REVIEW ARTICLE

RECENT ADVANCES IN DIAGNOSIS OF THALASSEMIA

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ABSTRACT

Mutations and/or deletions in the α-globin or β-globin genes cause hereditary hemoglobin disorders. Thalassemia is caused by haemoglobin structural abnormalities due to quantitative abnormalities, and hemoglobinopathies. With a rapid influx of people from endemic areas and marriages in blood relations, the incidence of thalassemia and hemoglobinopathy is growing. Therefore the disease knowledge is required. The α-thalassemias are induced by α-globin gene deletions, whereas β-thalassemias are associated with reduced β-globin synthesis due to mutations in the β-globin gene. Hemoglobinopathies include structural changes of hemoglobin in the α- or β-globin chains due to altered amino acid sequences. The next step is to detect hemoglobin abnormality using electrophoresis techniques, including high-performance liquid chromatography and mass spectrometry, if the patient is suspected of thalassemia / hemoglobinopathy from irregular complete blood count findings and/or family history. A more accurate molecular diagnosis of thalassemia / hemoglobinopathy is enabled by the advancement of innovative molecular genetic technologies, such as massively parallel sequencing. In addition, genetic testing for prenatal diagnosis allows the prevention of birth and pregnancy complications from thalassemia. The goal was to review the range and classification of diseases of thalassemia / hemoglobinopathy and diagnostic methods, including screening tests, molecular genetic tests, and prenatal diagnosis.

Keywords: Hemoglobinopathies, thalassemia, diagnosis, genetic testing, advances.

INTRODUCTION:

Biconcave disk-shaped cells without nuclei are red blood cells (RBCs) and are the most common cells present in the blood. RBCs provide peripheral tissues with oxygen and thus play a vital role in sustaining the life of organisms. Hemoglobin molecules in the cytoplasm of RBCs, which are highly advanced devices that bind, hold, and release oxygen, are used to transport oxygen. A hemoglobin molecule consists of 4 polypeptide globin chains (2-α and 2-β) each containing within a heme molecule that binds to oxygen. The hemoglobin molecules and RBCs are essential for the quantitative balance between the

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globin chains and their structural stability. Hemolytic anaemia occurs when the genes encoding the globin chains (HBA for α-globins and HBB for β-globins) have mutations (pathogenic variants) contributing to changes in the volume or structure of globins. Thalassemia is the most common type of human hemoglobinopathy and is caused by pathogenic variants leading to faulty protein synthesis, resulting in quantitative imbalance in globin chains. [1-2] Other pathogenic variants which cause structural abnormalities in globin chains cause hemoglobinopathies like sickle cell disease. [3]

Diagnosis of thalassemia / hemoglobinopathy starts with suspicion of the disorder in anaemic patients based on the findings of phenotype, family history and related laboratory test screening. Molecular genetic confirmation confirms the diagnosis by identifying pathogenic variants. In the past, the genetic variability of the disorder and the mutations which were overlooked by traditional sequence analyses complicated the molecular genetic diagnosis. However, the recent advent of novel molecular genetic technologies such as dosage mutation tests to detect significant deletion / duplication mutations and multiple massively parallel sequencing gene panel tests has allowed a more reliable molecular diagnosis of hereditary hemolytic anaemia and a deeper understanding of the disease's genetic / genomic mechanisms. [4-5] The spectrum and classification of thalassemia / hemoglobinopathy diseases and diagnostic methods, including screening tests, molecular genetic tests and prenatal diagnosis, were studied in this review.

CLASSIFICATION

Hemoglobinopathies are defects of hereditary hemoglobin caused by α-globulin or β-globulin gene mutations and/or deletions. These are classified into 2 major categories: structural hemoglobin variants and thalassemias. Thalassemias, classified as α- or β-thalassemia depending on the involved α- or β-globin chain, are caused by synthesis defects of hemoglobin chains. Structural variants of hemoglobin including sickle cell disease, hemoglobin C (HbC) disease, and hemoglobin E (HbE) disease are caused by gene defects that modify the structure of the hemoglobin. Furthermore, the symptoms of both thalassemia and structural types are combined in several mixed ways (Table 1). [6-7]

The α-thalassemia is almost the product of partial (α^{+}) deletions or complete (α^{0}) deletions of the α-globin gene. People with more deletions of the gene show more serious clinical symptoms. Fatal hemoglobin Bart's foetal hydrops, associated with homozygous α^{0} -thalassemia, are typically characterised by extreme hemolytic anaemia, hydrops and ascites in or shortly after birth, which may lead to foetal death. [8-9]

The β-thalassemias caused by β-globin gene mutations are associated with incomplete (β^{+-} β^{++}) or absent (β^{0}) synthesis of β-globin. The severity of symptoms is related to the degree of absent β-globin chain development. [10]

Due to the altered amino acid sequence in the α- or β-globin chains, irregular hemoglobins have structural defects, unlike thalassemias. Hemoglobin S (HbS), HbC, and HbE are among the common hemoglobin abnormalities. Of all hemoglobinopathies, HbS is the most dangerous. The sickle cells might cause vascular obliterations and infarctions in the main organs. [11] The path of HbC disease is close to progression of sickle cell disease but less fatal. The HbE condition, however, is like β-thalassemia. Other abnormal hemoglobins with structural detection are available.

Table 1: Classification of Hemoglobinopathies

Type		Diagnosis	Gene Type
Thalassemia	α-thalassemias	Heterozygous α^{+}-thalassemia	-α/αα
		Homozygous α^{+}-thalassemia	-α/-α
		Heterozygous α^{0}-thalassemia	--/αα
		Mixed heterozygosity, α^{2}/α^{2} - thalassemia	--/-α
		Homozygous α^{0}- thalassemia	--/--
	β-thalassemias	Heterozygous β-thalassemia	β^{++}/β, β^{+}/β, β^{0}/β
		Mild homozygous or compound heterozygous β – thalassemia	β^{+}/β^{+}, β^{+}/β^{++}, β^{+}/β^{0}, β^{0}/β^{0}
		Homozygous β – thalassemia	β^{+}/β^{+}, β^{0}/β^{0}
		Compound heterozygous β – thalassemia	β^{+}/β^{0}
Structural variants	HbS	HbS heterozygosity	HbAS
		Sickle Cell Disease	HbSS
	HbC	HbC heterozygosity	HbAC
		HbC disease	HbCC
	HbE	HbE heterozygosity	HbAE
		HbE disease	HbEE
Mixed variants	β-thalassemia + HbS or HbE	Sickle cell β^{+}-thalassemia	HbS β^{+}-thalassemia
		Sickle cell β^{0}-thalassemia	HbS β^{0}-thalassemia
		HbE β^{+}-thalassemia	HbE β^{+}-thalassemia
		HbE β^{0}-thalassemia	HbE β^{0}-thalassemia
	HbS + HbC	HbSC	HbSC disease

COMPLETE BLOOD COUNT

Mean corpuscular volume (MCV) of less than 80 fL and/or mean corpuscular haemoglobin (MCH) of less than 27 pg can typically be used for thalassemia screening as cut-off thresholds for a positive screening result. [12] These cut-off thresholds are derived from 2 standard deviations from the general population of the normal distribution of MCV and MCH. The benefit of MCV and MCH thalassemia screening is the achievement of quick, cost-effective, reproducible and precise results from automated hematology analyzers. However, microcytic anemias such as iron deficiency anemia (IDA) can also cause low MCV; variation in MCV from different automated blood cell counters has also been documented in comparison to MCH, which appears to be consistent between different automated hematology analyzers. [13] In addition, for HbE carriers and individuals with single α-globin gene deletion (-α3.7 and-α4.2) or non-deletional α-globin gene mutations [i.e., Hb Constant Spring (Hb CS) and Hb Quong Sze], low MCV is not suitable. [14] In addition, the association of heterozygous β-thalassemia with α-thalassemia alone or glucose-6-phosphate dehydrogenase deficiency may result in normal MCV and a false-negative result during thalassemia screening. [15] Thus, screening thalassemia using both MCV and MCH would be more appropriate than just using MCV; it would be very important to determine their cut-off levels using the automated hematology analyzer used in each laboratory.

The interpretation of peripheral blood smear using both MCV and MCH will be regarded as an important form of screening for thalassemia. Microcytosis, hypochromia, and anisopoikilocytosis consist of typical RBC morphology in thalassemia disease. By comparing the size of RBC with those of the nucleus of small lymphocytes, microcytes can be evaluated and hypochromic RBCs are characterised as having an increase in the central pallor diameter of RBCs, that

is, more than one-third of their diameter. Anisopoikilocytosis occurs from different anomalous RBC morphologies including schistocytes, microspherocytes, target cells, polychromasia, and nucleated RBCs. However, only certain forms of thalassemia from other causes of anemia, such as IDA or inflammatory anemia, can be indicated by peripheral blood smear findings, and it is not possible to identify a particular form of thalassemia based solely on RBC morphology. Red cell distribution width (RDW) is a measure of the degree of differences in red cell size, and an increase in RDW is characterised by certain causes of microcytic anaemia, most notably IDA. While thalassemia produces uniform microcytic red cells without a concomitant increase in RDW, this result is variable among the syndromes of thalassemia, including major increases in RDW in HbH disease and minor β-thalassemia. The RDW may also provide details that can be used as an adjunct to the diagnosis, but is not useful as a single screening predictor. [16] As a diagnostic adjunct, the RBC count is often useful because thalassemia causes microcytic anemia with an increase in the number of RBC, but IDA and chronic disease anemia are usually correlated with a decrease in the number of RBC that is proportional to the degree of anemia. However, as a sole screening method for thalassemia and hemoglobinopathies, the RBC count should not be used.

In view of all this, for the screening of thalassemia and hemoglobinopathies, different indices using complete blood count (CBC) components have been established, but none exceed the value of the combination of MCV and MCH in selecting cases for subsequent studies.

ELECTROPHORESIS AND ADVANCED METHODS

The International Committee for Standardization of Hematology suggested laboratory tests for 3 laboratory forms in 1978. [17] Screening laboratory should be in a position to conduct alkaline electrophoresis in that guideline. Rather complicated experiments such as citrate agar electrophoresis and globin electrophoresis had to be performed by the reference laboratory. These electrophoresis techniques involved manual steps from the preparation of reagents, electrophoresis, and data analysis during the hemoglobin analysis, and thus laboratory professional expertise was a key to successful identification. The launch of updated guidelines was motivated by the recent advancement of laboratory techniques and enhanced awareness of thalassemia and hemoglobinopathy. [18] The presumed identification of hemoglobins is recommended by the British Committee for Standards in Hematology on a minimum of 2 techniques and conclusive identification is known to be based on DNA analysis, mass spectrometry, or protein sequencing.

High Performance Liquid Chromatography (HPLC):

A tool used to distinguish compounds or molecules based on their chemical properties is the high-performance liquid chromatography (HPLC) technique. Several separation principles are available, such as size, affinity, and partition; ion-exchange chromatography is the most powerful and most commonly used for hemoglobin. The technique can also be manually controlled, but fully automated systems have recently become available. These systems can be used for hemoglobin analysis; however, systems that can switch between glycated diabetes hemoglobin analysis and variant hemoglobin analysis for thalassemia and hemoglobin variants may be more feasible to use in low prevalence areas. It is considered to be useful in the diagnosis of β-thalassemia, since HbA_2 can be quantified accurately. [18] Careful monitoring of analytical conditions

such as column temperature, flow rate, and buffer conditions is important, similar to other HPLC techniques.

Electrophoresis:

Electrophoresis is a method used in a gel and electrical field to distinguish molecules or compounds based on their migratory pattern. It is also commonly used for protein electrophoresis and differentiation of certain isoenzymes in clinical laboratories. In developed countries, manual preparation of gel and electrophoresis is rarely used as more sophisticated and automated techniques such as capillary electrophoresis are available. Electrophoresis of cellulose acetate is a representative technique of custom electrophoresis. Hb A, F, S / G / D, C / E, and H and other variants are known to allow identification. [18] In many automated HPLC systems, automated capillary electrophoresis is commonly used and has shown benefits in the detection of certain variants that are indistinguishable. [19]

Mass Spectrometry:

Based on their mass (molecular weight) to charge ratio, mass spectrometry is a technique to classify molecules. The strong advantage of the technique is that the molecules of interest use limited complex binding reagents. The basic analytical theory makes it possible to define less interference and more accurately. It is not easy to analyse hemoglobin with mass spectrometry because the laboratory should have both technological experience for analysing proteins and a very expensive instrument. Besides recognising hemoglobin based on the molecular weight of the intact molecule, it may also in some degree examine the sequence of amino acids. It is helpful for discovering new variants and checking the sequencing of DNA. [20]

MOLECULAR CHARACTERIZATION

In over 90% of cases, α-thalassemia is caused by gene deletion. A minority of cases of α-thalassemia are due to changes in sequence such as single nucleotide substitution, addition, or short addition / deletion. The α gene cluster consists of highly homologous genes and 2 HBA genes which encode identical proteins. Gene deletion is possibly caused during meiosis by unequal crossing between these homologous regions. So far, several breakpoints have been recorded including the most common deletion of 3.7 kb. [21]

About 90% of cases of β-thalassemia are caused by sequence differences relative to cases of α-thalassemia. The β-thalassemia is currently associated with more than 280 sequence variants. [21] Some β-thalassemia is caused by gene deletion which includes the HBB gene.

Numerous different molecular methods are used to identify mutations in the globine gene. Molecular techniques can be classified by mutation type to be targeted as follows: 1) Methods of detection for structural variations, such as gene deletion, duplication or triplication, and 2) Methods of detection for sequence variations, such as nucleotide substitution, insertion or short insertion / deletion. Gap polymerase chain reaction (PCR), specifically designed for the deletion concerned, can detect known gene deletions. For unknown gene deletions, southern blotting using named complementary gene probes can be used. Both known and unknown gene deletions can be identified by the multiplex ligation-dependent probe amplification (MLPA) process. MLPA is commonly used because it is highly sensitive, easy to use and can detect deletions of different kinds.

For certain ethnic groups, typical sequence variations can be identified in a cost-effective manner using techniques such as allele-specific PCR, reverse dot blotting, denaturing gradient gel electrophoresis, and refractory mutation system amplification. Rapid progress and cost

reduction of the sequencing technology made it possible in many laboratories to sequence the globin gene like promoter, 3' UTR, exon-intron boundaries and deep introns. In particular, for targeted genes, exomes, or even genomes, massively parallel sequencing technology can be applied.

PRENATAL DIAGNOSIS

Prenatal diagnosis involves screening of the carrier, genetic testing and genetic tests of the prenatal gene. To date, the prenatal diagnosis of thalassemia and hemoglobinopathy is one of the world's most commonly conducted genetic analyses. Hemoglobinopathies are widespread in many migrant countries as well as in endemic regions due to population migration. [22-25] The aim of prenatal diagnosis is to recognise and advise asymptomatic individuals whose offspring are at risk of inherited hemoglobinopathy, and to control complications throughout pregnancy. Clinical forms of hemoglobinopathies targeting prenatal diagnosis are associated with potentially serious sequelae and interfere with, for example, sickle cell disease, significant β-thalassemia arising from β-thalassemia homozygosity, and non-immune hydrops fetalis triggered by deletion or malfunction of all 4 α-globin genes. [26] Prenatal diagnosis is beneficial in this case, considering the early lethality of hydrops fetalis, since a large number of women carrying foetuses with this abnormality experience serious toxemia and extreme postpartum hemorrhage. [27] Therefore, a deep understanding of the association between genotype and phenotype, the impact of genetic modifiers and rare cases including dominantly inherited β-thalassemia, uniparental isodisomy and de novo mutation is warranted. [28]

Prenatal diagnosis includes the examination of foetal material in the maternal circulation from chorionic villi, amniotic fluid, cord blood and foetal DNA. Invasive prenatal diagnosis includes the success of first trimester chorionic villus sampling, and second trimester amniocentesis or cordocentesis. While analysis of foetal hemoglobin types is carried out successfully by automated HPLC, it can be determined by examining the foetal blood obtained through cordocentesis, and the procedure is prone to errors due to maternal tissue contamination. [29] In prenatal diagnosis, DNA analysis is particularly useful because irregular hematologic findings are observed and postnatal samples can be collected more easily. The detection of complex thalassemias and hemoglobinopathies seen in ethnically diverse populations has been improved by advances in molecular research. Universal screening services were introduced in Canada and in European countries to identify carriers and give prenatal diagnosis in pregnancies at risk of thalassemia. [30-31] Parental screening is not invasive and can be carried out without risks to the foetus growing. The recent invention of non-invasive prenatal diagnostic testing using maternal plasma cell-free foetal DNA enables active investigation of foetal genetic analysis to avoid invasive procedure. [32] Another benefit of this strategy is that earlier than using an invasive technique, foetal DNA can be separated from the maternal blood. [33] In order to classify foetal hemoglobinopathies, different methods have been used, such as mass spectrometry, next-generation sequencing, and genotyping assay. [34-36] The methods are still challenging, so further research are required to improve and validate them and eventually lead to efficient, accurate , and reliable non-invasive prenatal thalassemia and hemoglobinopathy diagnosis. [37]

CONCLUSION

Thalassemia and hemoglobinopathies are very common, and the incidence rises as people migrate from endemic areas and marriages become infectious. In addition to family hist-

ory, the doctors should closely study CBC, in particular MCV and MCH, to assume thalassemia and hemoglobinopathies in anemic patients. Using advanced electrophoresis techniques involving HPLC and mass spectrometry, quantitative and structural defects of hemoglobin can be detected more sensitively and precisely. Different molecular tests are used for thalassemia and hemoglobinopathy molecular diagnosis, and recent advances in sequencing technology, such as massively parallel sequencing technology, allow for more accurate genetic diagnosis. Also we should concentrate on prenatal diagnosis and genetic therapy on thalassemia and hemoglobinopathy to avoid births of thalassemia and complication of pregnancy. Disease suspicion by closely examining the screening test results and advanced molecular testing will assist in the early diagnosis and disease intervention. In addition, we expect thalassemia and hemoglobinopathies to establish treatment modality by understanding the molecular and protein characteristics.

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