polar body extrusion and pronuclear formation following ICSI in a zona-free human oocyte
PCR-based PGD analysis

Blastomere Biopsy → Freeze (-20°C >30 min) → Lab → Lysis / PK digestion

1\textsuperscript{st} round PCR (external primers) → 2\textsuperscript{nd} round PCR for DGGE analysis → LightCycler analysis (real-time PCR)
Melting curve analysis for the IVS I-110 mutation
DGGE analysis of 6 blastomeres during PGD
Preparation workup

- Strategy
- Training
- Setup of techniques on genomic DNA
- Tests on single cells (lymphocytes) >200
- Maximize amplification efficiency (>90%)
- Minimize allele dropout (<10%)
- Eliminate contamination factors
- Blastomere test from unused embryos
Determining factors for successful PGD

- Adequate number of ova
  - Not all will be fertilized successfully
- Adequate number of embryos
  - Not all will survive biopsy
  - Some may fail to develop normally
  - After analysis, ~25% expected not be suitable for transfer (affected)
  - A few may fail to amplify (5-10%) – no result
- Laboratory procedures
  - Biopsy techniques
  - Contamination control
  - Successful amplification of biopsy DNA
Sources of error

- Contamination
- Biopsy material (blastomere) actually not deposited in sample tube
- Cell fragmentation (bad quality embryos)
- Amplification efficiency
Contamination

- Embryo manipulation
  - Handling
  - Biopsy
- Biopsy manipulation
  - Transfer
  - PK digest
  - First PCR amplification
Contamination Control

- Blanks
  - Culture Medium Blanks
  - Biopsy Medium Blanks
  - Reagent Blanks

- Polymorphic Markers
  - D6S1056 (tetra-)
  - D15S652 (tri-)
Non Invasive Prenatal Diagnosis
Fetal Cells in Maternal Circulation

- A very small amount of fetal cells are present in the maternal circulation
- Methods for separating FNRBCs failed to recover a pure fetal cell population
- New technologies are now tested
  - Non-contact laser capture microdissection
  - Separation by electric field
Circulating Nucleic Acids

- First report 1948 (Mandel and Metais)
- Studies on Circulatory DNA focused on autoimmune diseases
- Diagnosis and prognosis of cancer 1977
- Discovery of fetal DNA in maternal plasma (Lo et al, 1997)
- NIPD offered for RHD and fetal sex for X-linked disorders
- NIPD under development for other single gene and chromosomal disorders
Properties of fetal DNA

- Possible source (placenta)
- Increased in a variety of pregnancy-related pathologies
- Fragmented (< 300 bp)
- 3-6% of plasma DNA
- Differentially methylated
Development of NIPD methods

Limitations

- Low quantity of fetal DNA
- Bad quality of fetal DNA
- The isolated DNA is mainly maternal (3-6% is fetal)
- Parents have the same mutations
NIPD Methodology

10ml peripheral blood

↓

4 ml Plasma

↓

Plasma DNA
(Maternal + Fetal)

↓

PCR based methods
NIPD for X-linked Disorders

- Test for the presence Y chromosome sequences in the maternal plasma

*Used for severe X-linked disorders:*
  - Duchene/Becker muscular dystrophy
  - X-linked agammaglobulinaemia
  - Hemophilia
  - Norrie disease (Episcopi blindness)
  - X-linked severe combined immunodeficiency (SCID)
Hemolytic Disease of the Newborn (HDN)

Mother: RHD-  
Father: RHD+

RhD-negative woman and RhD-positive man conceive a child

RhD-negative woman with Rh-positive fetus

Cells from RhD-positive fetus enter woman’s bloodstream

Woman becomes sensitized

Antibodies form to fight RhD-positive blood cells

In the next pregnancy RhD-positive pregnancy, maternal antibodies attack fetal red blood cells
NIPD for the RhD of the fetus

- For RhD-negative women
- Blood sample from the mother after the 16 weeks of gestation
- Analysis of the plasma DNA
- Diagnosis
β-Thalassaemia Non-Invasive Prenatal Diagnosis by Cell Free Fetal DNA

- The method is based on the detection of the paternally inherited fetal alleles
Selection/Analysis of SNPs for NIPD

- High degree of heterozygosity
- Informative SNPs
  - Mother A/A, Father A/B (determination of allele)
  - Mother A/A, Father B/B (confirmation of paternal allele)
Selection of SNPs with high degree of heterozygosity

- SNP genotyping analysis
- 75 random samples (Cyprus Population)
- Sequenom® MALDI-TOFF Mass Array
Analysis on 67 families at risk for β-thalassaemia for 42 SNPs

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<th>Informative SNPs</th>
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NIPD method using SNPs can be performed on 84% of families
Thalassochip
APEX (Arrayed Primer Extension)

- 54 beta thalassaemia mutations
- 6 Hb Variants
- 6 delta thalassaemia mutations
- 10 SNPs

- It was validated as a diagnostic tool for haemoglobinopathies (EC MedGeNet project)

Sensitivity/Specificity of APEX gen DNA SNP rs7480526 (g/t)

Gen DNA: t/t
1:1000
(1.5 ng/rxn)
1:10000
(0.15 ng/rxn)

Genotype 100% g/g
(15ng/rxn)

Genotype
95% g/g + 5% g/t
APEX analysis on maternal plasma rs10837631(a/t) mo: a/a, fa:a/t

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<th>C</th>
<th>G</th>
<th>T</th>
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NIPD on Family 22

rs 10768683 c/g
mother c/c
fetus c/c

rs 10837631 a/t
mother $\alpha / \alpha$
fetus $\alpha / t$

IVSII-745 c/g
mother c/c
fetus c/c
NIPD with SNPs (family 22)

NIPD: Normal or β-thal trait

Conclusions

- NIPD using SNPs analysis is possible
- Risk of error reduces to acceptable levels using three or more SNPs (The higher the number of SNPs the more efficient/reliable NIPD.
- APEX-promising technique, needs improvement
- Paternal allele of the fetus non-invasively detected