Genome Editing and Engineering
Prof. Utpal Bora
Department of Bioscience and Bioengineering
Indian Institute of Technology, Guwahati

Module - 08
Applications of genome editing in treating human diseases
Lecture - 28
Human cell engineering in diseases: Thalassemia - Part A

Welcome to my course on Genome Editing and Engineering. In this module number 8, we are going to discuss about the Applications of genome editing in treating human diseases. In this first lecture, we are going to discuss about Human cell engineering in diseases with respect to Thalassemia.

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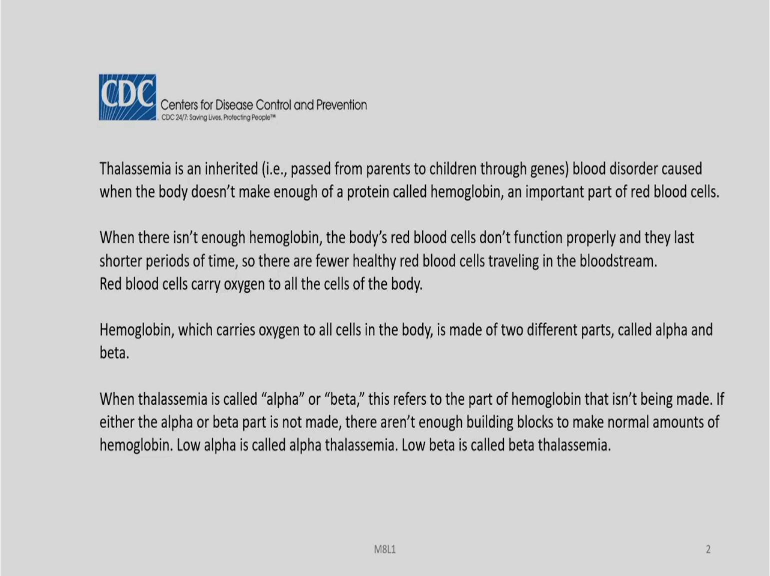

CDC Centers for Disease Control and Prevention
CDC 24/7: Saving Lives, Protecting People™

Thalassemia is an inherited (i.e., passed from parents to children through genes) blood disorder caused when the body doesn't make enough of a protein called hemoglobin, an important part of red blood cells.

When there isn't enough hemoglobin, the body's red blood cells don't function properly and they last shorter periods of time, so there are fewer healthy red blood cells traveling in the bloodstream.
Red blood cells carry oxygen to all the cells of the body.

Hemoglobin, which carries oxygen to all cells in the body, is made of two different parts, called alpha and beta.

When thalassemia is called "alpha" or "beta," this refers to the part of hemoglobin that isn't being made. If either the alpha or beta part is not made, there aren't enough building blocks to make normal amounts of hemoglobin. Low alpha is called alpha thalassemia. Low beta is called beta thalassemia.

So, this is the information is available in CDC regarding thalassemia which is an inherited disease, a blood disorder caused when the body does not make enough of a protein called hemoglobin, which is an important part of red blood cells. When there is not enough hemoglobin, the body's red blood cells do not function properly and they last shorter periods of time, so there are fewer healthy red blood cells traveling in the bloodstream. Red blood cells carry oxygen to all the cells of the body.

Hemoglobin, which carries oxygen to all cells in the body, present in the red blood cells is made of 2 different parts, called alpha and beta. When thalassemia is called alpha or beta this refers to the part of the hemoglobin that is not being produced by the body. If either the alpha or beta part is not made there are not enough building blocks to make normal amounts of hemoglobin. Low alpha is called alpha-thalassemia and low beta is called beta-thalassemia.

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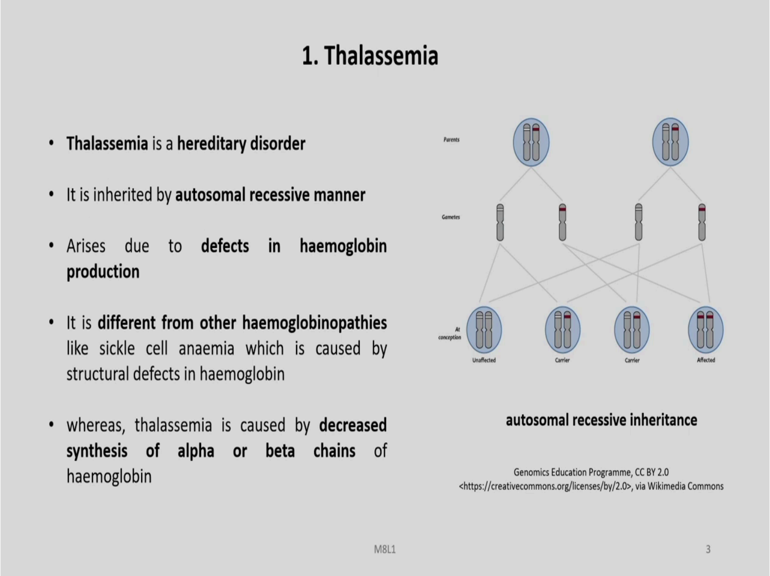

1. Thalassemia

- **Thalassemia** is a **hereditary disorder**
- It is inherited by **autosomal recessive manner**
- Arises due to **defects in haemoglobin production**
- It is **different from other haemoglobinopathies** like sickle cell anaemia which is caused by structural defects in haemoglobin
- whereas, thalassemia is caused by **decreased synthesis of alpha or beta chains** of haemoglobin

autosomal recessive inheritance

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So, now we know that thalassemia is a hereditary disorder and it is inherited by autosomal recessive manner. So, for example, we have 2 parents with heterozygous conditions to thalassemia. These parents appear to be normal and upon their marriage they may produce a very normal individuals because the thalassemia mutation is not being inherited in this case.

And they may also produce affected individuals because the gene which was there in recessive condition in both the parents has now become homozygous in these particular offspring and that causes the disease. And remaining population may be carriers and which are heterozygous as you can see from the pictorial representation.

These disease arises due to defects in the hemoglobin production as we have already discussed. It is different from other hemoglobinopathies like sickle cell anemia which is caused by structural defects in a hemoglobin whereas in thalassemia there is decreased synthesis of alpha or beta chains of the hemoglobin.

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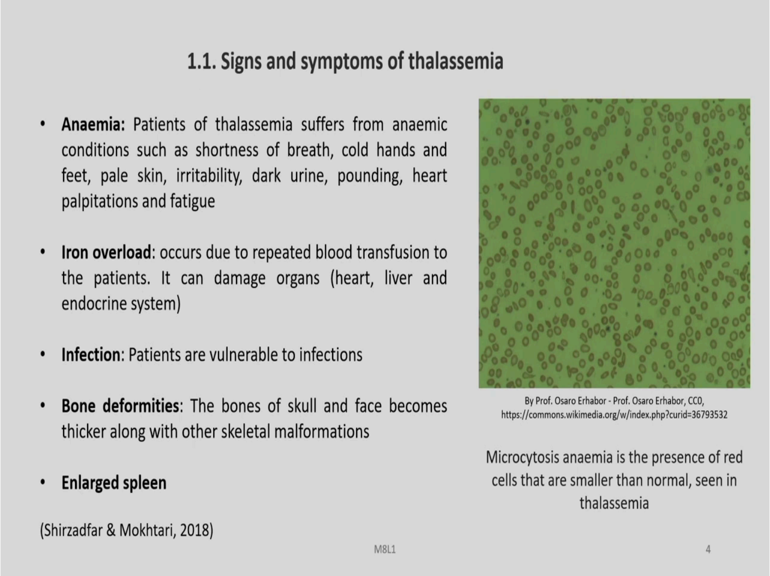

1.1. Signs and symptoms of thalassemia

- **Anaemia:** Patients of thalassemia suffers from anaemic conditions such as shortness of breath, cold hands and feet, pale skin, irritability, dark urine, pounding, heart palpitations and fatigue
- **Iron overload**: occurs due to repeated blood transfusion to the patients. It can damage organs (heart, liver and endocrine system)
- **Infection**: Patients are vulnerable to infections
- **Bone deformities**: The bones of skull and face becomes thicker along with other skeletal malformations
- **Enlarged spleen**

(Shirzadfar & Mokhtari, 2018)

By Prof. Osaro Erhabor - Prof. Osaro Erhabor, CC0, https://commons.wikimedia.org/w/index.php?curid=36793532

Microcytosis anaemia is the presence of red cells that are smaller than normal, seen in thalassemia

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What are signs and symptoms of this disease? Thalassemia patients with so, anaemia iron overload infection bone deformities and enlarged spleen. So, with respect to anaemia, thalassemia patients suffer from anaemic conditions such as shortness of breath, cold hands and feet, pale skin, irritability, dark urine, pounding, heart palpitations and fatigue.

There is always iron overload which occurs due to repeated blood transfusion to the patients it can damage organs and then patients are vulnerable to various infections. The bones of skull and face become thicker along with other skeletal malformations.

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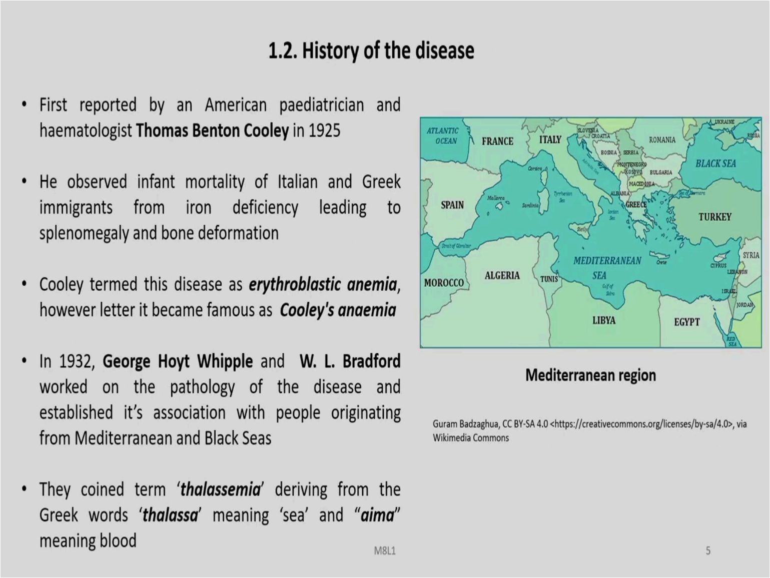

1.2. History of the disease

- First reported by an American paediatrician and haematologist **Thomas Benton Cooley** in 1925
- He observed infant mortality of Italian and Greek immigrants from iron deficiency leading to splenomegaly and bone deformation
- Cooley termed this disease as ***erythroblastic anemia***, however letter it became famous as ***Cooley's anaemia***
- In 1932, **George Hoyt Whipple** and **W. L. Bradford** worked on the pathology of the disease and established it's association with people originating from Mediterranean and Black Seas
- They coined term '***thalassemia***' deriving from the Greek words '***thalassa***' meaning 'sea' and "***aima***" meaning blood

Mediterranean region

Guram Badzaghua, CC BY-SA 4.0 <https://creativecommons.org/licenses/by-sa/4.0>, via Wikimedia Commons

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If you look into the history of this disease, it was first reported by an American Paediatrician Thomas Benton Cooley in 1925. He observed infant mortality of Italian and Greek immigrants from iron deficiency leading to splenomegaly and bone deformation.

Cooley termed this diseases as erythroblastic anemia; however, letter it became famous as Cooley's anaemia. In 1932 George Hoyt Whipple and Bradford worked on the pathology of the disease and established its association with people originating from Mediterranean and Black Sea.

They coined the term thalassemia deriving from the Greek words thalassa meaning sea and aima meaning blood.

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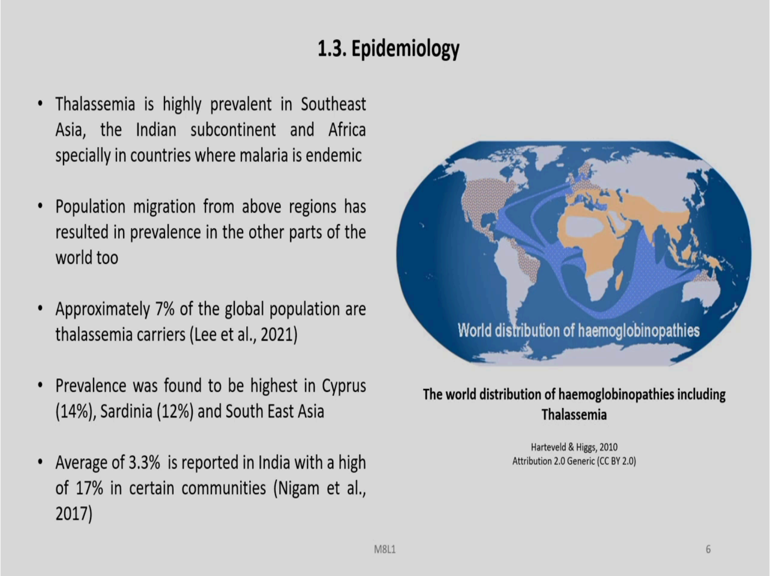

1.3. Epidemiology

- Thalassemia is highly prevalent in Southeast Asia, the Indian subcontinent and Africa specially in countries where malaria is endemic
- Population migration from above regions has resulted in prevalence in the other parts of the world too
- Approximately 7% of the global population are thalassemia carriers (Lee et al., 2021)
- Prevalence was found to be highest in Cyprus (14%), Sardinia (12%) and South East Asia
- Average of 3.3% is reported in India with a high of 17% in certain communities (Nigam et al., 2017)

The world distribution of haemoglobinopathies including Thalassemia

Harteveld & Higgs, 2010
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Thalassemia is highly prevalent in South Asia as you can see from this map, the Indian subcontinent and then Africa especially countries where malaria is endemic. Population migration from above regions has resulted in prevalence in the other parts of the world too. Approximately 7 percentage of the global population are thalassemia carriers as reported by Lee. Prevalence was found to be highest in Cyprus, Sardinia and South East Asia.

Average of 3.3 percent is reported in India with a high of 17 percent in certain communities as reported by Nigam.

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- Thalassemia is predominantly found in tropical and sub-tropical areas
- In southeast Asia, thalassemia genes are observed in about 75% of the population (carrier), where malaria is also highly prevalent (Vlok et al., 2021)
- Even though the mechanism is not clear and still debated, it is believed that α-thalassemia is a natural selection to provide protection to the carriers against malaria (*Plasmodium falciparum*) (Farashi & Harteveld, 2018)

scientific reports

OPEN **Forager and farmer evolutionary adaptations to malaria evidenced by 7000 years of thalassemia in Southeast Asia**

Melandri Vlok[1], Hallie R. Buckley[1], Justyna J. Miszkiewicz[2], Meg M. Walker[2], Kate Domett[3], Anna Willis[7], Hiep H. Trinh[4], Tran T. Minh[4], Mai Huong T. Nguyen[4], Lan Cuong Nguyen[4], Hirofumi Matsumura[5], Tianyi Wang[6], Huu T. Nghia[4] & Marc F. Oxenham[2]

Thalassemias are inherited blood disorders that are found in high prevalences in the Mediterranean, Southeast Asia and the Pacific. These diseases provide varying levels of resistance to malaria and are proposed to have emerged as an adaptive response to malaria in these regions. The transition to agriculture in the Holocene has been suggested to have influenced the selection for thalassemia in the Mediterranean as land clearance for farming encouraged interaction between *Anopheles* mosquitos, the vectors for malaria, and human groups. Here we document macroscopic and microscopic skeletal

It is predominantly found in the tropical and subtropical areas as we have seen from the graph earlier illustration. In Southeast Asia thalassemia genes are observed in about 75 percent of the population and their carriers where malaria is also highly prevalent. Even though the mechanism is not clear and still debated, it is believed that alpha-thalassemia is a natural selection to provide protection to the carriers against a malaria.

This paper in 'Scientific Reports' reports about the forager and farmer evolutionary adaptations to malaria evidenced by 7,000 years of thalassemia in South Asia.

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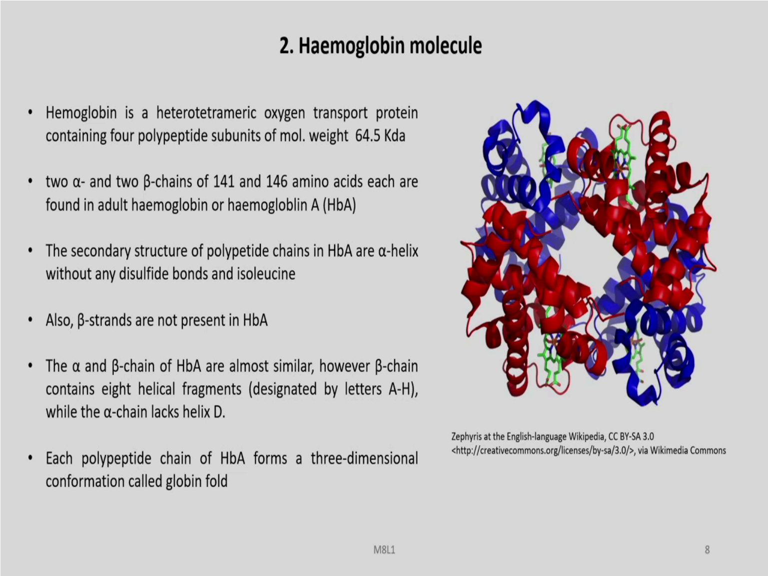

2. Haemoglobin molecule

- Hemoglobin is a heterotetrameric oxygen transport protein containing four polypeptide subunits of mol. weight 64.5 Kda
- two α- and two β-chains of 141 and 146 amino acids each are found in adult haemoglobin or haemoglobin A (HbA)
- The secondary structure of polypeptide chains in HbA are α-helix without any disulfide bonds and isoleucine
- Also, β-strands are not present in HbA
- The α and β-chain of HbA are almost similar, however β-chain contains eight helical fragments (designated by letters A-H), while the α-chain lacks helix D.
- Each polypeptide chain of HbA forms a three-dimensional conformation called globin fold

Zephyris at the English-language Wikipedia, CC BY-SA 3.0 <http://creativecommons.org/licenses/by-sa/3.0/>, via Wikimedia Commons

Let us have a look at the hemoglobin molecule which carries oxygen and is responsible for these disorder. This is a heterotetrameric oxygen transport protein containing four polypeptide subunits of molecular weight 64.5 kilo Dalton. 2 alpha and 2 beta chains of 141 and 146 amino acids each are found in the adult hemoglobin or hemoglobin A.

The secondary structure of polypeptide chains in HbA are alpha helix without any disulfide bones and isoleucine. Also beta strands are not present in these HbA. The alpha and beta chain of HbA are almost similar; however, beta chain contains 8 helical fragments designated by the letter A-H while the alpha chain lacks the helix D. Each polypeptide chain of HbA forms a three dimensional conformation called globin fold.

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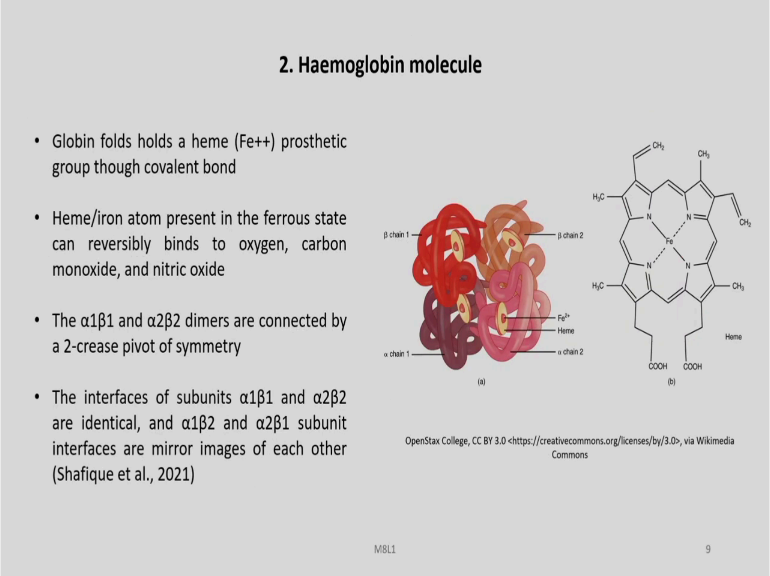

2. Haemoglobin molecule

- Globin folds holds a heme (Fe++) prosthetic group though covalent bond
- Heme/iron atom present in the ferrous state can reversibly binds to oxygen, carbon monoxide, and nitric oxide
- The α1β1 and α2β2 dimers are connected by a 2-crease pivot of symmetry
- The interfaces of subunits α1β1 and α2β2 are identical, and α1β2 and α2β1 subunit interfaces are mirror images of each other (Shafique et al., 2021)

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Globin fold holds a heme prosthetic group through covalent bond. The heme atom present in the ferrous state can reversibly bind to oxygen carbon monoxide and nitric oxide. The alpha 1 beta 1 and alpha 2 beta 2 dimers are connected by a 2 crease pivot of symmetry.

The interfaces of subunits alpha 1 beta 1 and alpha 2 beta 2 are identical and alpha 1 beta 2 and alpha 2 beta 1 subunit interfaces are mirror images of each other and you can see this iron being acting as a prostatic group here.

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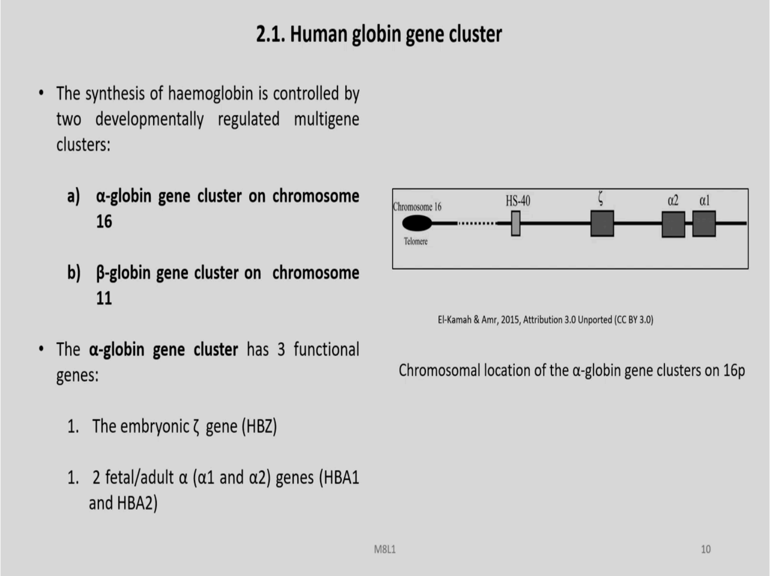

2.1. Human globin gene cluster

- The synthesis of haemoglobin is controlled by two developmentally regulated multigene clusters:

  a) **α-globin gene cluster on chromosome 16**

  b) **β-globin gene cluster on chromosome 11**

- The **α-globin gene cluster** has 3 functional genes:

  1. The embryonic ζ gene (HBZ)

  1. 2 fetal/adult α (α1 and α2) genes (HBA1 and HBA2)

El-Kamah & Amr, 2015, Attribution 3.0 Unported (CC BY 3.0)

Chromosomal location of the α-globin gene clusters on 16p

The synthesis of hemoglobin is controlled by 2 developmentally regulated multigene clusters; alpha-globin gene cluster on chromosome 16, beta-globin gene cluster on chromosome 11. The alpha-globin gene cluster has 3 functional genes; the embryonic zeta gene, 2 fetal/adult alpha (alpha 1 and 2) genes or HBA 1 and HBA 2.

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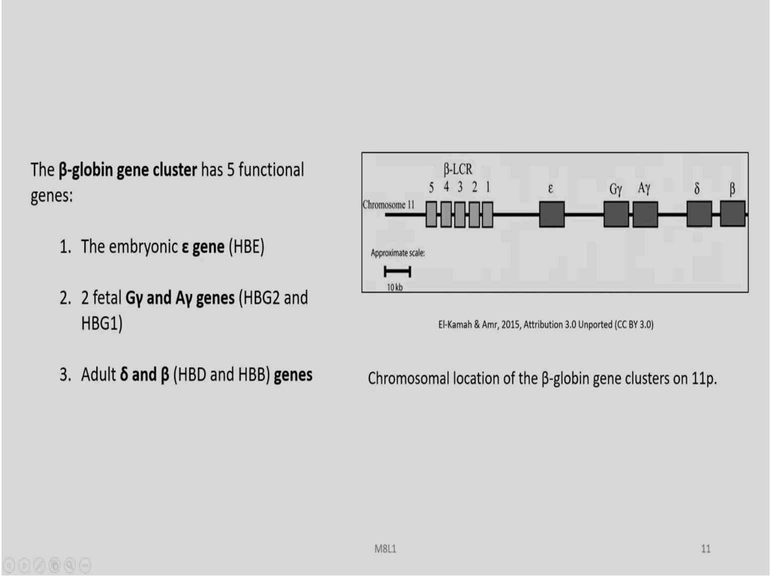

The **β-globin gene cluster** has 5 functional genes:

1. The embryonic **ε gene** (HBE)
2. 2 fetal **Gγ and Aγ genes** (HBG2 and HBG1)
3. Adult **δ and β** (HBD and HBB) **genes**

El-Kamah & Amr, 2015, Attribution 3.0 Unported (CC BY 3.0)

Chromosomal location of the β-globin gene clusters on 11p.

The beta-globin cluster has 5 functional genes; the embryonic epsilon gene, the 2 fetal G gamma and A gamma genes and HBG1, adult delta and beta HBD and HBB genes as shown in this diagram.

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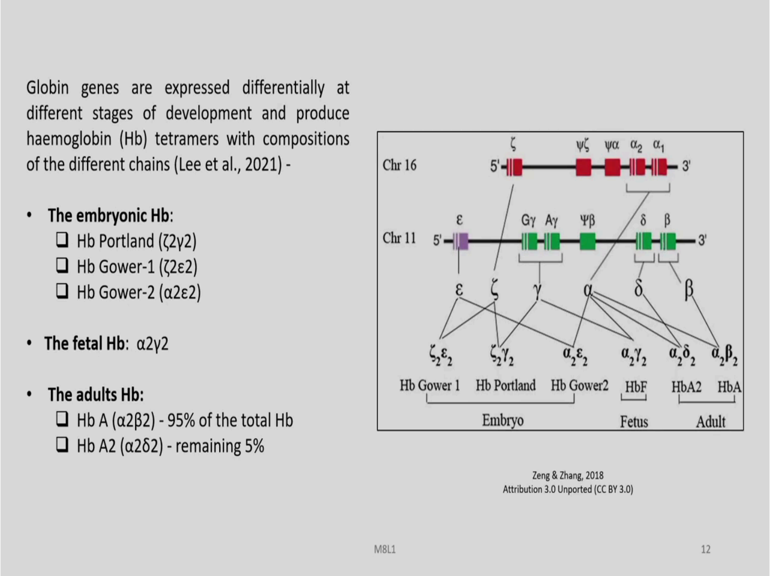

Globin genes are expressed differentially at different stages of development and produce haemoglobin (Hb) tetramers with compositions of the different chains (Lee et al., 2021) -

- **The embryonic Hb:**
  - ❑ Hb Portland (ζ2γ2)
  - ❑ Hb Gower-1 (ζ2ε2)
  - ❑ Hb Gower-2 (α2ε2)
- **The fetal Hb:** α2γ2
- **The adults Hb:**
  - ❑ Hb A (α2β2) - 95% of the total Hb
  - ❑ Hb A2 (α2δ2) - remaining 5%

Zeng & Zhang, 2018
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Globin genes are expressed differentially at different stages of development and produce hemoglobin tetramers with compositions of the different chains; the embryonic Hb - Hb Portland, Hb Gower-1 and Hb Gower-2 where you can see the compositions like alpha 2 epsilon 2 or zeta 2 epsilon 2 or zeta 2 gamma 2 and so on.

The fetal Hb has alpha 2 gamma 2, the adults Hb has Hb A and Hb A2 with alpha 2 beta 2 and alpha 2 delta 2 respectively and majority 95 percent of the total comes under Hb A and the remaining 5 percent belongs to HbA2. There are cis acting regulatory elements present upstream of globin gene clusters and regulate globin gene expression.

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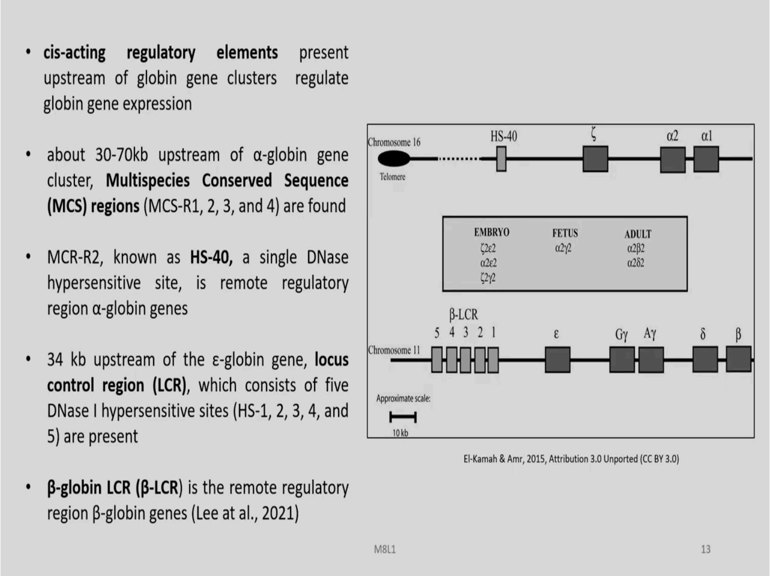

- **cis-acting regulatory elements** present upstream of globin gene clusters regulate globin gene expression
- about 30-70kb upstream of α-globin gene cluster, **Multispecies Conserved Sequence (MCS) regions** (MCS-R1, 2, 3, and 4) are found
- MCR-R2, known as **HS-40,** a single DNase hypersensitive site, is remote regulatory region α-globin genes
- 34 kb upstream of the ε-globin gene, **locus control region (LCR)**, which consists of five DNase I hypersensitive sites (HS-1, 2, 3, 4, and 5) are present
- **β-globin LCR (β-LCR)** is the remote regulatory region β-globin genes (Lee at al., 2021)

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About 30 to 70 kilo base upstream of alpha-globin gene cluster, multi species conserved sequence regions like MCS-R1, 2, 3 and 4 are found. MCR-R2 known as HS-40 a single DNase hypersensitive site is remote regulatory region alpha-globin genes. 34 kilo base upstream of the epsilon globin gene, locus control region or LCR, which consists of five DNase I hypersensitive sites HS 1, 2, 3, 4 and 5 are present and as shown in this figure.

A beta-globin LCR/beta LCR is the remote regulatory region beta-globin genes. What are the various types of thalassemia? This classification is done depending on the type of polypeptide chain whose synthesis has been impaired.

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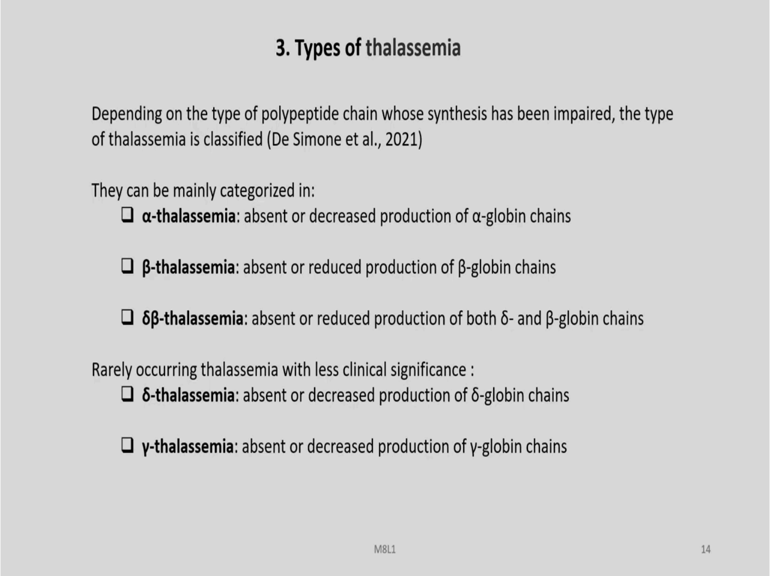

3. Types of thalassemia

Depending on the type of polypeptide chain whose synthesis has been impaired, the type of thalassemia is classified (De Simone et al., 2021)

They can be mainly categorized in:

- ☐ **α-thalassemia**: absent or decreased production of α-globin chains
- ☐ **β-thalassemia**: absent or reduced production of β-globin chains
- ☐ **δβ-thalassemia**: absent or reduced production of both δ- and β-globin chains

Rarely occurring thalassemia with less clinical significance :

- ☐ **δ-thalassemia**: absent or decreased production of δ-globin chains
- ☐ **γ-thalassemia**: absent or decreased production of γ-globin chains

The type of thalassemia are as follows- like alpha-thalassemia, beta-thalassemia or delta beta-thalassemia. In alpha-thalassemia, there is absence or decrease production of alpha-globin chains and in beta, there is absence or reduced production of beta-globin chains, in delta beta, there is absence or reduced production of both delta and beta-globin chains.

Rarely occurring thalassemia with less clinical significance are delta-thalassemia where there is absence and decrease production of delta globin genes and gamma-thalassemia where there is absent or decreased production of gamma globin chains.

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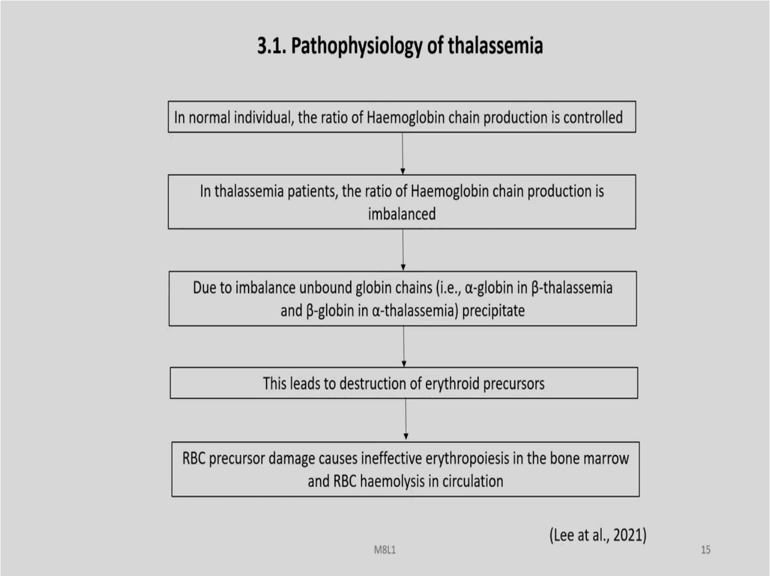

3.1. Pathophysiology of thalassemia

In normal individual, the ratio of Haemoglobin chain production is controlled

In thalassemia patients, the ratio of Haemoglobin chain production is imbalanced

Due to imbalance unbound globin chains (i.e., α-globin in β-thalassemia and β-globin in α-thalassemia) precipitate

This leads to destruction of erythroid precursors

RBC precursor damage causes ineffective erythropoiesis in the bone marrow and RBC haemolysis in circulation

(Lee at al., 2021)

Let us look into the pathophysiology of thalassemia. In normal individual the ratio of hemoglobin chain production is controlled. In thalassemia patients the ratio of hemoglobin chain production is imbalanced. Due to imbalance unbound globin chains, that is alpha-globin in beta-thalassemia and beta-globin in alpha-thalassemia precipitate. This leads to destruction of erythroid precursors.

RBC precursor damage causes ineffective erythropoiesis in the bone marrow and RBC haemolysis in circulation.

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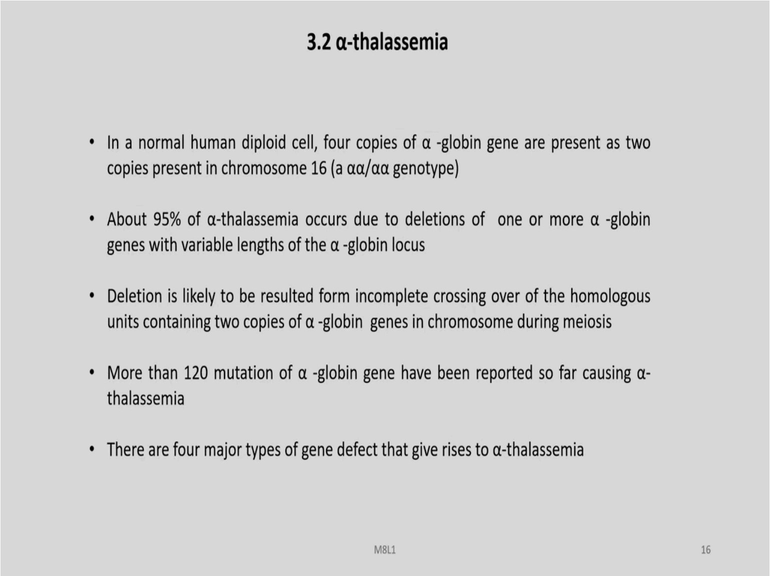

3.2 α-thalassemia

- In a normal human diploid cell, four copies of α -globin gene are present as two copies present in chromosome 16 (a αα/αα genotype)
- About 95% of α-thalassemia occurs due to deletions of one or more α -globin genes with variable lengths of the α -globin locus
- Deletion is likely to be resulted form incomplete crossing over of the homologous units containing two copies of α -globin genes in chromosome during meiosis
- More than 120 mutation of α -globin gene have been reported so far causing α-thalassemia
- There are four major types of gene defect that give rises to α-thalassemia

Alpha-thalassemia: In a normal human diploid cell, four copies of alpha-globin genes are present as two copies present in chromosome 16. About 95 percent of alpha-thalassemia occurs due to deletions of one or more alpha-globin genes with variable lengths of the alpha-globin locus.

Deletion is likely to be resulted from incomplete crossing over of the homologous units containing two copies of alpha-globin genes in chromosome during meiosis. More than 120 mutations of alpha-globin genes have been reported so far which causes alpha-thalassemia. There are four major types of gene defect that gives rise to alpha-thalassemia.

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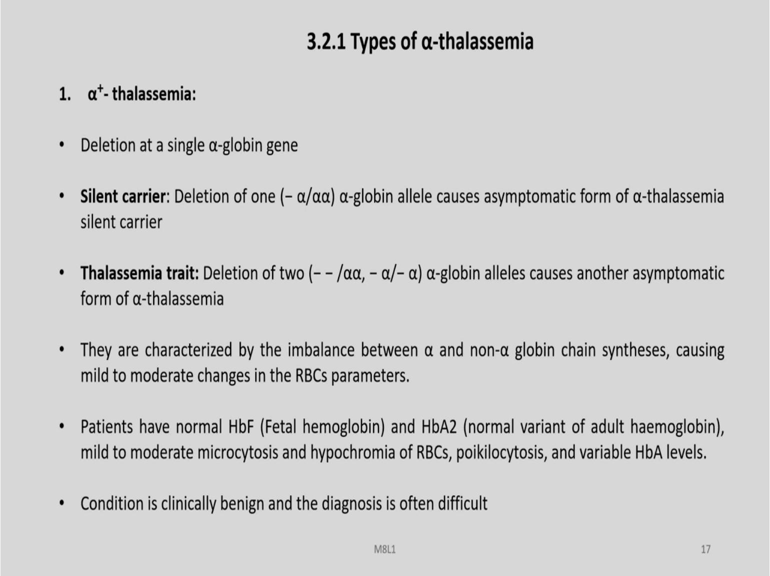

3.2.1 Types of α-thalassemia

1. **α^{+}- thalassemia:**

- Deletion at a single α-globin gene
- **Silent carrier**: Deletion of one (– α/αα) α-globin allele causes asymptomatic form of α-thalassemia silent carrier
- **Thalassemia trait:** Deletion of two (– – /αα, – α/– α) α-globin alleles causes another asymptomatic form of α-thalassemia
- They are characterized by the imbalance between α and non-α globin chain syntheses, causing mild to moderate changes in the RBCs parameters.
- Patients have normal HbF (Fetal hemoglobin) and HbA2 (normal variant of adult haemoglobin), mild to moderate microcytosis and hypochromia of RBCs, poikilocytosis, and variable HbA levels.
- Condition is clinically benign and the diagnosis is often difficult

So, we have the alpha plus thalassemia, there is a deletion at a single alpha-globin gene. It becomes a silent carrier deletion of one- dash alpha and alpha alpha; alpha-globin allele causes asymptomatic form of alpha-thalassemia silent carrier. And there is a thalassemia trait deletion of two- dash dash oblique alpha alpha and dash alpha oblique dash alpha; alpha-globin alleles causes another asymptomatic form of alpha-thalassemia.

They are characterized by the imbalance between alpha and non-alpha globin chain synthesis, causing mild to moderate changes in the RBCs parameters. Patients have normal HbF fetal hemoglobin and HbA2 a normal variant of adult hemoglobin, mild to moderate microcytosis and hypochromia of red blood cells, poikilocytosis and variable HbA levels. Condition is clinically benign and the diagnosis is often difficult.

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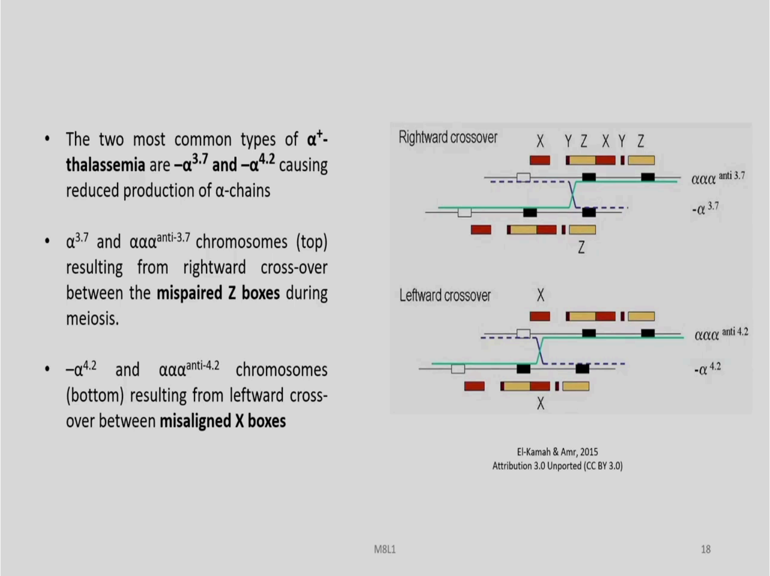

- The two most common types of **α^{+}-thalassemia** are **$-\alpha^{3.7}$ and $-\alpha^{4.2}$** causing reduced production of α-chains
- $\alpha^{3.7}$ and $\alpha\alpha\alpha^{anti\text{-}3.7}$ chromosomes (top) resulting from rightward cross-over between the **mispaired Z boxes** during meiosis.
- $-\alpha^{4.2}$ and $\alpha\alpha\alpha^{anti\text{-}4.2}$ chromosomes (bottom) resulting from leftward cross-over between **misaligned X boxes**

El-Kamah & Amr, 2015
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The two most common types of alpha plus thalassemia are alpha 3.7 and alpha 4.2 causing reduced production of alpha chain. Alpha 3.7 and triple a triple alpha anti 3.7 chromosomes as you can see in the top resulting from rightward cross over between the mispaired Z boxes during meiosis.

Alpha 4.2 and triple alpha triple alpha anti 4.2 chromosomes as shown in the bottom result from leftward crossover between misaligned X boxes. Deletions of one alpha gene are giving rise to alpha plus thalassemia.

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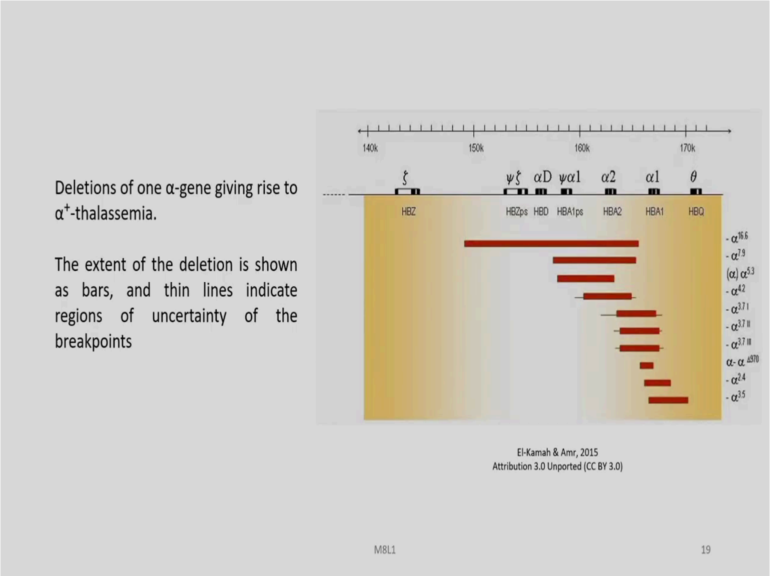

Deletions of one α-gene giving rise to α^{+}-thalassemia.

The extent of the deletion is shown as bars, and thin lines indicate regions of uncertainty of the breakpoints

El-Kamah & Amr, 2015
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The extent of the deletion is shown as bars and thin lines indicate regions of uncertainty of the breakpoints as shown in this figure.

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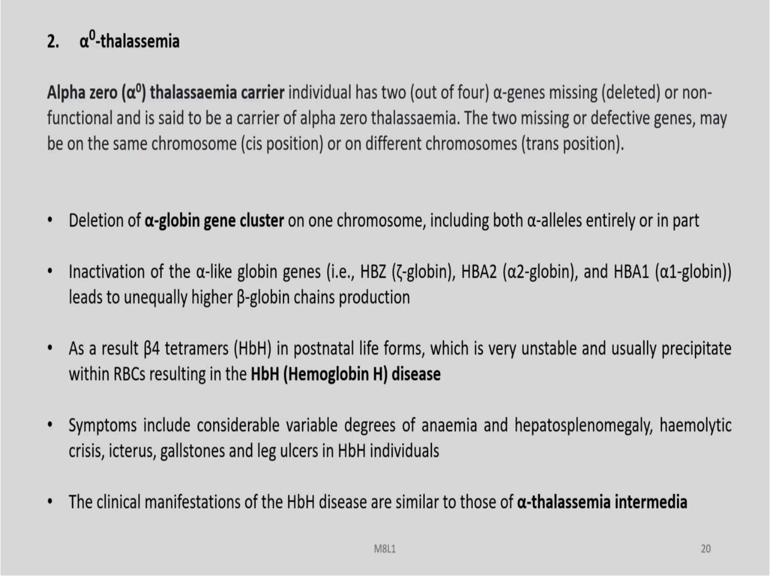

2. α^0-thalassemia

Alpha zero (α^0) thalassaemia carrier individual has two (out of four) α-genes missing (deleted) or non-functional and is said to be a carrier of alpha zero thalassaemia. The two missing or defective genes, may be on the same chromosome (cis position) or on different chromosomes (trans position).

- Deletion of **α-globin gene cluster** on one chromosome, including both α-alleles entirely or in part
- Inactivation of the α-like globin genes (i.e., HBZ (ζ-globin), HBA2 (α2-globin), and HBA1 (α1-globin)) leads to unequally higher β-globin chains production
- As a result β4 tetramers (HbH) in postnatal life forms, which is very unstable and usually precipitate within RBCs resulting in the **HbH (Hemoglobin H) disease**
- Symptoms include considerable variable degrees of anaemia and hepatosplenomegaly, haemolytic crisis, icterus, gallstones and leg ulcers in HbH individuals
- The clinical manifestations of the HbH disease are similar to those of **α-thalassemia intermedia**

Alpha zero thalassemia carrier individual has two out of four alpha genes missing or deleted or non-functional and is said to be a carrier of alpha zero thalassemia. The two missing or defective genes may be on the same chromosome cis position or on different chromosomes called trans position.

So, gene deletion of alpha-globin gene cluster on one chromosome, including both alpha allelles entirely or in part. The inactivation of the alpha like globin genes leads to unequally higher beta-globin chains production. As a result beta 4 tetramer in postnatal life forms which is very unstable and usually precipitate out within RBCs resulting in the hemoglobin H disease.

Symptoms include considerable variable degrees of anemia and hepatosplenomegaly, haemolytic crisis, icterus, gallstones and leg ulcers in HbH or hemoglobin H individuals. The clinical manifestations of the hemoglobin H disease are similar to those of alpha-thalassemia intermedia.

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In fetal life, the homozygous type of α^0 -thalassaemia (– – /– –) leads to an excess of γ-chains and the formation of γ4 tetramers (Hb Bart)

This condition is known as **Hb Bart's hydrops fetalis** is associated with intrauterine or neonatal death

A further abnormal Hb composed of four δ-globin chains has been detected in two individuals with the "**rare HbH disease**"

In fetal life the homozygous type of alpha zero thalassemia dash dash oblique dash dash leads to an excess of gamma chains and the formation of gamma 4 tetramers. This condition is known as Hb Bart's hydrops fetalis is associated with intrauterine or neonatal death. A further abnormal Hb composed of four delta-globin chains has been detected in two individuals with the rare HbH disease.

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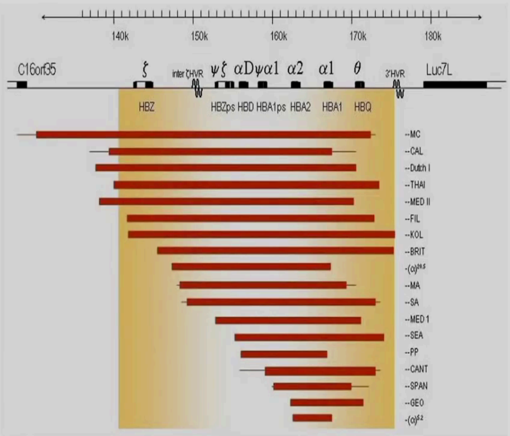

El-Kamah & Amr, 2015
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Deletions of two α-genes involved in α^0-thalassaemia. The MC, CAL etc. represents different deletions that have reported. For example, the specific deletions first reported in Southeast Asia and Mediterranean are represented as SEA and MED1

In this picture you can see deletions of two alpha genes involve in alpha zero thalassemia. The MC, CAL etcetera represents different deletions that have been reported. For example, the specific deletions first reported in Southeast Asia and Mediterranean are represented as SEA and MED1.

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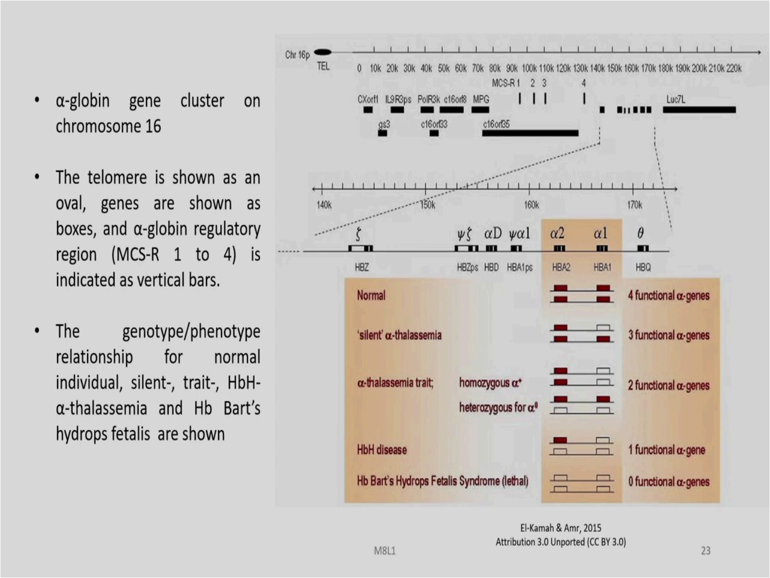

- α-globin gene cluster on chromosome 16
- The telomere is shown as an oval, genes are shown as boxes, and α-globin regulatory region (MCS-R 1 to 4) is indicated as vertical bars.
- The genotype/phenotype relationship for normal individual, silent-, trait-, HbH-α-thalassemia and Hb Bart's hydrops fetalis are shown

El-Kamah & Amr, 2015
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In this picture you can see alpha-globin gene cluster on chromosome 16 here. And the telomere is shown as an oval genes are shown as boxes and alpha-globin regulatory region MCS-R1 to 4 are shown as vertical bars. The genotype oblique phenotype relationship for normal individual, then silent, trait, HbH-alpha-thalassemia and Hb Bart's hydrops are also shown in this particular figure below.

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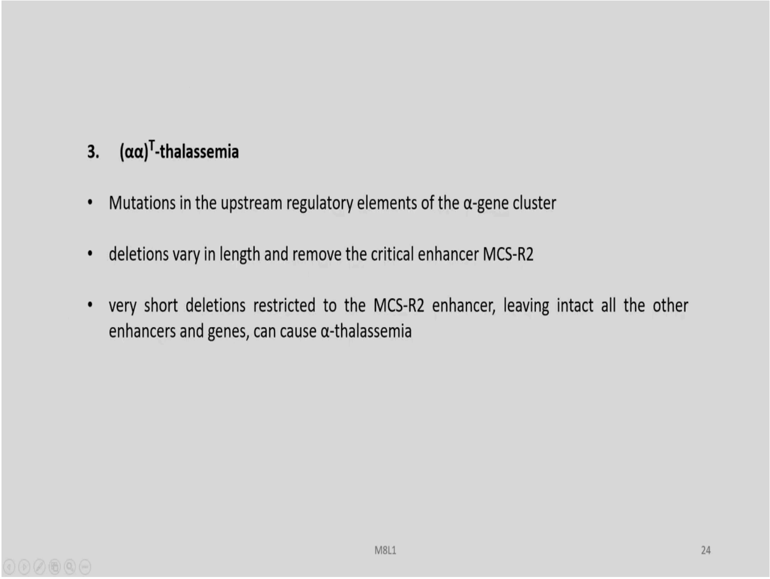

3. $(\alpha\alpha)^T$-thalassemia

- Mutations in the upstream regulatory elements of the α-gene cluster
- deletions vary in length and remove the critical enhancer MCS-R2
- very short deletions restricted to the MCS-R2 enhancer, leaving intact all the other enhancers and genes, can cause α-thalassemia

Alpha alpha T thalassemia - mutations in the upstream regulatory elements of the alpha gene cluster happens, deletions vary in length and remove the critical enhancer MCS-R2, very short deletions restricted to the MCS-R2 enhancer leaving intact all the other enhancers and genes and can cause alpha-thalassemia.

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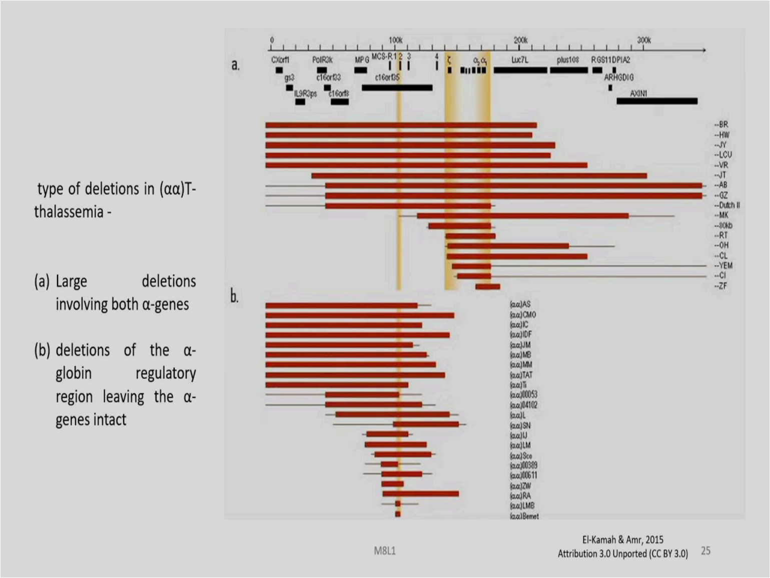

type of deletions in (αα)T-thalassemia -

(a) Large deletions involving both α-genes

(b) deletions of the α-globin regulatory region leaving the α-genes intact

El-Kamah & Amr, 2015
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In this picture we can see the types of deletion since alpha alpha T thalassemia. In figure a, large deletions involving both alpha genes and in b, deletions of the alpha-globin regulatory region leaving the alpha genes intact.

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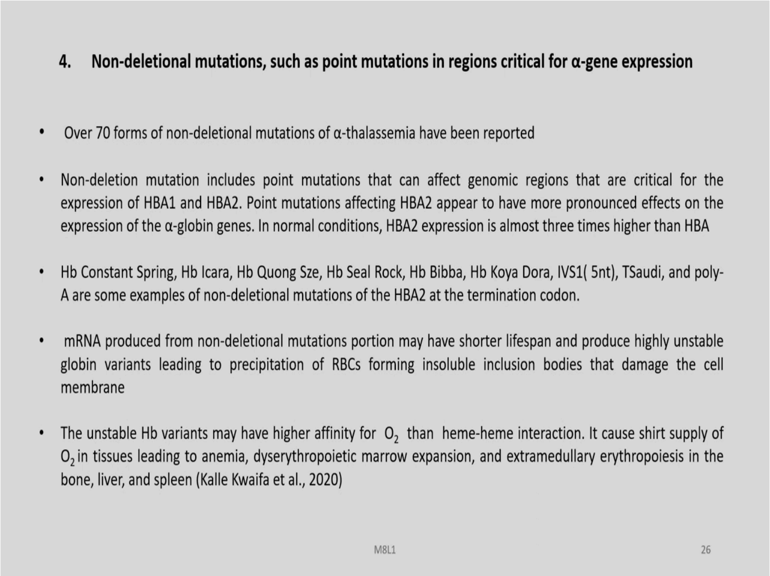

4. Non-deletional mutations, such as point mutations in regions critical for α-gene expression

- Over 70 forms of non-deletional mutations of α-thalassemia have been reported
- Non-deletion mutation includes point mutations that can affect genomic regions that are critical for the expression of HBA1 and HBA2. Point mutations affecting HBA2 appear to have more pronounced effects on the expression of the α-globin genes. In normal conditions, HBA2 expression is almost three times higher than HBA
- Hb Constant Spring, Hb Icara, Hb Quong Sze, Hb Seal Rock, Hb Bibba, Hb Koya Dora, IVS1(5nt), TSaudi, and poly-A are some examples of non-deletional mutations of the HBA2 at the termination codon.
- mRNA produced from non-deletional mutations portion may have shorter lifespan and produce highly unstable globin variants leading to precipitation of RBCs forming insoluble inclusion bodies that damage the cell membrane
- The unstable Hb variants may have higher affinity for O_2 than heme-heme interaction. It cause shirt supply of O_2 in tissues leading to anemia, dyserythropoietic marrow expansion, and extramedullary erythropoiesis in the bone, liver, and spleen (Kalle Kwaifa et al., 2020)

Non-deletional mutations such as point mutations in regions critical for alpha gene expressions also occur. Over 70 forms of non-deletional mutations of alpha-thalassemia has been reported. Non-deletion mutations include point mutations that can affect genomic regions that are critical for the expression of HBA1 and HBA2. Point mutations affecting HBA2 appear to have more pronounced effects on the expression of the alpha-globin genes.

In normal conditions HBA2 expression is almost three times higher than HBA. Hb Constant Spring, Hb Icara, Hb Quong Sze, Hb Seal Rock, Hb Bibba, Hb Koya Dora and polyA are some examples of non deletional mutations of HBA2 at the termination codon.

mRNA produced from non-deletional mutation portions may have shorter lifespan and produce highly unstable globin variants leading to precipitation of RBCs forming insoluble inclusion bodies that damage the cell membrane. The unstable Hb variants may have higher affinity for oxygen than heme-heme interaction. It causes a short supply of oxygen tissues leading to anemia and several other problems in bone, liver and spleen.

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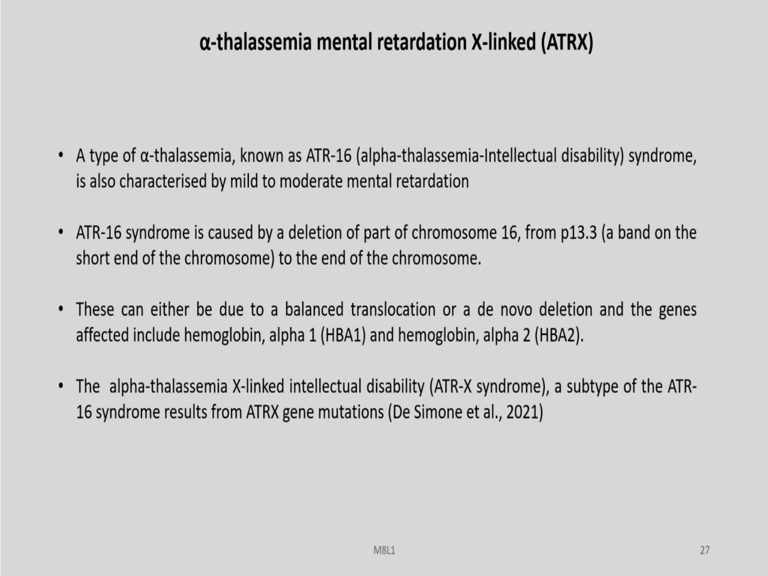

α-thalassemia mental retardation X-linked (ATRX)

- A type of α-thalassemia, known as ATR-16 (alpha-thalassemia-Intellectual disability) syndrome, is also characterised by mild to moderate mental retardation
- ATR-16 syndrome is caused by a deletion of part of chromosome 16, from p13.3 (a band on the short end of the chromosome) to the end of the chromosome.
- These can either be due to a balanced translocation or a de novo deletion and the genes affected include hemoglobin, alpha 1 (HBA1) and hemoglobin, alpha 2 (HBA2).
- The alpha-thalassemia X-linked intellectual disability (ATR-X syndrome), a subtype of the ATR-16 syndrome results from ATRX gene mutations (De Simone et al., 2021)

A type of alpha-thalassemia, known as ATR-16 alpha-thalassemia-intellectual disability syndrome is also characterized by mild to moderate mental retardation. So, this is known as the alpha-thalassemia mental retardation X-linked or ATRX. ATR-16 syndrome is caused by a deletion of part of chromosome 16, from p13.3 a band on the short end of the chromosome to the end of the chromosome.

These can either be due to a balanced translocation or a de novo deletion and the genes affected include hemoglobin alpha and hemoglobin alpha2. The alpha-thalassemia X-linked intellectual disability ATR-X syndrome, is subtype of the ATR-16 syndrome results from ATRX gene mutations as reported by Simone.

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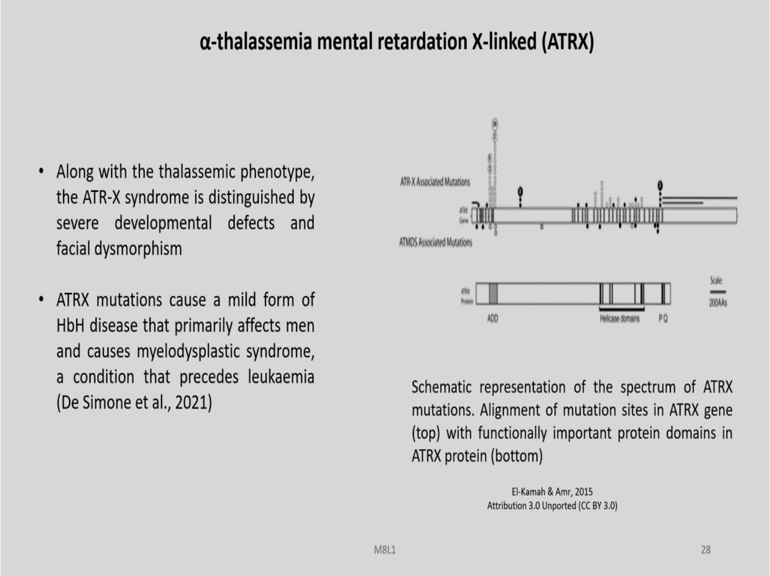

α-thalassemia mental retardation X-linked (ATRX)

- Along with the thalassemic phenotype, the ATR-X syndrome is distinguished by severe developmental defects and facial dysmorphism
- ATRX mutations cause a mild form of HbH disease that primarily affects men and causes myelodysplastic syndrome, a condition that precedes leukaemia (De Simone et al., 2021)

Schematic representation of the spectrum of ATRX mutations. Alignment of mutation sites in ATRX gene (top) with functionally important protein domains in ATRX protein (bottom)

El-Kamah & Amr, 2015
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Along with the thalassemia phenotype, the ATR-X syndrome is distinguished by severe developmental defects and facial dysmorphism. ATRX mutations cause a mild form of HbH disease that primarily affects men and causes myelodysplastic syndrome a condition that precedes leukaemia.

In this figure you can see the schematic representation of the spectrum of ATRX mutations. Alignment of mutation sites in ATRX genes on the top with functionally important protein domains in ATRX protein as shown below.

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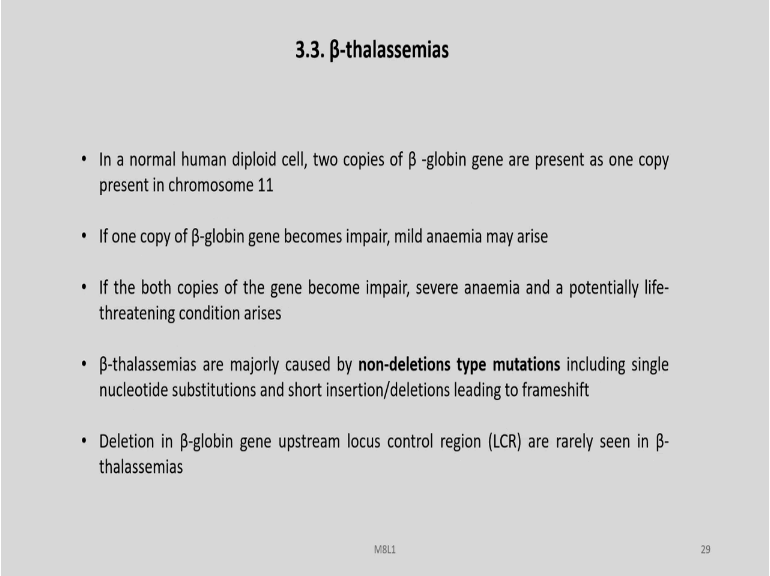

3.3. β-thalassemias

- In a normal human diploid cell, two copies of β -globin gene are present as one copy present in chromosome 11
- If one copy of β-globin gene becomes impair, mild anaemia may arise
- If the both copies of the gene become impair, severe anaemia and a potentially life-threatening condition arises
- β-thalassemias are majorly caused by **non-deletions type mutations** including single nucleotide substitutions and short insertion/deletions leading to frameshift
- Deletion in β-globin gene upstream locus control region (LCR) are rarely seen in β-thalassemias

Let us now have a look at the beta-thalassemias. In a normal human diploid cell, two copies of beta-globin gene are present as one copy present in chromosome 11. If one copy of beta-globin gene becomes impaired mild anaemia may arise. If both copy of the gene becomes impaired, severe anemia and a potentially life threatening condition arises.

Beta-thalassemias are majorly caused by non-deletions type mutations including single nucleotide substitutions and short insertion deletion leading to frameshift. Deletion in beta-globin gene cluster upstream locus control region are rarely seen in beta-thalassemias.

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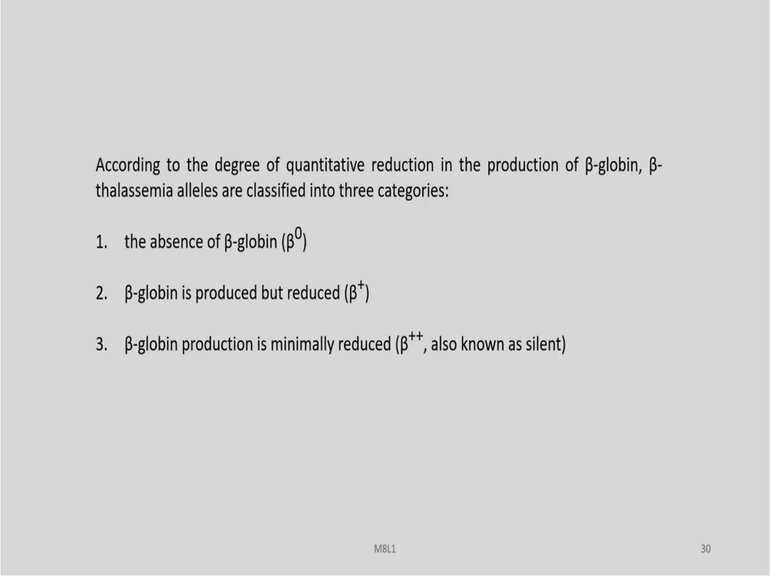

According to the degree of quantitative reduction in the production of β-globin, β-thalassemia alleles are classified into three categories:

1. the absence of β-globin (β^0)
2. β-globin is produced but reduced (β^+)
3. β-globin production is minimally reduced (β^{++}, also known as silent)

According to the degree of quantitative reduction in the production of beta-globin, beta-thalassemia alleles are classified into three categories. The absence of the beta-globin or beta 0; beta-globin is produced but reduced, beta plus; beta-globin production is minimally reduced beta plus plus, also known as a silent.

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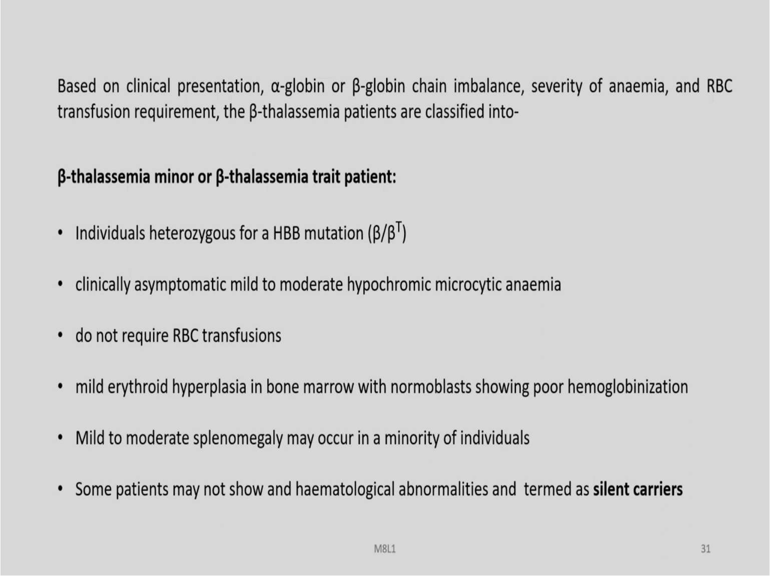

Based on clinical presentation, α-globin or β-globin chain imbalance, severity of anaemia, and RBC transfusion requirement, the β-thalassemia patients are classified into-

β-thalassemia minor or β-thalassemia trait patient:

- Individuals heterozygous for a HBB mutation (β/β^T)
- clinically asymptomatic mild to moderate hypochromic microcytic anaemia
- do not require RBC transfusions
- mild erythroid hyperplasia in bone marrow with normoblasts showing poor hemoglobinization
- Mild to moderate splenomegaly may occur in a minority of individuals
- Some patients may not show and haematological abnormalities and termed as **silent carriers**

Based on clinical presentations, alpha-globin or beta-globin chain imbalance, severity of anemia and RBC transfusion requirement, the beta-thalassemia patients are classified into beta-thalassemia minor or beta-thalassemia trait patient: Individuals heterozygous for a HBB mutation beta oblique beta T, clinically asymptomatic mild to moderate hypochromic microcytic anaemia.

And they do not require RBC transfusions. This is a there is a mild erythroid hyperplasia in bone marrow with normoblasts showing poor hemoglobinization. Mild to moderate splenomegaly may occur in a minority of individuals. Some patients may not show and haematologic abnormalities and are termed as silent carriers.

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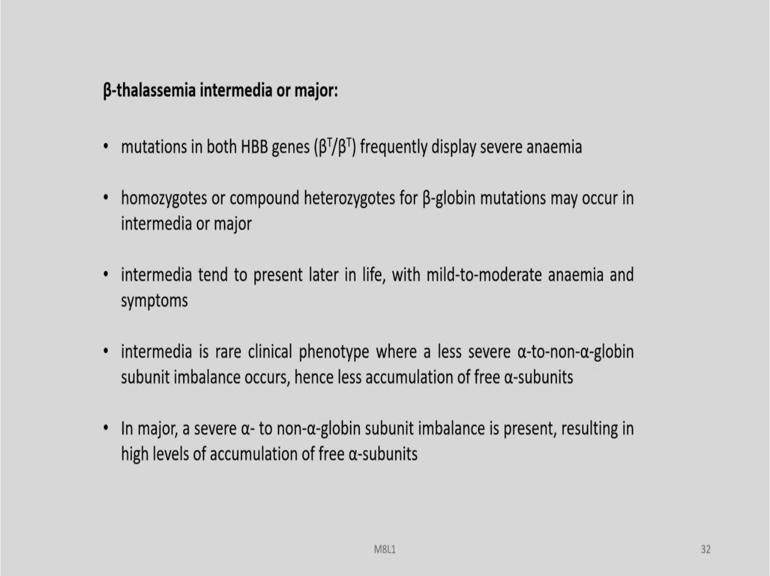

β-thalassemia intermedia or major:

- mutations in both HBB genes (β^T/β^T) frequently display severe anaemia
- homozygotes or compound heterozygotes for β-globin mutations may occur in intermedia or major
- intermedia tend to present later in life, with mild-to-moderate anaemia and symptoms
- intermedia is rare clinical phenotype where a less severe α-to-non-α-globin subunit imbalance occurs, hence less accumulation of free α-subunits
- In major, a severe α- to non-α-globin subunit imbalance is present, resulting in high levels of accumulation of free α-subunits

Beta-thalassemia intermedia or major: Mutations in both HBB genes beta T oblique beta T frequently display severe anaemia. Homozygotes or compound heterozygotes for beta-globin mutations may occur in intermedia or major. Intermedia tends to present later in life with mild to moderate anaemia and symptoms.

Intermedia is rare clinical phenotype where a less severe alpha–to-non-alpha-globin subunit imbalance occurs hence less accumulation of free alpha subunits. In major, a severe alpha-to-non-alpha-globin subunit imbalance is present resulting in high levels of accumulation of free alpha-subunits.

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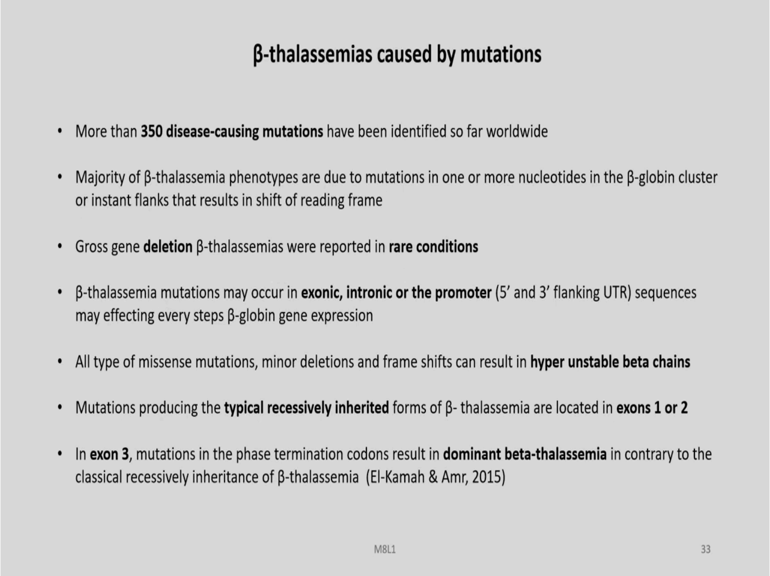

β-thalassemias caused by mutations

- More than **350 disease-causing mutations** have been identified so far worldwide
- Majority of β-thalassemia phenotypes are due to mutations in one or more nucleotides in the β-globin cluster or instant flanks that results in shift of reading frame
- Gross gene **deletion** β-thalassemias were reported in **rare conditions**
- β-thalassemia mutations may occur in **exonic, intronic or the promoter** (5′ and 3′ flanking UTR) sequences may effecting every steps β-globin gene expression
- All type of missense mutations, minor deletions and frame shifts can result in **hyper unstable beta chains**
- Mutations producing the **typical recessively inherited** forms of β- thalassemia are located in **exons 1 or 2**
- In **exon 3**, mutations in the phase termination codons result in **dominant beta-thalassemia** in contrary to the classical recessively inheritance of β-thalassemia (El-Kamah & Amr, 2015)

M8L1

So, we already have some ideas about beta-thalassemias which are caused by mutations. We know about a large number of disease causing mutations globally. The majority of beta-thalassemia phenotypes are due to mutations in one or more nucleotides in the beta-globin cluster or instant flanks that result in shifts of reading frame. Gross gene deletion beta-thalassemia is reported in rare conditions.

Beta-thalassemia mutations may occur in exonic, intronic or the promoter 5 prime and 3 prime flanking UTR sequences may affect every steps of beta-globin gene expression. All types of missense mutations, minor deletions and frame shifts can result in hyper unstable beta chains.

Mutations producing the typical recessively inherited forms of beta-thalassemia are located in exons 1 or 2. In exon 3, mutations in the phase termination codon results in dominant beta-thalassemia in contrast to the classical recessively inheritance of beta-thalassemia.

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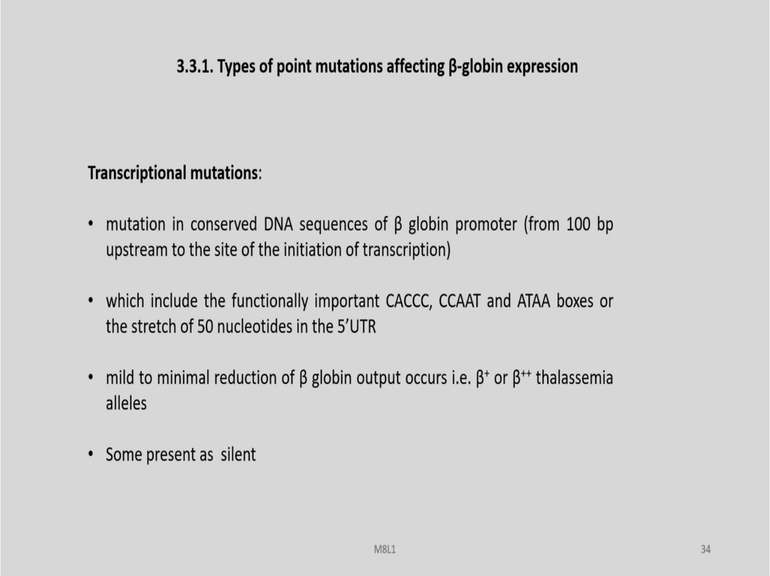

3.3.1. Types of point mutations affecting β-globin expression

Transcriptional mutations:

- mutation in conserved DNA sequences of β globin promoter (from 100 bp upstream to the site of the initiation of transcription)
- which include the functionally important CACCC, CCAAT and ATAA boxes or the stretch of 50 nucleotides in the 5′UTR
- mild to minimal reduction of β globin output occurs i.e. β^{+} or β^{++} thalassemia alleles
- Some present as silent

What are the types of point mutations that affect beta-globin expression? Number one is a transcriptional mutations. Mutation in observed DNA sequences of beta-globin promoter from 100 base pairs upstream to the site of the initiation of transcription occurs, which include the functionally important CACCC, CCAAT, and ATAA boxes or the stretch of 50 nucleotides in the 5 prime UTR. Mild to minimal reaction of beta-globin output results that is beta plus or beta plus plus thalassemia alleles. And some are present as silent.

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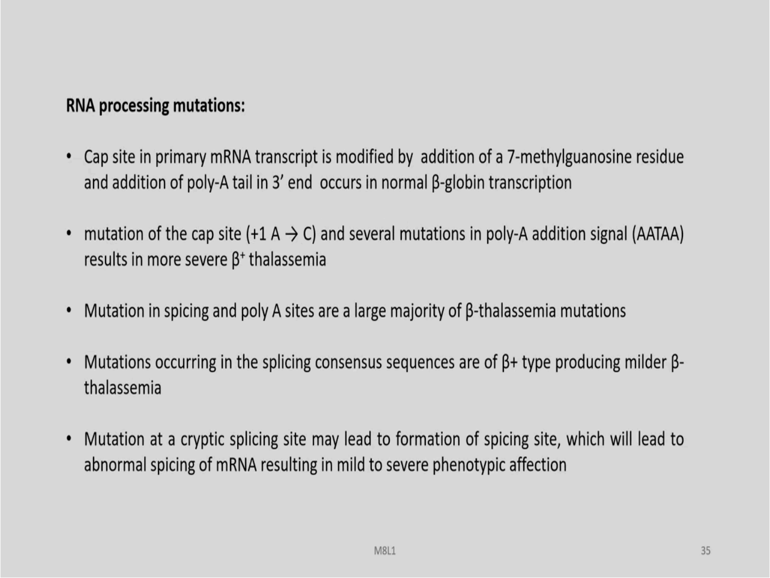

RNA processing mutations:

- Cap site in primary mRNA transcript is modified by addition of a 7-methylguanosine residue and addition of poly-A tail in 3′ end occurs in normal β-globin transcription
- mutation of the cap site (+1 A → C) and several mutations in poly-A addition signal (AATAA) results in more severe β^{+} thalassemia
- Mutation in spicing and poly A sites are a large majority of β-thalassemia mutations
- Mutations occurring in the splicing consensus sequences are of β+ type producing milder β-thalassemia
- Mutation at a cryptic splicing site may lead to formation of spicing site, which will lead to abnormal spicing of mRNA resulting in mild to severe phenotypic affection

RNA processing mutations: Cap site in primary mRNA transcript is modified by addition of a 7-methylguanosine residue and addition of poly-A tail in 3 prime end in normal beta-globin-transcription. Mutation of the cap site plus A to C and several mutations in poly-A additional addition signal AATAA results in more severe beta-thalassemia.

Mutations in spicing and poly A sites are large are a large majority of beta-thalassemia mutations. Mutations occurring in the splicing consensus sequence are of beta plus type producing milder beta-thalassemia. Mutation at a cryptic splicing site may lead to formation of splicing site, which will lead to abnormal splicing of mRNA resulting in mild to severe phenotypic affection.

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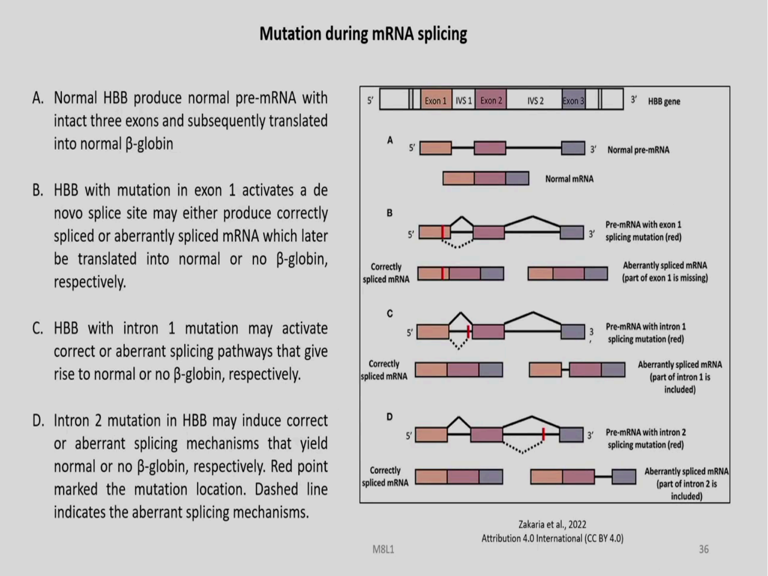

Mutation during mRNA splicing

A. Normal HBB produce normal pre-mRNA with intact three exons and subsequently translated into normal β-globin

B. HBB with mutation in exon 1 activates a de novo splice site may either produce correctly spliced or aberrantly spliced mRNA which later be translated into normal or no β-globin, respectively.

C. HBB with intron 1 mutation may activate correct or aberrant splicing pathways that give rise to normal or no β-globin, respectively.

D. Intron 2 mutation in HBB may induce correct or aberrant splicing mechanisms that yield normal or no β-globin, respectively. Red point marked the mutation location. Dashed line indicates the aberrant splicing mechanisms.

Zakaria et al., 2022
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Mutations during mRNA splicing; You can see here a HBB gene with exons 1, 2, 3 and IVS 1 and 2 and this is a normal pre-mRNA and the normal mRNA where exon 1, 2 and 3 are together one after the other. A pre-mRNA with exon 1 splicing mutations may occur as shown in this with the red color and then apparently spliced mRNA become part of exon 1 which is missing.

Then you we have pre-mRNA with intron 1 splicing mutations again shown in red and aberrantly spliced mRNA which is part of intron 1 is also included. Then pre-mRNA with intron 2 splicing mutations happening as shown in red in number D. Then we have correctly spliced mRNA here and aberrantly spliced mRNA which is part of intron 2 is also included.

So, we know now that normal HBB produces normal pre mRNA with three intact exons and subsequently translated into normal beta-globin. HBB with mutation in exon 1 activates a de novo splice site may either produce correctly spliced or aberrantly spliced mRNA which later on translated into normal or no beta-globin respectively.

HBB with intron 1 mutations may activate correct or aberrant splicing pathways that give rise to normal or no beta-globin respectively. And in the case of D, intron 2 mutations in HBB may induce correct or aberrant splicing mechanisms that yields normal or no beta-globin respectively. Red points marked the mutation locations, dashed lines indicate the aberrant splicing mechanisms.

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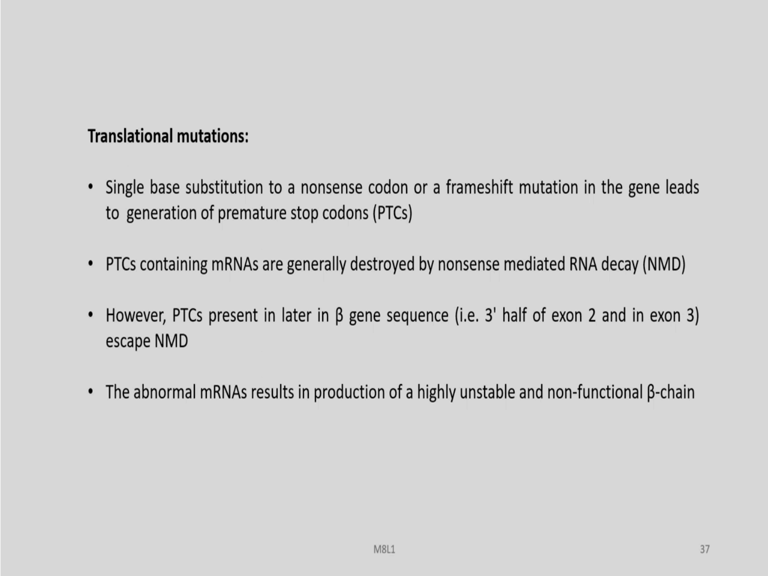

Translational mutations:

- Single base substitution to a nonsense codon or a frameshift mutation in the gene leads to generation of premature stop codons (PTCs)
- PTCs containing mRNAs are generally destroyed by nonsense mediated RNA decay (NMD)
- However, PTCs present in later in β gene sequence (i.e. 3' half of exon 2 and in exon 3) escape NMD
- The abnormal mRNAs results in production of a highly unstable and non-functional β-chain

Translational mutation: Single base substitution to a nonsense codon or a frameshift mutation in the gene leads to generation of premature stop codons called PTCs. PTCs containing mRNAs are generally destroyed by nonsense mediated RNA decay or NMD. However, PTCs present in later in beta gene sequence escape the nonsense mediated RNA decay or NMD.

The abnormal mRNAs results in production of a highly unstable and non-functional beta-chains.

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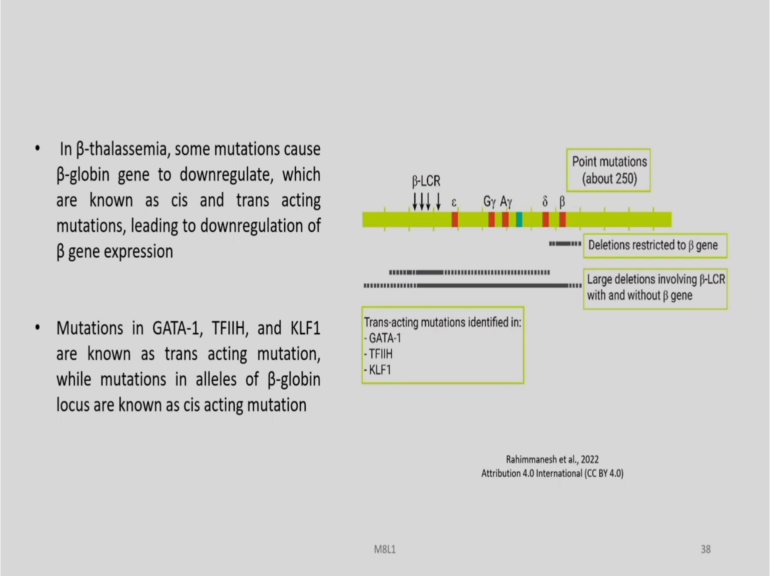

- In β-thalassemia, some mutations cause β-globin gene to downregulate, which are known as cis and trans acting mutations, leading to downregulation of β gene expression
- Mutations in GATA-1, TFIIH, and KLF1 are known as trans acting mutation, while mutations in alleles of β-globin locus are known as cis acting mutation

Rahimmanesh et al., 2022
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In beta-thalassemia, some mutations cause beta-globin gene to downregulate, which are known as cis and trans acting mutations, leading to downregulation of beta gene expression. Mutations in GATA-1, TFIIH and KLF1 are known as transacting mutations, while mutations in alleles of beta-globin locus are known as cisacting mutations.

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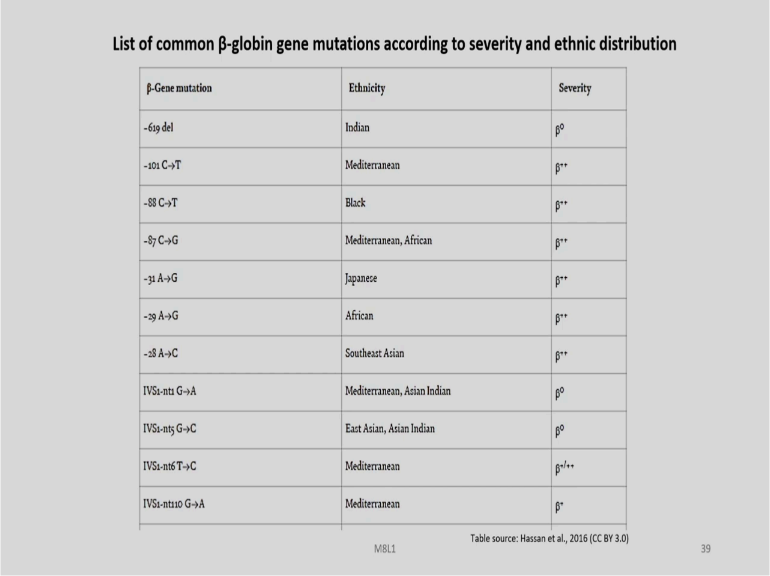

List of common β-globin gene mutations according to severity and ethnic distribution

β-Gene mutation	Ethnicity	Severity
-619 del	Indian	β^0
-101 C→T	Mediterranean	β^{++}
-88 C→T	Black	β^{++}
-87 C→G	Mediterranean, African	β^{++}
-31 A→G	Japanese	β^{++}
-29 A→G	African	β^{++}
-28 A→C	Southeast Asian	β^{++}
IVS1-nt1 G→A	Mediterranean, Asian Indian	β^0
IVS1-nt5 G→C	East Asian, Asian Indian	β^0
IVS1-nt6 T→C	Mediterranean	$\beta^{+/++}$
IVS1-nt110 G→A	Mediterranean	β^+

Table source: Hassan et al., 2016 (CC BY 3.0)

Here are some of the list of common beta-globin gene mutations according to the severity and ethnic distribution. Some found in Indian, some in Mediterranean, some in Black, also in Japanese and then African and so on. So, this is the minus 619 del beta-gene mutation and you can see here the severity as shown by the beta 0, beta plus plus and so on, beta plus oblique plus plus and here the various beta gene mutations namely 101 C to T, 88 C to T, 29 A to G and so on and so forth.

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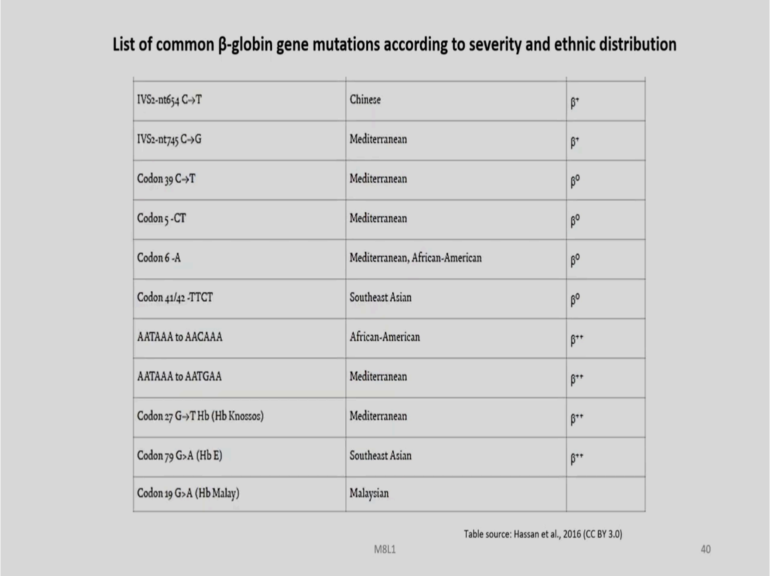

List of common β-globin gene mutations according to severity and ethnic distribution

IVS2-nt654 C→T	Chinese	β^{+}
IVS2-nt745 C→G	Mediterranean	β^{+}
Codon 39 C→T	Mediterranean	β^{0}
Codon 5 -CT	Mediterranean	β^{0}
Codon 6 -A	Mediterranean, African-American	β^{0}
Codon 41/42 -TTCT	Southeast Asian	β^{0}
AATAAA to AACAAA	African-American	β^{++}
AATAAA to AATGAA	Mediterranean	β^{++}
Codon 27 G→T Hb (Hb Knossos)	Mediterranean	β^{++}
Codon 79 G>A (Hb E)	Southeast Asian	β^{++}
Codon 19 G>A (Hb Malay)	Malaysian	

Table source: Hassan et al., 2016 (CC BY 3.0)

And this is the list of the same beta-globin mutations and here we have the severity and have we have the ethnic distribution. With this we come to end of this part. We will continue our discussion on thalassemia and we will try to discuss how gene editing techniques are used to address these problem.

Thank you.