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Molecular Diagnostic Approaches For Sickle Cell Anemia

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Abstract. Sickle cell anemia (SCA) is a common genetic disease caused by gene mutations, affecting a large population worldwide, with newborns accounting for up to 79%. With the continuous development of molecular biology and molecular diagnostic technology, a variety of molecular diagnostic methods have been widely used in clinical practice in recent years, including polymerase chain reaction (PCR), high-resolution melting curve analysis (HRM), Sanger sequencing, next-generation sequencing (NGS), and digital PCR. This article introduces the pathological basis of SCA and its related gene mutations, and systematically summarizes several commonly used molecular diagnostic techniques, aiming to provide a reference for the optimization and improvement of early molecular diagnostic methods for the disease. As the core tool for achieving precise management, molecular diagnostic technology includes both traditional methods (such as Sanger sequencing) and emerging methods (such as CRISPR-Dx), and the two should complement each other in clinical practice. Future research should focus on simplifying technical processes, reducing testing costs, and widespread application worldwide to promote early diagnosis and effective management of SCA.

Keywords: Molecular diagnostic; sickle cell anemia; next-generation sequencing.

1. Introduction

SCD affects a large global population, with 306,000 babies born annually with the condition, primarily in sub-Saharan Africa, which accounts for 79% of these births. Sickle cell anemia (SCA) is a common autosomal dominant genetic disease. Sickle-shaped red blood cells lose their normal plasticity and deformability, and are easily destroyed and hemolyzed. SCA is the consequence of abnormal haemoglobin production due to an inherited point mutation in the β-globin gene [1]. SCA is caused by point mutations in the Hemoglobin subunit beta (HBB) gene, which codes for β-subunit, where adenine is substituted by thymine (Glutamic acid replaces valine.) at codon 6 of the HBB gene [2]. Clinically, there are three types of Sickled hemoglobin, Hbs disease: homozygous, heterozygous, and mixed heterozygous. Laboratory tests for SCA include sickled cells that causes hemolysis of blood cells, anemia, painful episodes, organ damage, and in some cases death. Early diagnosis is essential for the treatment. Different kinds of molecular diagnostic methods, such as Polymerase Chain Reaction (PCR), restriction fragment length polymorphism (RFLP), Next-Generation Sequencing (NGS), have specificity and sensitivity for SCA. DNA sequencing technologies include the gold standard method Sanger sequencing and pyrose sequencing. SNP chips and customized microarrays are also used in the diagnosis of SCA. These methods can be used for screening. Emerging molecular diagnostic approaches such as single-base editing based on the CRISPR/Cas system are used to break through diagnostic validation in repair studies. New sensor-based technologies also offer precise diagnostic options by detecting specific biological markers, advancing both diagnosis and treatment strategies [3]. However, molecular diagnostics are more demanding for laboratories and laboratory personnel. Molecular diagnostics can be applied in clinical scenarios such as prenatal diagnosis, carrier screening, and newborn screening.

Nowadays, there is more emphasis on the complementary application of traditional molecular diagnostic methods for SCA, such as Sanger sequencing, emerging technologies such as CRISPR-DX. Clinical application scenarios are also very wide. Therefore, in this paper, the author hopes to systematically summarize the development status and potential of molecular diagnostic technology.

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2. Fundamentals of Molecular Pathology

Hemoglobin S (HbS) results from the replacement of glu-tamic acid by valine in the sixth position of the β-globin chain of hemoglobin [4]. Hbs include three types, homozygous, heterozygous, and mixed heterozygous. Hbs and normal hemoglobin are consistent in their ability to bind oxygen, but the difference between the two is that HbS forms red blood cell hemolysis, which leads to a decrease in the number of red blood cells in the patient's body. Red blood cells after hemolysis are unable to transport oxygen properly. The main clinical manifestations are pain attacks and damage to end organs such as the heart, lungs, cerebrovascular system, and kidneys [4].

Although SCA is caused by a single point mutation (βS) in the HBB gene, its clinical manifestations are significantly diverse. Phenotypic differences between patients are often confusing, ranging from asymptomatic mild anemia to recurrent vaso-occlusive crises (VOCs), multi-organ failure, and even early death. This phenotypic heterogeneity not only reflects the complexity of genotype-phenotype associations, but also reveals the dynamic interaction between genetic modifiers, epigenetic regulatory networks, and environmental factors. For example, a major genetic modifier is the patient's fetal he hemoglobin (HbF) level [5]. Some patients with βS homozygous have significantly milder clinical symptoms than typical cases due to concomitant α-thalassemia mutations or persistent HbF high expression [6].

The hemoglobin molecule is a tetrameric protein consisting of 2 pairs of polypeptidyglobin chains, each of the 4 chains attached to a heme group consisting of porphyrins and Fe atoms. The homozygous condition HbS is the most common and severe form of SCD and is also known as SCA. Notably, HbS can coexist with other aberrant β-globin variants and copolymerize with them [5]. Previously study showed that BCL11A single nucleotide polymorphisms (SNP) highly associated with HbF levels. In nonanemic populations (Northern Europeans and Sardi nians), whom HbF expression levels are typically 1%. Previously research discoveries that these SNPs are located in the intergenic region between the BCL11A gene on chromosome 2 and the HBS1L-MYB gene on chromosome 6. These preliminary findings were observed in a non-anemic population. This indicates that this genetic polymorphism may be an important genetic modulator of disease severity [7]. Environmental stresses such as infection, hypoxia, or dehydration may accelerate erythrocyte deformation and induce acute complications。 In addition to genetic variation, Environmental factors are also worthy of attention. Exposure to cold and windy weather seems to trigger acute complications in many individuals, although these effects are unpredictable and vary by geographical location. Low hemoglobin, low hemoglobin F percentage, and high reticulocyte counts during childhood are associated with poorer outcomes, although these effects are also relatively weak and inconsistent [8].

The diagnosis and classification of SCA rely on the precise identification of disease-specific biomarkers. These markers not only include classic gene mutations and abnormal hemoglobin but also encompass multi-dimensional information such as epigenetic modifications, metabolic disorders, and cellular mechanical properties. SCA is genetically caused by two copies of a mutated form of the gene that encodes the β chain of the adult hemoglobin, leading to the translation of valine instead of glutamic acid at position 6 of the protein. Under hypoxic conditions, HbS molecules form rigid polymers through hydrophobic interactions, leading to erythrocyte sickling, hemolysis, and microvascular occlusion [9]. Using the HPLC detection method, distinguish HbS from other hemoglobin variants (HbA, HbC, HbF) based on retention time differences and quantify the proportion of HbS. Or utilize CRISPR-Cas9 technology for HbS mutation detection [3]. However, the simple quantification of HbS alone cannot reflect the phenotypic heterogeneity (such as the symptom differences in patients with coexisting α-thalassemia or high expression of HbF). The condition should be comprehensively evaluated by combining the clinical phenotype with other markers (such as reticulocyte count). HbF is an important regulatory factor in the severity of the disease because its γ chains can inhibit the aggregation of HbS. The level of HbF is significantly negatively correlated with clinical phenotype, meaning that the higher the HbF, the milder the symptoms. However, the expression of HbF exhibits significant individual variability, which is

primarily determined by genetic factors (such as genes like BCL11A, HBS1L-MYB) and environmental factors [10]. Quantify its proportion in total hemoglobin utilizing the resistance of HbF to strong bases (such as NaOH). The frequency of pain crises in patients with HbF >20% is reduced by 50% to 80%. HbA2, a tetramer of a- and d-globin chains, accounts for 2%-3% of total hemoglobin in adults and has no actual physiological function, but its increased level aids in diagnosing β-thalassemia [11].

3. Molecular Diagnostic Methods

References are cited in the text just by square brackets [1]. (If square brackets are not available, slashes may be used instead, e.g., /2/.) Two or more references at a time may be put in one set of brackets [3, 4]. The references are to be numbered in the order in which they are cited in the text and are to be listed at the end of the contribution under a heading *References*, see our example below. Molecular diagnostic technology uses DNA or RNA as the diagnostic object. The fundamental principle is to detect whether there are changes in the structure of DNA or RNA, the quantity present, and whether the expression function is abnormal, in order to determine whether the subject has any genetic-level abnormalities. The prevention, prediction, diagnosis, treatment, and prognosis of SCA are of significant importance. In the late 1970s, a group of scientists including Kan successfully performed genetic diagnosis for SCA using liquid-phase DNA molecular hybridization. SCA is caused by a point mutation in the HBB gene, which encodes the beta subunit, where adenine is replaced by thymine (GAG > GTG) at codon 6 of the HBB gene [2].SCD occurs when two mutant alleles βS/βS are inherited (homozygous) or when mixed heterozygous alleles of different types are inherited, such as sickle cell β-thalassemia (HbSβ-thalassemia), sickle cell hemoglobin C disease (HbSC), and other combinations. The detection of hemoglobin S and the diagnosis of SCA now rely heavily on clinical laboratories for molecular diagnosis. high-performance liquid chromatography (HPLC), full count of blood cells, and Hb electrophoresis, are organised gold standerd methods.

3.1. PCR: (Clinical Pros Cons Optimize)

PCR is a molecular biology technique used to amplify specific DNA fragments. PCR is the based technique of detecting SCD. There are several PCR-based techniques that can be used to detect beta mutations, such as high-resolution melting (HRM) analysis, which is simple, sensitive and cost-effective, Large-scale screening of SCD genotypes [2].

3.2. HRM

HRM is the method based on PCR. The properties of inserting specific dsDNA dyes into the minor groove of DNA double strands (PCR products) can be utilized. By real-time monitoring of the melting process during temperature increases, the unwinding of dsDNA occurs, leading to the release of the fluorescent dye and a corresponding decrease or disappearance of the fluorescent signal. This process allows for the high-resolution recording of the melting curve, thereby enabling the detection of samples [12]. Compared to basic Sanger sequencing, HRM is characterized by rapid processing and high specificity. However, it demands high DNA quality and instrument specifications, and it is unable to distinguish between βs variants [13]. HRM is generally used for noninvasive prenatal diagnosis (NIPD) of Hbs [14]. HRM is currently widely used for general prenatal screening and basic clinical diagnosis. In the future, portable devices can be developed for rapid disease diagnosis in remote areas.

3.3. RFLP

RFLP is used to detect SCA by restriction enzymes, RFLP utilizes restriction endonucleases to cleave DNA molecules at specific sites, producing fragments characterized by restriction patterns, resulting in enzymatic fragments of varying lengths. In the diagnose of SCA, due to βs mutation destroyed the restriction enzyme cleavage sites. Thus, following enzyme digestion, differentiation can be made based on the length variations. For example, in normal gene βA, include the Ddel I restriction enzyme

cleavage site (CCTGAGG). However, in Mutated genes, it turns to CCTTAGG, which caused the Ddel unabled to identify and cut [15]. RFLP has significantly increased the content and relative specificity of the target DNA, and the typing is clear with good repeatability. The results are intuitive, as the fragment differences can be directly observed through gel electrophoresis, and the costs are low. However, RFLP only applicable to mutations at the cleavage site (βs), unable to detect other types. Moreover, the lengthy duration makes it difficult to observe small fragments during routine electrophoresis. Therefore, in the future, automated electrophoresis systems can be applied to save operation time for PCR, enzyme digestion, and electrophoresis. The combined use of various restriction enzymes to detect compound mutations.

3.4. Sanger

Sanger technique which has become widely used in the diagnostic community, is regard as Gold Standard [16]. The core principle of Sanger sequencing is the chain-termination method. Utilizing double-deoxynucleotide random termination for DNA continuation. Subsequently, separate different length fragments in electrophoresis to determine the base sequence. This method usually was used to confirm the c.20 A > T mutation in HBB gene [17]. Or to detect modified genes (BCL11A) SNPs. The advantage of Sanger sequencing lies in its high accuracy, which allows for the identification of new types of mutations. However, the long duration and high cost of detecting individual samples are unavoidable.

3.5. Emerging Molecular Diagnostics

NGS technology is a high-throughput method for DNA sequencing and has become a widely adopted tool in SCA diagnostics. The "next generation" in NGS refers to a revolutionary technological leap that enables the large-scale parallelization of the DNA fragment sequencing process, similar to running millions of individual Sanger sequencing experiments simultaneously. This high-throughput "shotgun" solution can rapidly sequence entire genomes [18]. In SCA diagnostics, NGS is often utilized for targeted sequencing. The target genes are the HBB gene（βS mutations）, gamma-globin, and BCL11A. This method is also commonly used for painless prenatal diagnosis. Avoid invasive procedures that may lead to miscarriage. Compared to Sanger sequencing, NGS offers significant advantages such as high throughput and high sensitivity, and it is widely used in clinical settings for the diagnosis of complex cases and multi-gene screening. Although NGS has become the current standard method due to its comprehensive diagnostic capabilities, its high cost limits its widespread use, especially in resource-limited settings. In contrast, digital PCR (dPCR) offers comparable accuracy at a lower complexity and cost, making it a promising alternative for SCA detection. dPCR achieves absolute quantification by partitioning the reaction system into thousands of independent micro-reaction units (such as droplets or microwells), with each unit containing zero, one, or multiple target DNA molecules. Through endpoint detection techniques, it directly counts the number of positive reaction units and employs Poisson distribution to calculate the absolute copy number of the target sequence, without the need to rely on standard curves or reference genes. dPCR can detect extremely low concentrations of fetal cell-free DNA (cffDNA) in maternal plasma, accurately identifying even when the fetal DNA proportion is as low as 4% [19]. In dPCR for NIPT, cfDNA is analyzed to identify βs mutations.

4. Conclusion

In conclusion, in recent years, molecular diagnosis of sickle cell anemia has occupied an important position in clinical testing. With the continuous improvement of molecular diagnostic technology and the research level of molecular biology, the diagnosis of sickle cell anemia has become increasingly accurate. Molecular diagnosis has also gained more trust from clinical laboratories due to its high sensitivity and accuracy. Whether it is the previous PCR, hrm or the further developed NGS and digital PCR, they all play an important role in areas such as NIPT and early neonatal screening. This article systematically expounds the pathogenesis and molecular diagnostic techniques of SCA,

clarifying the development prospects and optimization directions of SCA molecular diagnosis. However, there are still some aspects that need further optimization in the current SCA molecular diagnosis. For example, addressing the inconvenience of carrying out screening work in remote areas, shortening the laboratory workflow time, and reducing the cost of individual samples. This article does not compare with traditional laboratory diagnostic methods such as blood cell counting, and does not clearly explain the complementary aspects of molecular diagnosis and traditional methods. Future research on SCA molecular diagnosis will move towards high-throughput, short-time, and low-cost directions.

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