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Review Article

New Progress in Laboratory Detection of Abnormal Hemoglobinopathies

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Abstract

Abnormal hemoglobinopathies are a group of hereditary diseases caused by Hb variants with new molecular structure changes due to globin defects and changes in the primary structure of globin. It is one of the most common birth defects and ranks third in global incidence [1]. Compared with thalassemia, most abnormal hemoglobinopathies are clinically asymptomatic and have a low incidence rate, which makes the awareness of the disease not widespread enough. Therefore, there is no mature and independent screening and diagnosis program at present, and abnormal hemoglobinopathies are often It is accompanied by abnormalities found in thalassemia screening, and then can be diagnosed by genetic sequencing.

Keywords: Abnormal Hemoglobinopathies; Gene Sequencing Detection; Hb Electrophoresis

Overview Foreword

The HbVar database of human hemoglobin variants and thalassemia mutations records more than 700 Hb variants. According to Sun Manna's research results, the population carrier rate of abnormal hemoglobinopathy in Guangxi is 0.38%, except for thalassemia (HbCS, HbE), 9 abnormal Hbs were also detected, among which Hb New York and Hb Q-Thailand ranked first and second [2], while Lou Jiwu et al. [3] showed that the population carrying rate of abnormal hemoglobinopathies in Guangdong was 0.4%. In addition to the abnormal Hb mentioned by Sun Manna, Hb G-Honolulu and HbS were also reported, which provided the detailed prevalence and molecular characteristics of abnormal HbS in the population of Dongguan. Therefore, the screening and diagnosis of abnormal hemoglobinopathies is of great significance for the guidance of marriage and childbirth and prenatal diagnosis. Abnormal Hb has the characteristics of wide distribution and regional characteristics. It is hidden because it has no clinical phenotype. Most of them are discovered when abnormal Hb variants appear in hemoglobin electrophoresis during thalassemia screening. There is no mature reagent yet. Box can be detected. Most of the carriers of abnormal Hb that have been found have no clinical symptoms, and a few have obvious abnormal physiological functions. Some abnormal Hb (such as HbCS, HbE, etc.) combined with thalassemia can lead to moderate to severe anemia. Therefore, improving the detection method of abnormal Hb variants is of great significance for the prevention and control of abnormal hemoglobinopathies in the future. In recent years, the separation technology of Hb has been continuously updated. With the development of high-performance liquid chromatography, fully automatic capillary electrophoresis, and mass spectrometry, people generally support the combination of mass spectrometry, HPLC and CE to detect and screen abnormal Hb, because the application of blood Routine examination and Hemoglobin (Hb) analysis and screening have certain defects in the diagnosis of hemoglobinopathies. Some electrophoresis cannot be clearly distinguished, which reduces the reliability of electrophoresis results, and the diagnostic accuracy is low. It can no longer meet the needs of clinical diagnosis, and some have been eliminated. Some can only be used for screening. With the advancement of science and technology and the development of experimental diagnostic technology, the diagnostic technology of abnormal hemoglobinopathies has been gradually replaced by Sanger sequencing and high-throughput sequencing.

Abnormal hemoglobinopathies are changes in the normal function of the body caused by changes in hemoglobin, and are caused by abnormal peptide chain structure or synthesis due to genetic mutations in globin (including α-globin gene defects, β-globin gene defects, and δ-globin gene defects). arising from obstacles. A group of diseases caused by the partial or complete replacement of normal hemoglobin by abnormal hemoglobin, collectively referred to as abnormal hemoglobinopathies, is also the most common single-gene genetic disease in the world, posing a major public health challenge in the world [4]. Because most abnormal hemoglobinopathies have no hematological phenotype and anemia, only abnormal protein bands are found during hemoglobin electrophoresis. Therefore, the detection of abnormal hemoglobinopathies often relies on laboratory tests. Usually, the detection of abnormal hemoglobinopathies is performed by routine blood or hemoglobin electrophoresis, just like screening for thalassemia. The current research progress of the commonly used screening and laboratory diagnosis methods for abnormal hemoglobinopathies is summarized as follows.

Blood Routine Screening

Most abnormal hemoglobinopathies have no hematological phenotype. The study by Wang Yefei et al. [5] showed that, except for Hb E, Hb Youngstown and Hb M-Boston, most Hb variants themselves did not cause obvious clinical manifestations in patients. Hemolysis, anemia and other symptoms often appear when combined with other diseases, especially when combined with thalassemia, which can cause moderate or severe anemia. Therefore, large-scale screening for abnormal blood protein diseases is mainly carried out with the screening of thalassemia. Routine blood screening is one

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of the most common methods for thalassemia. At present, blood routine testing is carried out by an automatic blood cell analyzer, which mainly uses the principles of resistance method and light scattering method, as well as cytochemical techniques such as special cell staining, and VCS combined detection and other cutting-edge methods, which can accurately measure blood samples for blood cell parameters. The main parameters of blood routine screening for thalassemia are: Mean Corpuscular Volume (MCV) and Mean Corpuscular Hemoglobin (MCH), usually MCV<82fL and/or MCH<27pg as screening positive, but Chen Yabin et al [6] considered that MCV <82.1fL and/or MCH<27.3pg should be considered as a suspected case of thalassemia. Under the same detection conditions, MCH is less affected and more stable than MVC. Xu Shanshan et al [7] believed that MCH as an indicator of thalassemia screening has high sensitivity. Routine blood testing can quickly and cost-effectively provide testing and screening results, and is suitable for large-scale premarital and pregnancy testing for abnormal hemoglobinopathy screening in couples.

Hemoglobin (Hb) Analysis and Screening

Because most abnormal hemoglobinopathies only have abnormal Hb variants without clinical phenotype, with the development of Hb analysis technology, Hb analysis and screening has become the main method for abnormal hemoglobinopathies screening. Commonly used Hb analysis includes Cellulose Acetate Film (CAC) electrophoresis, citric acid agar electrophoresis, agarose electrophoresis, Isoelectric Focusing Electrophoresis (IEF), high performance liquid chromatography, automatic capillary electrophoresis, mass spectrometry, etc.

Cellulose Acetate Film (CAC) Electrophoresis

Zone electrophoresis using CAC as a support medium. The principle of its application is that the negatively charged Hb molecules move to the anode at different rates in the electric field. Due to the different rates, the zones of various Hbs on the CAC are different, so that the different Hbs can be qualitatively and quantitatively analyzed. Because CAC media electrophoresis has the advantages of not adsorbing proteins, short time-consuming, no tailing, clear and transparent images, and can be stored for a long time [8], it can not only quickly separate common hemoglobin components, but also does not require expensive instruments, and the number of specimens is small, easy to elute and quantify. However, in actual clinical work, the freshness of the sample and the sampling point of the sample, the quality of the staining solution, the number of times of use and the staining time, the quality of the cellulose film, and the current intensity can all affect the analysis results of electrophoresis [9]. The A zone and the hemoglobin E and A2 zones cannot be separated because they have the same mobility, and many other hemoglobin's cannot be clearly distinguished, which reduces the reliability of the electrophoresis results and has been gradually eliminated.

Citrate Agar Electrophoresis

In citrate agar electrophoresis, hemoglobin appears to bind reversibly to sulfated polysaccharide agarose, a natural component of Difco bacterial agar. At pH 6.2 there is only a weak positive charge, while carbohydrates have a net negative charge. Electroendosmosis, on the other hand, proceeds in the cathodic direction, and these opposing flows separate hemoglobin in order of its affinity for agaragglutinin. An agaragglutinin binding site on hemoglobin was identified by computer-aided modeling and its association with hemoglobin variants that exhibited abnormal citrate agar mobility was determined. Winter W believed that agar agglutinin has anti-gelling effect on Hb [10], so citrate agar electrophoresis should be used to distinguish HbS and HbC.

Agarose Electrophoresis

Since 1959, when gel was used as a support medium for electrophoresis, a new chapter in electrophoresis was opened. The principle of agarose gel electrophoresis is that different Hb molecules have different charges and different isoelectric points under alkaline conditions, and they flock to the cathode or anode to separate different Hb bands. The agarose gel has a large pore size and does not adsorb. Proteins, with neat bands without tailing, good repeatability and higher resolution [11], gradually replaced cellulose acetate film electrophoresis with the emergence of automatic electrophoresis instruments. Wang Hong et al [12] believed that automatic agarose gel electrophoresis can not only quantitatively detect the contents of HbA, HbF, and HbA2, but also detect various abnormal hemoglobin's, such as HbH, HbE, HbG, etc. The research of He Yajun et al. [13] showed that automatic agarose gel electrophoresis has extremely high resolution, can distinguish extremely small amounts of abnormal Hb, and accurately analyze those with abnormally increased HbA2 and HbF, which provides accurate and reliable diagnosis for thalassemia. data. However, as with cellulose acetate film electrophoresis, hemoglobin E and A2 still cannot be distinguished, because the bands of Hb H and Hb Barts are very close to each other, so it is difficult to separate them.

Isoelectric Focusing Electrophoresis (IEF)

It uses a special buffer (ampholyte) to create a pH gradient in a gel (commonly used polyacrylamide gel), and each protein will migrate to its equivalent during electrophoresis. Electrophoresis of the zone formed at the pH of the electric point (pI). Schaefer et al. [14] analyzed dried blood spots collected from nearly 100,000 infants and young children in all 10 districts and 112 districts of the Republic of Uganda using an IEF surveillance study, and isolated Hb A, Hb F, Hb S, Hb C, Hb Stanleyville II, Hb G-Pest, Hb O-Arab, Hb P-Nilotic, Hb Kenya and other hemoglobin variants, it can be seen that IEF is a rapid and reproducible method for screening abnormal hemoglobin. Compared with cellulose acetate film electrophoresis, IEF has the advantages of no migration, low concentration detection and rapid separation of Hb, but it is labor-intensive and the quantification of HbA2 is not accurate enough.

High Performance Liquid Chromatography (HPLC)

HPLC uses ion exchange resin as a separation column to efficiently separate and purify hemoglobin according to different physical and chemical properties of hemoglobin and different residence time in the separation column. HPLC has the characteristics of high sensitivity and rapidity, no need for pre-washing and hemolysis, and high resolution and specificity. Now it has been used as a routine hemoglobin analysis method in most laboratories. HPLC can separate and identify hemoglobin variants quickly, sensitively,

and high-throughput, and quantitatively determine Hb A and Hb F. It is a targeted quantitative quality control material. In 2012, it was recommended by ICSH for the separation and identification of hemoglobin variants and quantitative determination of Hb A. and the standard method for HbF [15]. The most prominent feature is that Hb E and Hb A2 can be completely separated and accurately quantified. The study by Wei Shuofeng et al. [16] showed that the coincidence rate of HPLC detection of Hb E with genetic diagnosis was 93.2%. HPLC can not only accurately distinguish and quantify Hb A2 and Hb F, but also accurately quantify HbF at low concentrations, and can also separate abnormal hemoglobin's such as HbE, HbQ, and HbCS, and even some bands that cannot be distinguished by traditional Hb electrophoresis can also be separated. come out. Xie Jianhong et al [17] believed that HPLC could quickly and accurately identify various abnormal hemoglobin, and it was an ideal tool for screening abnormal hemoglobinopathies. The study by Wang Yefei et al. [18] showed that the combined detection of β-thalassaemia, HbH disease and abnormal hemoglobinopathies by HPLC and routine blood test has high sensitivity and specificity, a high coincidence rate with the results of gene analysis, and is easy to operate, fast, suitable for clinical rapid screening. Although HPLC has a high separation rate and good repeatability, it needs to be calibrated before each sample loading, and the determination of the results of samples with too low or too high hemoglobin content is not accurate, and the quality of the chromatographic separation column is also required.

Capillary Electrophoresis (CE)

CE is a separation technique that combines classical electrophoresis and modern microcolumn separation. In automatic capillary electrophoresis, the liquid that automatically dissolves red blood cells at the anode is separated by electrophoresis in a capillary tube with high pressure, and the hemoglobin is measured at 415nm at the cathode, and the image is analyzed by the Phoresis program. Fully automatic capillary electrophoresis has less sample consumption, high automatic operation, fast detection speed (8 channels can detect 8 samples in a few minutes), high sensitivity and resolution (can identify the 20 most common hemoglobin variants), these Its characteristics make it rapidly developed into the most commonly used method for laboratory detection of abnormal hemoglobin variation. The Phoresis program divides the electropherogram into 15 zones, z1-z15. Xu Weihua et al. [19,20] used automatic capillary electrophoresis to detect 232 cases of abnormal Hb, including common HbH, HbBart's, HbCS; Hb E, Hb K, Hb Q, Hb G, Hb J and high content of HbF confirm that automatic capillary electrophoresis has important application value in the detection of various common abnormal Hb, and can be used for the screening of thalassemia and hemoglobinopathies and diagnosis to provide a fast and accurate basis for diagnosis. Hb New York was not separated from Hb A by HPLC, but was 100% detected by automatic capillary electrophoresis. The study by Li YQ et al. [21] believed that in the high-incidence area of Hb New York, automatic capillary electrophoresis was used for hemoglobinopathy screening. Preferred method. However, the automatic capillary electrophoresis currently only has the indoor quality control material for the normal value of HbA2, and there is no indoor quality control material for other Hb variants, so it is impossible to monitor the precision of other Hb variants; for some hemoglobin variants homozygotes Or compound heterozygotes, because HbA is missing, the variant must be diluted with normal whole blood at 1:1 to be dissolved and then electrophoresed to locate the variant; and due to the high precision of the instrument, it has high requirements on the environment, and changes in dust and temperature and humidity can affect the instrument. run and checkout of results.

Mass Spectrometry (MS) Analysis

A technique for identifying molecules based on the ratio of their mass (molecular weight) to charge, and is widely used. The specific binding of reagents to target molecules reduces interference and allows for more accurate identification, which is a powerful advantage of mass spectrometry. With the development of mass spectrometry, combined with liquid chromatography, Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) technology with liquid chromatography as the high-throughput sampling system and mass spectrometry as the detection system has also developed rapidly [22] stand up. LC-MS/MS combines the high separation power of liquid chromatography with the advantages of mass spectrometry, which can provide relative molecular mass and structural information. Abnormal Hb disease is due to globin gene mutation to produce new abnormal Hb, its Molecular Weight (M) has changed. LC-MS/MS detects hemoglobinopathies by cleaving Hb with trypsin to form peptide fragments, and the peptide fragments are separated and arranged according to different mass-to-charge ratios, and a mass spectrum containing mass-to-charge ratio and ion peak intensity information is obtained, Changes in the synthesis amount and molecular mass of globin chains before and after mutation were analyzed [23], and by analyzing the mass-to-charge ratio of the appearance, disappearance or signal change of ion peaks in the mass spectrogram, the marker peptides of hemoglobinopathies were screened and identified, and the Hb variant was determined. Type; combined with the internal standard of peptide isotope, it can qualitatively and quantitatively analyze normal Hb and abnormal Hb at the same time. Yu Chaowen [24] research shows that the new method of LC-MS/MS technology system for hemoglobinopathies, especially thalassemia screening, the sensitivity and specificity are higher than 98%, The sampling speed is fast (60 seconds/case), the detection throughput is high, and the cost is low. In addition to identifying hemoglobin, mass spectrometry can also analyze amino acid sequences to a certain extent, which is very helpful for identifying new variants and confirming DNA sequencing. Lee Young Kyung et al. [25] considered the use of LC-MS/MS electrophoresis technology. The detection of hemoglobin helps in more precise molecular diagnosis of hemoglobinopathies. However, mass spectrometry analysis of hemoglobin requires technical expertise in analyzing proteins and very expensive equipment, which limits its application.

Gene Sequencing Diagnosis

Because the application of blood routine examination and Hemoglobin (Hb) analysis to screen and diagnose hemoglobinopathies has certain defects, some electrophoresis cannot be clearly distinguished, which reduces the reliability of electrophoresis results, and the diagnostic accuracy rate is low, which can no longer meet the needs of clinical diagnosis. has been eliminated. Some can only be used for screening. With the advancement of science and technology and the development of experimental diagnostic technology, the

diagnostic technology of abnormal hemoglobinopathies has been gradually replaced by Sanger sequencing and high-throughput sequencing.

Sanger Sequence Determination

DNA sequence determination was reported as early as 1953, but it was not developed rapidly until Sanger invented end termination sequencing in 1977. DNA sequence determination (Sanger method), also known as first-generation sequencing, is based on the use of a DNA polymerase (deoxynucleotide triphosphate dNTP) to extend the primer bound to the template to be determined until the chain termination nucleotide (dideoxynucleoside triphosphate ddNTP). Sanger sequence determination can design primers for the globin gene sequence mutation site for PCR sequencing, and determine the mutation position and mutation nature [26,27], which is currently the main force in the identification and diagnosis of abnormal Hb. However, the DNA sequencing method is cumbersome to operate, and there are disadvantages such as insufficient throughput of single-sequence sequencing, which limits its practical clinical application.

High-throughput Sequencing Technology

Also known as second-generation sequencing technology, it is a sequencing technology with a new principle developed after first-generation sequencing. Next-generation sequencing is that it can simultaneously sequence millions of DNA molecules in a single reaction in parallel [28], and can detect point mutations, small indels, large fragment deletions, and copy number gains at one time. High-throughput sequencing has become one of the most promising technologies in many clinical fields due to its simplicity, speed, higher resolution, and high-throughput. High-throughput sequencing enables high-throughput sequencing of the full-length gene sequences of α-globin and β-globin, and has been widely used for molecular screening and prenatal diagnosis of thalassemia since 2016 [29]. In 2018, Pu Jiajie [30] used next-generation sequencing to diagnose a case of abnormal hemoglobinopathy caused by a spontaneous dominant mutation in the HBB exon. In 2020, Zhao Tingting used next-generation sequencing to diagnose 13 different abnormal hemoglobins, including 5 alpha-globin chain variants and 8 beta-globin chain variants [31], indicating that next-generation sequencing plays an important role in the detection and diagnosis of abnormal hemoglobinopathies. Compared with first-generation sequencing, multiple sequences can be sequenced in parallel, which shortens the research cycle and cost, and is suitable for the study of a large number of samples, but the sequencing fragments are limited to 250-300 bp, and some sequences with a small amount cannot be amplified and lost. Information about the probability of mismatched bases.

Summary

The blood routine test is carried out by an automatic blood cell analyzer, mainly using the principles of resistance method and light scattering method, cytochemical techniques such as special cell staining, and VCS combined detection and other cutting-edge methods, which can accurately measure blood samples for blood cell parameters. As a result, the Mean Corpuscular Volume (MCV) <82fL and/or the mean corpuscular hemoglobin (MCH) <27pg are usually used as positive screening results. Routine blood testing can provide rapid and cost-effective screening results, which are suitable for large-scale premarital examinations. The screening of abnormal hemoglobinopathies in couples with pregnancy test and pregnancy test, but there is no mature kit for the detection of abnormal Hb directly. With the development of Hb analysis technology, Hb analysis and screening have become the main means of screening abnormal hemoglobinopathies. Compared with routine blood testing, Hemoglobin (Hb) analysis and screening have made progress, especially High-Performance Liquid Chromatography (HPLC), Capillary Electrophoresis (CE) and Mass Spectrometry (MS) analysis, which have fast sampling and detection speed, sensitivity, specificity However, these detection methods still have shortcomings. For example, capillary electrophoresis requires high precision of instruments, high environmental requirements, and changes in dust and temperature and humidity. can affect the operation of the instrument and the detection of results. Mass spectrometry analysis of hemoglobin requires technical expertise in protein analysis and very expensive instruments. Sanger sequence determination can design primers for the mutation site of the globin gene sequence for PCR sequencing, and determine the mutation position and mutation nature. It is the current method for identifying and diagnosing abnormal Hb. main force. However, the DNA sequencing method is cumbersome to operate, and there are disadvantages such as insufficient throughput of single-sequence sequencing. Next-generation sequencing is that it can simultaneously sequence millions of DNA molecules in a single reaction [28], and can analyze point mutations, Small indels, large deletions, and copy number gains are detected. High-throughput sequencing has become one of the most promising technologies in many clinical fields due to its simplicity, speed, higher resolution, and high-throughput. At present, DNA detection methods are constantly developing. With the development trend of multi-ethnic society, DNA detection methods have developed from targeted population-specific methods to general methods, such as Sanger sequencing (point mutation) and next-generation sequencing technology. While not yet fully ready for routine use, next-generation sequencing may soon become a laboratory diagnosis for some hemoglobinopathies. It is believed that in the near future, abnormal hemoglobinopathies will have more perfect screening technology and genetic diagnosis technology platform.

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