Molecular diagnosis of hemoglobin disorders
الفريق الطبي الأكاديمي

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**NOTE:** the doctor starts with a general explanation about this lecture in this page, in the next pages he'll talk about them in details. ^-^**

In the previous lecture we talked about types of HB disorders, now we'll talk about molecular diagnosis of these disorders. We mentioned the **HBA2** is raising in **βTH** and **HBF** above normal physiological level in **sickle cell** disease.

* How we could know if HBF or HBA2 or HBS are high above normal concentration? *By measuring how many grams of HB you have, normally is above 12-15 mg/dl.*

So if you suspect **sickle cell anemia** you ask for **CBC** tests, they'll take blood sample and values about **HB, BCV, MCV, RBCs, WBCs…**; supposedly you saw **low values** of HB, MCV, BCV and you want to know what is the problem, is it iron deficiency or blood genetic disease, so to exclude if it's iron deficiency you ask for iron level; if it's **low** you **confirm** your suspects. You could also go to ferritin to test the storage ferritin of iron in our cells; if iron or ferritin is **normal** so you have to make **farther investigation** which begin with **{HB Electrophoresis}** take a drop of blood from the patient and hemolysis it and run it on a gel or stripe of paper under electrical field, that drop of blood contain many types of HBs and HBs are proteins; according to different charges they carry they'll migrate differently, so HBA will migrate and gives you a band different from HBA2, HBF, HBS, HBC, HBH, HBE. Once you have these results you could tell what that patient suffers. Supposedly even with these values you **didn't reach to a conclusive decision;** in that case you have to do **DNA tests (molecular tests)** to know exactly what **gene** is involved in the problem.
HB Electrophoresis:

Slides:
- Hemoglobin electrophoresis is used as a screening test to identify variant and abnormal hemoglobin, including hemoglobin A1 (HbA1), hemoglobin A2 (HbA2), hemoglobin F (HbF; fetal hemoglobin), hemoglobin C (HbC), and hemoglobin S (HbS).
- Separation of hemoglobin is based on variable rates of migration of charged hemoglobin molecules in an electrical field.

Indications and applications of hemoglobin electrophoresis include the following:
- Evaluation of unexplained hemolytic anemia
- Microcytic anemia unrelated to iron deficiency, chronic disease, or lead toxicity
- A peripheral smear with abnormal red cell features (eg, target cells or sickle cells)
- Positive family history of hemoglobinopathy
- Positive neonatal screen results
- Positive results on sickle cell or solubility test
Diagnosis of Hemoglobin disorders

This is called **electrohemograph** and as you see this is a stripe of **cellulose acetate paper** (المستطيل الذي يتضمن الخطوط). You put a **drop of blood** after **hemolysis** at this site or here for different patients who you want to investigate their HB. Then you apply an **electrical field** to help for these HBs for migration and you run a **standard lane** for different types of HBs in order to know where each type of HB will be **migrated**.

**Ex.** At this site (A2) **HbA2** will appear migrate to this limit, **HbS** will migrate to this limit (HbS), **HBF…**, **HBA…**, **HBH…**, after you have your electrogram you stain it with specific stains in order to see these bands and the **stain** called (**ponceau S**) and the bands will appear very clear to you as you see them here. **So each band represent the type of HB that the patient has.**

**Ex.** This (**اول مستطيل**) represent the normal adult HB electrophoresis pattern, so you see a band under A2 and another band under A that very thick comparable to this band (**باقي الخطوط بنفس المستطيل**) this is the electrophoresis pattern for a normal person. You could quantitate how much of this relative to this (**مقارنة حجم الخطوط ببعضها**) by **scanning**, you take this paper put it under scanner and it'll give you amounts of **how much of each** (HB).
So normally you'll see very little of A2, F and more than 97% will be HBA.

**Student question:** can we use the molecular weight in this test?  
**Dr. Answer:** yes we can work on M.W but we unite the charges because they are different.

**Dr. asked:** why they are different even they all are HB?  
**Answer:** because they are different proteins even if it's just one different amino acid ex. HbS.

**Q:** And why this difference in one amino acid make difference in migration between A and S?  
**Answer:** because there was change between glutamic acid (negative charge) and valine (no charge), so difference in charges make this difference in migration.
Now **sickle cell trait** → you'll see traces of \( A_2 \) some HbS, little HbF, low HbA (but more intense or concentrate than S) in this case \( A > S \)→ Sickle trait "heterozygotes".

**Sickle cell disease** → S, F and no A → because no synthesis of normal \( \beta \) globin due to both genes are defected \( \beta^s \beta^s \), so most of Hb are HbS and nothing of A. and why HbF appears here? Due to compensation because there is no normal \( \beta \) so some \( \gamma \) genes will switched on.

\[ \beta \text{ TH trait} \] → some \( A_2 \) (thicker than normal), F > normal, A< normal, so \( A_2, F \) are compensating for decrease one of the \( \beta \) globin genes.

\[ \beta \text{ TH major} \] → both \( \beta \) globin genes are defected, could be \( \beta^+ \) or \( \beta^0 \) → high \( A2/F \), very little of A (but here you can't know if node or +, so you need some DNA tests).

**Sickle cell \( \beta \) TH** → high \( A2, S, F \) and low A (very severe like \( \beta \) TH major), you will see a lot of these of double heterozygotes compound conditions of sickle and TH this is called "**sickle cell \( \beta \) Thal**".

- A lot of people are missing this diagnosis, it's different from \( \beta \) TH major → counselling, genetic counselling, management will be different. So it's important to know exactly the diagnosis by these techniques. If you have like this (no S, low F, 50% of A and H) so low \( \alpha \) globin→ HbH disease (a lot of \( \beta \),tetramers of \( \beta \), nonfunctional anemia).

- There are different types of HB electrophoresis, in order to differentiate between variants of HB upon their migration under electrical field sometimes you have to change the PH in order to differentiate between for example A2 and C because in some systems they'll migrate together, so you need to change PH to give different charges to these similar migrated molecules to differentiate between them.
Diagnosis of Hemoglobin disorders

**Slides:**

- **Sickle cell trait** • (HbAS)
  - Sickle α- thalassemia
- **Sickle cell anemia** • (HbSS)
  - Sickle beta 0- thalassemia
  - Sickle–HPFH
- **Sickle β+ thalassemia**
  - HbS > HbA and HbS> HbF
  - Sickle beta + - thalassemia
- **HbC trait** (HbAC)
  - HbC < HbA
  - HbC > HbA
  - HbC and HbC
- **HbC disease** (HbCC)
  - HbC –beta 0 - • thalassemia (HbC- HPFH
  - HbC beta + - • thalassemia
  - HbSC disease

**Additional diagnosis:**

- **HbH disease** •
- **Increased HbA2** •
  - Beta-thalassemia minor
- **Increased HbF** •
  - Hereditary persistence of fetal hemoglobin
  - Sickle cell anemia•
  - Beta-thalassemia•
  - HbC disease•
  - HbE disease•
If you couldn’t reach to a conclusive decision you have to go to DNA tests but it’s not wise to begin with DNA testing, first you have to go to CBC then HB electrophoresis then to DNA testing.

**DNA testing:**
You take blood sample put it in a tube that have **anticoagulant** like (EDTA) ethylene diamine tetra acetate, you take 1 mL of blood sample put it in that tube, shack it to prevent coagulation then prepare the DNA from the blood (we know RBCs don’t contain any nucleus or DNA) so we preparing the DNA from the WBCs, there are available kits (ways) to prepare that DNA, once you have DNA it’s very very low concentration, you can’t do with that amount any testing, so the solution is **PCR** that amplify, suppose you have one copy of DNA from that patient, if you run the PCR as we explained previously you could increase that copy to million copies and you’ll have Nano grams of that DNA, now you could use these amounts for testing. The most important test after amplify the region in which the mutation isolated is **sequencing**?

How can I know that region?
1. **Family history** → if they have any type of mutation for any type of sickle or TH.
2. **ethnic group** (مجموعة عرقية) if they are Asian or Caucasian (European region) or different regions → high spread mutation.

Relay on these we **do amplification** → **sequencing** → determine mutated region → put primers in different directions and strands → put the enzyme that do amplification (TAC)

**Preview of PCR:**
We take the **double strands** that we separates from blood sample → **denaturation** on 95 cm integrin → **single strand** → detect **amplifying region** → put primer on one strand in one direction and primer on the other strand in the other direction → **trap amplifying region** → put the enzyme → reaction start in 2 direction → do 30 cycle to make million copy → we take the product → **DNA sequence** (the technique for this sequencing by a machine “**sequencer**” that give us the sequence direct) → compare the resulting sequence with the wild type (sequence normal) → if there is any **difference** even in one nucleotide → there is a **mutation** in this region
Slides:
Sources of DNA
- The main source of DNA is peripheral leucocytes obtained from peripheral blood anticoagulated, preferably with EDTA.
- Fetal DNA is mainly isolated from chorionic villi.
- Fetal DNA can also be prepared from amniotic fluid cells directly or after culture.
- Noninvasive methods of prenatal diagnosis utilize DNA from fetal cells in maternal circulation (Cheung, Goldberg & Kan, 1996).

Overview of technique and methodology:
- Almost all the methods for DNA analysis of the hemoglobinopathies used today are based on the polymerase chain reaction (PCR) (Saiki et al., 1985).
- Therefore whether a mutation is a deletion, a rearrangement or a point mutation a similar test will be performed with the variability and specificity coming from the primers used.
- The sensitivity and specificity of PCR has revolutionized the molecular diagnostic field.

The PCR-Based Techniques used in Hemoglobin Diagnostics include:

A. Known mutations
- Allele-specific oligonucleotide (ASO) hybridization or dot-blot analysis,
- Allele-specific priming or amplification refractory
mutation system (ARMS),
- Restriction enzyme analysis, amplification created restriction analysis, (RFLP)
- Gap-PCR.
- The ultimate method of mutation identification is by direct sequence analysis of specifically amplified DNA.

B. Unknown mutations: (SSCP)

GLOBIN GENES
• The majority of the mutations causing β-thalassaemia are concentrated in two regions –
  (i) 5' promoter and 5' untranslated region (UTR), exon 1, intron 1, exon 2 and the flanking intron 2 sequences
  (ii) 3' intron 2 sequences, exon 3 and the 3' UTR

The arrow indicates the location of a heterozygote where two signals are read at the same location with half the signal intensity of a homozygous signal.

(a) [Line diagram illustrating the α2 and α1 globin genes and positions of the amplification primers.]

(b) [Sequence analysis of the α2-globin gene at the exon 1/intron 1]

Direct sequence analysis of beta globin genes

DNA sequence gives colored peaks and reads them then compares them with the wild type to detect the differences. Each peak is a nucleotide (ex: green peak → A, blue peak → C) and at somewhere there are 2 peaks are overlapped each other (like the state at the arrow in the picture above and we notice that the machine gives us the letter N → that means there is a mutation here and it's a heterozygote; because it's between 2 different sequences like the heterozygote defect in β globin gene or α globin gene or any gene we're testing)

(a) Line diagram illustrating the α2 and α1 globin genes and positions of the amplification primers.
(b) Sequence analysis of the α2-globin gene at the exon 1/intron 1
junction. One base (G) after the end of exon 1 the sequencing peaks of the chromatogram are reduced in height with double peaks in some locations.

- This picture is typical of a frameshift due either to an insertion or deletion.
- Comparison of the frameshifted sequence with that of the wild type indicates a 5 bp deletion from IVS1-1 position (α2 IVS1-5 bp). The mutation is confirmed by Hph I restriction analysis.

Detection of the α2 IVS1–5 bp del (αHphα/) thalassaemia variant.

Here please follow the record (29:26-30:06) with the picture.

(c) Confirmation of the α2 IVS1-5 bp deletion by Hph I restriction analysis.

The deletion destroys the Hph I site resulting in a larger restriction fragment of 676 bp in the affected individual. Lanes 1 and 4 are DNA size markers (markers X and IX respectively, Roche), lane 2 is a heterozygous individual and lane 3 is a wild type control.
In β thalassemia the mutations focus on promoter region, exon(1 mainly), intron 1 and some of exon 2 and intron 2.

Approximately there are more than 1000 mutations that cause β thalassemia but there are very limited types of mutations that are the most prevalent in all thalassemic patients, usually we look for the most prevalent ones if we don’t see them by using these primers we do the test for the whole sequence to compare it with the wild type and detect the mutation.

In the picture: we notice that there are short peaks that overlap with each other; that means there is deletion of few bases that cause changes in the reading of the DNA or mRNA; this mutation is called (FRAME SHIFT) mutation.

**Allele-specific priming – ARMS-PCR:**

- Principle: a perfectly matched primer is much more efficient at annealing and directing primer extension than a mismatched primer.
- Allele-specific amplification relies on the specificity of the 3’ terminal nucleotide.
- Two reverse allele-specific primers, one complementary to the mutant sequence, the other to the normal DNA sequence.
- Presence of the mutant allele will generate a PCR product in the tube containing the mutation-specific primer, and vice versa.
The principle of ARMS: we have a patient’s DNA and we want to know the mutation so we use 2 types of primers (one is normal & another is mutant).

*Normal primer can’t make amplification for mutant and vice versa.

ARMS-PCR analysis

ARMS-PCR analysis for:
(a) Hb S
(b) Hb C
• Conventional ARMS-PCR is performed in two separate reactions using a common primer with a primer specific either for the wild type (N) or mutant (Mt) sequence.
• All PCRs are monitored by a control reaction (C) which amplifies a different part of the genome. Lanes labelled Mr are DNA size markers (Marker IX, Roche).

We use agarose or polyacrylamide gel after the amplification of DNA.
*Agarose: it’s slap, thin and has grooves to put the DNA inside them.
And there is a marker of many bands that tell us the size of each band (ex: 1kb, 7kb).
Normal DNA → expected fragments (bands) and there isn’t any amplified fragments.
Mutant DNA → any amplified fragment in the DNA so there is a mutation.
*If there are normal and mutant in the gene → heterozygote.
*If there isn’t normal and there is mutant → homozygote.

EX: in testing β globin to detect the sickle cell anemia → homozygote … بدنا نتأكد من المعلومة من الدكتور.
Gap-PCR:

Principle:
- PCR primer pairs are designed to flank a known deletion generating a unique amplicon that will be smaller in the mutant sequence compared with the wild type (Faa et al., 1992).
- For small deletions such as the 619 bp deletion, a common cause of β-thalassaemia in Asian Indians, differential amplification products are generated in the mutant and wild type.

Usually it’s used when there is a deletion; mostly the deletion in thalassemia occurs in α thalassemia (deletion for 1, 2 or 3 α globin genes).

Q: How can we use the PCR to tell us that there is a deletion in the patient’s DNA?!  
A: let’s take α globin gene as an example; we bring primers that are complementary to a specific region and put them around the testing region that we are expecting deletion in.

Gap-PCR for α-thal 1:
This is an example on Gap PCR: here we have α globin chromosome or DNA and we want to know if these are deleted or not.  
We put 2 primers (pink color) then we do the amplification if that gives us:
- Amplified DNA fragments (length=314bp)→this is normal and we don’t have any deletion.
- Amplified DNA fragments (length =188bp)→this is mutation and we have deletion.
Then we take the amplification result and look for 188bp strand that indicates the deletion or for 314bp strand that indicates no deletion; then we take the family and see who has the 188bp or 314bp if we:

- find that both present → heterozygote (one gene is normal & another is mutant).
- find that only 188bp presents → homozygote.

**Restriction enzyme analysis of PCR product:** (Restriction fragment length polymorphism)

- Restriction enzymes cut double-stranded DNA at specific recognition sequences;
- some mutations naturally create or abolish restriction enzyme sites..
- Genomic DNA containing the mutation, the ‘target’, is amplified by PCR and the product is then digested by the diagnostic restriction enzyme and the resulting DNA fragments separated on gels.
- The presence or absence of the recognition site is determined from the pattern of the PCR digest hence an alternative term for this technique is restriction fragment length polymorphism (RFLP) analysis.)
Restriction enzyme analysis of PCR product: Detection of βS mutation by Dde I restriction analysis.

(a) Line diagram of the β globin gene showing locations of the PCR primers (horizontal arrows) and the βS mutation (in red) in codon 6, exon 1. Exons are shown in striped boxes and introns in open boxes.

(b) Several Dde I restriction sites are found in this PCR amplicon, the Dde I site in codon 6 is removed by the βS mutation, creating the largest restriction fragment of 308 bp. The additional and constant Dde I sites act as internal control for complete deletion Dde I digestion.

(c) Electrophoresis of the Dde I restriction digest. Lane 1 is the DNA size marker, marker V Roche; lane 2 is a homozygous affected control (Hb SS), lane 3 is the heterozygous control (Hb AS) and lane 4, the wild type control (Hb AA). Lanes 5, 6 and 7, are patient samples found to be Hb SS, Hb SS and Hb AS, respectively.
Here the mutation either produces a site of the restriction enzyme or cancels one. 

*Restriction enzyme: enzyme that will breaks the double DNA strands.
The breaking can’t occur at any place it must occur at a recognition sequence; if there is a mutation in this recognition sequence so the enzyme can’t bind and cut at that sequence (canceling); but if the recognition sequence is already not present then the mutation produces it and the enzyme will cut there (production).
So this technique depends on the production or canceling a recognition site by mutation.
For example (the picture above): we have a piece of DNA from a patient and we want to test for βs mutation; so we make amplification around the gene; if:
- the restriction enzyme cuts from the red lines → normal
- the first restriction enzyme is deleted because of the mutation that damages the recognition site so there is no cutting but the other cutting sites are normal → βs mutation.
In electrophoresis:
- 201bp → normal → indication for normal allele.
- 308bp → patient → indication for βs mutation.

Diagrammatic representation of an RFLP analysis for the presence of the sickle-cell locus.
Genomic DNA is isolated and digested with the restriction enzyme MstII. One MstII site is lost at the sickle-cell locus. The DNA is then Southern blotted and analyzed with a β-globin-specific probe corresponding to sequences at the 5’-end of the gene. Individuals homozygous for the normal globin genes will exhibit a single hybridization band since both maternal and paternal genes are unaffected. Heterozygotes will exhibit the normal band and the larger sickle-cell gene band. Homozygous sickle-cell individuals will exhibit a single larger hybridization band.

**MstII restriction sites around β-globin gene**

5' → lost in sickle cell → 3’
frgments

1.2kbp
1.4kbp

**Possible RFLP Data**

<table>
<thead>
<tr>
<th>Normal</th>
<th>Carrier</th>
<th>Sickle Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4kbp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2kbp</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This is also for the sickle cell:
- normally → we have 1.2 kbp fragment.
- mutation → cutting 1.4 kbp (case of illness sickle cell).
- carrier → the 2 fragments are present (heterozygote).
We use 2 probes one is normal another is mutant. 
*Probe: piece of DNA that we hybridize it. (مجس). 
*If there is hybridization that means the patient has the mutant--