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Clinical Applications of Molecular Technologies in Hematology

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Abstract

The rapid emergence of molecular diagnostic platforms has revolutionized the diagnostic approaches in hematology laboratory. Fluorescence *in-situ* hybridization, polymerase chain reaction and DNA sequencing are common techniques used in routine clinical laboratories for the diagnosis of hematological diseases. Different molecular techniques are indicated in different situations. This paper describes the utility of common molecular techniques. Fluorescence in-situ hybridization is specific for detection of chromosomal abnormalities using fluorescent labeled targeting probe. Polymerase chain reaction amplifies target DNA and reverse transcription polymerase chain reaction amplifies target RNA for the analysis of gene and its expression level. Real-time polymerase chain reaction is highly sensitive for detection of minimal residual disease in hemic malignancies. Gap-polymerase chain reaction is often employed for diagnosis of large deletions such as in alpha thalassemia. Allele-specific polymerase chain reaction is commonly used for single nucleotide polymorphism detection which is common in beta thalassemia, myeloproliferative neoplasm and acute leukemia. Inverse shifting-polymerase chain reaction can be employed for the detection of large genetic rearrangements such as those seen in hemophilia A. For genetically complex diseases such as hemophilia A, which involves a great variety of mutations in large genes, high resolution melting analysis can be used to scan for point mutations. Any suspected mutations are confirmed using post-PCR technologies, such as DNA sequencing. Although conventional diagnostic methods are able to provide a basic analysis in most cases, molecular technologies generate valuable genetic information that can refine diagnosis, better predict prognosis and facilitate disease monitoring.

Keywords: Molecular diagnosis; hematological malignancies; FISH; PCR; HRM

Abbreviations: FISH: Fluorescence *In-Situ* Hybridisation; PCR: Polymerase Chain Reaction; RT-PCR: Reverse Transcription-Polymerase Chain Reaction; Q-PCR: Quantitative Polymerase Chain Reaction; IS-PCR: Inverse Shifting- Polymerase Chain Reaction; HRM: High Resolution Melting

Introduction

While labor-intensive manual tests and high-volume automations are routinely employed for clinical diagnostics, we are heading towards the era of personalized therapy using molecular platforms. With the recent advances in molecular medicine, hematological malignancies, hemoglobinopathies, and congenital hematological disorders such as coagulating factor deficiencies can now be diagnosed genetically. Over the past decade, laboratory diagnostic approaches have been developed from the comparatively more laborious manual methods, which usually take hours to perform, to a rapid, robust and accurate molecular diagnostic platform. To provide the best patient care, an accurate and expeditious diagnosis is essential for clinical management of patients with hematological disorders. Complete blood counts and morphological examinations are fundamental and play important roles during diagnosis. Conventional cytogenetics is another time-honoured assay for the detection of global and specific chromosomal abnormalities, such as translocations, partial deletions and aneuploidies [1,2]. Marrow cells and at least 20 banded metaphases that are required for cytogenetics examination, however, are difficult to be obtained. Moreover, other difficulties in cytogenetics include poor quality metaphases, small sample size and the fact that only large size alterations are revealed all contribute to its low sensitivity. In addition, the need for cell culture and metaphase analysis, which are both labor intensive and technically demanding, have also limited its application [3,4]. Emerging molecular-cytogenetic techniques, such as fluorescence *in situ* hybridization (FISH), and other molecular technologies have overcome many of the drawbacks of conventional diagnostic methods. In this review paper, the utility and application of common molecular diagnostics in clinical hematology are discussed.

Fluorescence in situ hybridization (FISH)

FISH was developed in early 80s and became one of the most sensitive assays for localization of specific nucleic acid sequences and detection of numerical chromosome abnormalities, structural chromosomal rearrangements and cryptic abnormalities [5]. Bone marrow aspirate and peripheral blood smears can be used for FISH analysis [2,4]. FISH makes use of fluorescent probes which hybridize only to complementary sequences and the resulting signals are examined under a fluorescent microscope. The utility of specific FISH

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probes provides intense signals and multiple targets can be visualized simultaneously by using probes labled with different fluorescent colors. Varieties of fluorescent probes are commercially available and commonly used probes in clinical routine laboratory include centromeric enumeration probes (CEP), chromosome painting probes, dual-color dual fusion probes (D-FISH) and dual-color break-apart probes. CEP is used for enumeration of chromosomes by hybridizing to the unique alpha (or beta) satellite repeats within the centromeric regions. Chromosome painting probes are used to highlight the entire chromosome of interest. D-FISH are used to detect chromosomal translocations. The development of D-FISH is significant for diagnosis and treatment monitoring. D-FISH probe detects cryptic rearrangement and has a low false-positive rate. It employs large probes that span the upstream and downstream of the target breakpoints on both chromosomes, and each probe is labeled with a different color, such as red and green. There are two red signals and two green signals in normal cells. In affected cells where translocations present, two fused red/green signals from the translocated chromosomes, together with a single red and green signal from the unaffected counterparts, would be observed during metaphases (Figure 1) [4]. Cryptic insertion of the *BCR* to *ABL* gene at 9q34, t(9;22) variant in chronic myeloid leukemia (CML), and three-way translocation in CML and acute promyelocytic leukemia (APL) have been reported and can be identified by D-FISH [3].

FISH is pivotal in the diagnosis and clinical management of hematological malignancies. Other applications include the demonstration of t(11;18)(q21;q21) in mucosa-associated lymphoid tissue (MALT) lymphoma [6]; the detection of clonal abnormalities (deleted 5q, trisomy 8 and monosomy 7) in myelodysplastic syndromes (MDS) with minimal dysplasia; 11q23 abnormalities in acute myeloid leukemia (AML) [7]; the demonstration of hyper- or hypo-diploidy and occult translocations in acute lymphoid leukemia (ALL), which is hard to grow in metaphase and is one of the difficulties of cytogenetic detections [8]; myeloproliferative neoplasms (MPN) with eosinophilia and interstitial deletion on short arm of chromosome 4; chronic lymphocytic leukemia (CLL) with trisomy 12 and cryptic t(15;17) variant – ins(17;15) in APL [9].

In comparison to cytogenetics, FISH detects cryptic *BCR-ABL1* translocations. FISH also detects all molecular variants in CML and minimal residual disease (MRD) at low leukemic cell counts. Moreover, both metaphase and interphase cells can be used in FISH. The utility of interphase cells minimizes laborious procedures of growing metaphase cells in cytogenetics. However, result interpretations can be challenging when there are too much signals overlapping [2,10]. In clinical diagnosis, FISH is often used in parallel with cytogenetics and/or other molecular techniques for diagnostics, confirmations and treatment monitoring.

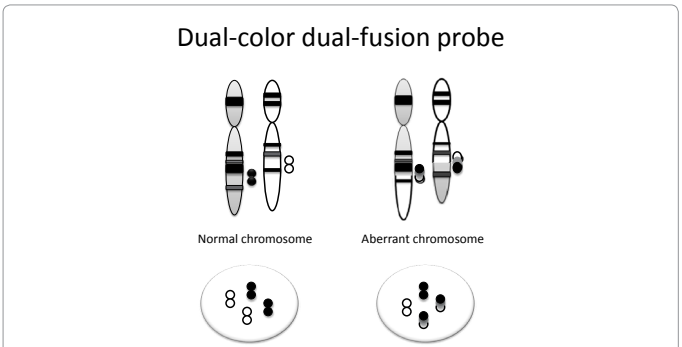

Figure 1: The diagram illustrates the utility of dual-color dual-fusion (D-FISH) probe. It employs two large probes that span over the breakpoints. In normal metaphases, two signals each for the wild-type alleles are generated. In positive metaphases, one signal each for the wild-type alleles and two fused signals are generated. Black dot represents a D-FISH probe for one chromosome. Circle represents different colored D-FISH probe for another chromosome of interest. Black/grey/white dot represents the fusion signals.

Polymerase Chain Reaction (PCR)

PCR is commonly used for detection of known mutations at the DNA level. PCR procedure involves 20-40 thermal cycles which is comprised of denaturation, annealing, and elongation in each cycle. Post-PCR procedures include gel electrophoresis, sequencing and melting curve analysis. Both bone marrow aspirate and peripheral blood can be used. Unlike FISH, PCR and its variants only detect breakpoints covered by designated specific primers. Examples of the application of PCR include *JAK2 V617F* mutations; *FLT3, NPM1*, and *CEBPA* mutations in acute myeloid leukemia with normal cytogenetics (CN-AML) [11]; and gene rearrangements such as Ig heavy chain gene rearrangement for B-cell malignancies and T-cell receptor gamma chain gene rearrangement for T-cell malignancies [12]. Various derivatives of PCR technology are widely used in hematological laboratories, including RT-PCR, Q-PCR, multiplex PCR, gap-PCR, ARMS and IS-PCR.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR is commonly used in routine laboratory to examine target gene expression that is significant to the pathogenesis of hematological malignancies. RT-PCR procedure is comprised of complementary DNA (cDNA) synthesis using target RNA as a template and cDNA PCR amplification. One-step RT-PCR, where the entire reaction occurs in a single tube, is widely used nowadays. In diagnostic services, RT-PCR is very useful in identification of disease associated transcripts that are often crucial for making clinical decisions. For example, the reciprocal translocation of chromosome 9 and 22 (t9;22) in CML has 4 combinations (e1a2, b2a2 or b3a2, e19a2) that generate p190, p210, and p230 *BCR-ABL1* transcripts respectively. The p210 is commonly found in CML whereas the p190, which has almost identical constitutive tyrosine kinase activity as the p210, is more commonly seen in ALL [8,13]. RT-PCR can accurately distinguish the breakpoint in variant transcripts at the level of gene expression. Moreover, RT-PCR detects translocations such as the presence of *AP12-MALT1* transcripts that is indicative of MALT-lymphoma [6]; *RUNX1-ETO* transcripts in AML [14]; *PML-RARα* transcripts in APL [15]; and *ETV6-RUNX1* transcripts in ALL [8]. RT-PCR also detects gene fusion resulting from interstitial deletion such as *PDGFRA*-associated chronic eosinophilic leukemia [16].

RT-PCR is pivotal in detection and monitoring of specific breakpoint, for example the *BCR-ABL1* in CML, however, is not sensitive enough for monitoring *BCR-ABL1* transcript level during treatment [2,17]. Comparatively, quantitative RT-PCR (Q-RT-PCR) is suggested to have high sensitivity up to 0.001-0.0001% and is commonly used for treatment monitoring nowadays [2]. Despite the fact that RT-PCR is useful in diagnosis, it fails sometimes because of RNA degradation, leading to false-negative results. Cross-contamination is another issue leading to false-positive results in all PCR technologies [18].

Furthermore, multiplex RT-PCR, in which multiple pairs of primers are added in one PCR reaction, is used for simultaneous detection of multiple gene transcript targets. Pallisgaard et al. have successfully established a multiplex RT-PCR protocol with 8 parallel PCR reactions for simultaneous detection of 29 chromosomal aberrations in leukemia,

for example, *BCR-ABL* (e1a2, b2a2, b3a2) chimeric gene for ALL and CML; MLL/AF6, MLL/AF6, MLL/AF6, MLL/AF6 in AML; and NPM-RARA in APL [19]. Recently, simplified multiplex RT-PCRs for common translocations in leukemia have been developed [20]. This has massively lowered labor costs and significantly improved turnaround-time. In addition to CML cases, multiplex RT-PCR has greatly improved the detection of typical and atypical *BCR-ABL* fusion transcripts. Apart from the three predominant fusion transcripts of ela2, e13a2 and e14a2, a number of atypical transcripts such as e1a3, e13a3, e14a3, e19a2, e6a2 and e8a2, can also be analysed simultaneously by multiplex RT-PCR [21]. Multiplexing technology has enhanced the speed and reliability of fusion transcript detection by PCR.

Real-Time Quantitative Polymerase Chain Reaction (Q-PCR)

Q-PCR and Q-RT-PCR allow the quantification of DNA and RNA molecules, respectively. Q-PCR makes use of fluorochrome-labeled sequence-specific probes such as adjacent hybridization probe and TaqMan probe to measure the quantity of target amplicons in real-time. Besides, a known amount of target or adjacent sequence is co-amplified in the PCR reaction. This acts both as an internal control and a reference standard to determine the amount of target molecules. For example in CML, DNA/cDNA of *ABL* is co-amplified as an internal marker with the target DNA/cDNA of chimeric *BCR-ABL*. Commonly used TaqMan probes consist of a reporter at 5'-end and a quencher at 3'-end . It hybridizes to complementary target DNA and is cleaved by Taq polymerase, which has 5' to 3' exonuclease activity, during primer elongation and emits fluorescent signal. The quantity of target DNA, which is proportional to the fluorescent signals emitted, is determined with reference to a standard curve. The standard curve is a serial dilution of the internal control sequence with a known concentration [2,22]. The establishment of relative or absolute (standard curve) quantification provides a well presentation of data. Besides, international scale also allows inter-laboratory comparison [23].

Among all commonly used molecular techniques, Q-PCR is the most sensitive and is routinely used for treatment monitoring and detection of MRD such as in CML [24,25]. The quantification of *BCR-ABL* transcript is important for determining disease status and progression. In MRD, a complete remission requires less than 5% of leukemic blasts found in bone marrow. In order to achieve the requirement, conventional cytogenetics requires analysis of a large number of metaphases. The costly and laborious procedure has limited its uses. Although FISH is comparatively easier for interpretation by counting 100-200 cells, it requires in-depth knowledge and technical expertise [1,2]. Q-PCR is thereby suggested to be the most sensitive and fastest method for the detection of MRD with no post-PCR procedures required, thus dramatically reducing the risks of cross-contamination.

Gap-PCR

Gap-PCR detects deletion that might be missed by DNA sequencing. Specific primers are designed to flank a known deletion. The principle of gap-PCR is based upon the inability of the primers to generate a PCR product unless a deletion joins the flanking sequences together. If a deletion is present, PCR amplification will occur and the product is examined by electrophoresis.

In hematology, gap-PCR is commonly used specifically as a routine method for alpha-thalassemia (α-thalassaemia) genotyping. α-thalassaemia, which is an inherited recessive disorder, is predominantly diagnosed with common deletions and in some cases with non-deletional mutations, leading to a reduced or impaired production of alpha globin chains. There are two major types of α-thalassaemia: 1) Hemoglobin Bart's syndrome, which is the severe form and can be fatal and 2) Hemoglobin H disease, which is of moderate severity. Gap-PCR is useful in diagnostic laboratories for a simple and rapid detection of common α-thalassaemia mutation. Universal detection primers that target the common deletion sites can be used [26,27]. Less common globin disorders caused by deletions that can be detected by gap-PCR include delta-beta deletions (HPFH-1, HPFH-2, HPFH-3, HPFH-7, Lepore) and beta-globin deletions (beta-FL, -619bp, Asian-Indian inversion) [28]. Multiplex gap-PCR can be used to detect multiple target deletions simultaneously. For example, HbH disease is detected using multiple specific primers for the most common α-thalassaemia deletions [29].

Amplification Refractory Mutation System (ARMS)

ARMS, also named allele specific PCR, is a diagnostic technique for the detection of single base mutation including both germ-line and somatic mutations. It is simple and reliable for detection of single nucleotide polymorphisms (SNPs). ARMS is performed by using a pair of PCR primers that are specific to either the mutant or the wild-type sequences. The polymorphic nucleotides should be present at the 3' end of the primers to enhance the specificity of the reaction. Extension would only occur if the 3' end of the allele specific primer is bound to the complementary target sequences. Polymerases with no 3' endonuclease activities are required to prevent correction of terminal mismatch. The genotype of SNPs is determined by analysis of PCR products with gel electrophoresis [22]. A positive PCR amplification with the wild-type-specific primers indicates a homozygous wild-type genotype. On the other hand, positive PCR with both wild-type and mutant primers indicates heterozygous whereas a positive PCR with mutant primers indicates a homozygous mutant genotype.

Among all PCR technologies, ARMS is one of the most specific techniques to detect SNPs. It allows not only detection but also rapid genotyping. Its ability to detect SNPs or single base mutation has accelerated the introduction of ARMS to the clinical setting. Applications of ARMS include detection of sickle cell anemia, *JAK2* mutation for MPNs [30], A gamma- and G gamma- promoter mutations which are associated with non-deletional hereditary persistence of fetal hemoglobin (HPFH), non-deletional α-thalassemia and beta-thalassemia (β-thalassaemia).

Inverse Shifting-Polymerase Chain Reaction (IS-PCR)

IS-PCR is a variation of inverse PCR for rapid screening of large known rearrangements such as large duplications, large inversions, large deletions and chromosomal translocations. In clinical laboratory, IS-PCR improves the turnaround-time significantly in diagnosis of hemophilia A (HA) which is characterized by *factor VIII (F8)* gene disruption. HA is an X-linked coagulation disorder, affecting one in every 5000 males [31]. A majority of severe HA cases are diagnosed with DNA inversions in the *F8* gene, which is large and complex. Intron 22 inversion (Inv22) of *F8* comprises 40-50% of all mutations and is the most prevalent mutation [32]. Inversion in intron 1 (Inv 1), comprising 5% - 7% of severe HA, is another common hotspot [33]. Both Inv22 and Inv1 are result from non-allelic meiotic recombination [31]. The remaining cases are caused by different mutations in the *F8* gene. For example, one of the large deletions in the *F8* gene is suggested to be Del22, which is found in heterozygous females with no bleeding but with a certain degree of fertility impairment. Del22 carriers can be directly detected by IS-PCR [31].

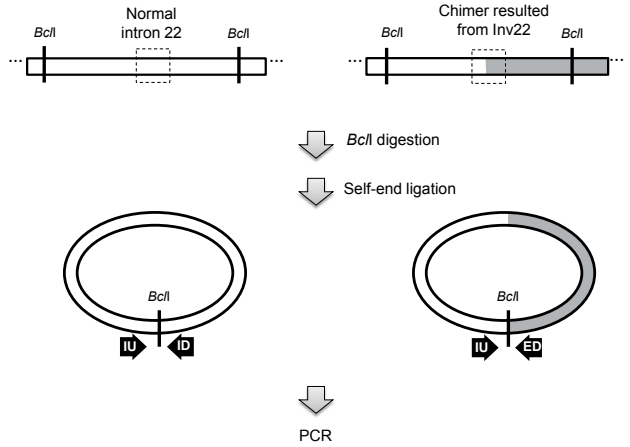

Figure 2: Principle of IS-PCR as illustrated by Inv22 mutation detection in haemophilia A. Genomic DNA is digested by *BclI*, followed by self-end ligation. Ligated circles corresponded to the normal *F8* gene and the Inv22 mutant chimer are differentiated by a multiplex reaction containing three primers, namely intragenic upstream primer (IU), intragenic downstream primer (ID) and extragenic downstream primer (ED). The resultant PCR products of different sizes are resolved by gel electrophoresis. The dotted rectangle denoted the region of homologous recombination.

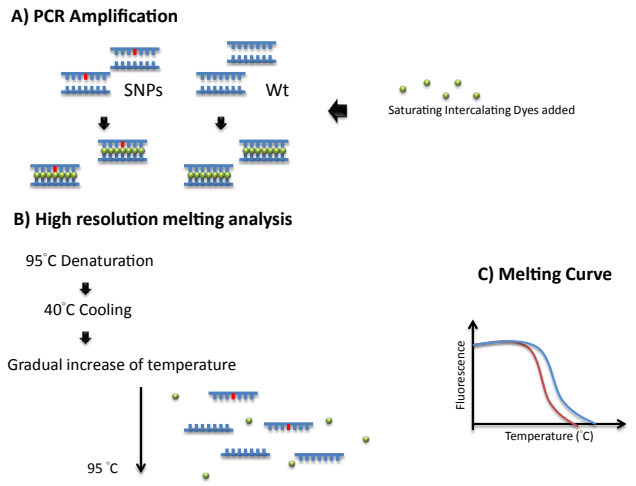

Figure 3: The diagram illustrates the principle of HRM analysis. A) The DNA of interest is amplified by PCR. B) High resolution melting analysis is performed after PCR amplification. It begins with DNA denaturation at 95˚C and cooling down to 40˚C, followed by a gradual increase of temperature from around 65˚C up to 95˚C. Amplicons separate when the melting temperature is reached and thus the intercalating dyes are released and fluorescent signals decrease. C) The signal changes are detected upon laser excitation during analysis and melting curve are recorded accordingly.

IS-PCR involves three main steps: restriction enzyme digestion, self-ligation and PCR analysis (Figure 2). To detect Inv22 mutation in HA, genomic DNA sample is first digested by *BclI*. This is followed by self-end ligation of *BclI* fragments. While irrelevant ligation products are produced as background, some ligation events generate relevant circles that contain the normal *F8* intron 22 sequences, or the Inv22 chimers resulting from the intrachromosomal recombination between *F8* intron 22 and its homologous copies outside the *F8* gene. PCR primers are designed to target the *BclI* spanning regions of these relevant circles. The targeted region of the "normal circle" is amplified by a pair of intragenic upstream and downstream primer, while that of the "Inv22 circle" is amplified by an intragenic upstream and an extragenic downstream primer. The two PCR products are of different sizes, which are resolved by gel electrophoresis [34].

IS-PCR is an alternative approach to Southern blot analysis and long distance-PCR (LD-PCR), which were commonly used for the detection of Inv22 and Inv1 in HA. However, these conventional methods are labor-intensive, complex and costly. On the other hand, IS-PCR is more rapid, user-friendly and capable of genotyping all structural variants derived from the hotspots. IS-PCR does not require PCR amplification of long DNA sequences and is a robust technique for screening large populations. IS-PCR is a reliable technique for diagnosis, prognosis and prenatal check up and has been introduced to service for severe HA.

High Resolution Melting (HRM) Analysis

High resolution melting (HRM) is a powerful post-PCR technique that reveals and distinguishes point mutations according to the difference in melting temperature (Tm) and is subsequently confirmed using sequencing. HRM makes use of intercalating dyes such as LCGreen, which emit light when intercalated into double-stranded DNA (ds DNA) but only fluoresce at low level in the absence of dsDNA. Unlike SYBR green, LCGreen does not inhibit amplification. The melting analysis is first introduced with the Light-Cycler and is reported to be performed on other HRM instruments. HRM is performed after PCR, where the amplicons undergoes DNA denaturation at 95˚C and cooling down to 40°C, followed by a gradual increase of temperature from around 65˚C up to 95˚C. During the ramping process, double-stranded DNAs are denatured at their melting temperature and thus the intercalating dyes are released and the fluorescent signals decrease. The signal change is detected upon laser excitation and a melting curve is recorded accordingly (Figure 3) [35,36]. The double-stranded DNAs containing the wild-type and mutant alleles have different melting temperatures due to their nucleotide differences. Such the difference could be differentiated under the melting curve.

HRM has become a promising method for screening SNPs. Examples of applications include β-thalassaemia, which is another common hereditary disease' and HA. In addition to large insertions/deletions, point mutations can also result in HA. According to the FVIII HAMSTeRS (The Hemophilia A Mutation, Structure, Test and Resource Site) database, there are more than 1209 mutations within the *F8* coding and untranslated regions [37]. Therefore, HRM analysis could be a potential screening test in the clinical setting. Among all molecular techniques, HRM is fast, convenient, cost effective and sensitive in screening point mutations and genotyping multiple samples simultaneously [38]. Unlike conventional PCR thermocycler, which is block-based PCR, HRM is usually performed with a centrifuge-like holder and capillary sample format that requires smaller amount of samples. It allows better temperature control and therefore a shorter turnaround time and every tube is measured constantly and evenly [38]. Molecular testing in HA and thalassemia have a great clinical value not only for diagnosis but also prognosis, genetic counseling for at-risk families, and prenatal diagnosis.

DNA Sequencing

DNA sequencing is a post-PCR analysis and a confirmation method for mutations. Applications include non-deleted α thalassaemia, *F8* mutation in HA, AML with normal karyotype, AML with *NPM1* exon 12 mutation, AML with mutated *CEBPA* (not common), *FLT3* exon 14 tandem duplication and other mutated genes – *KIT, MLL, WT1, NRAS, KRAS;* and enquiries on normal or specific karyotypes [11,31].

Future Perspectives

There have been rapid developments in molecular techniques ever since the introduction of PCR and Sanger sequencing. Over the past decade, the cost of sequencing per base has been driven down significantly as a result of the emergence of novel technologies such as massively parallel sequencing, or so called next generation sequencing (NGS). The completion of the human genome project in 2003, the Encyclopedia of DNA Elements (ENCODE) project [39] and studies of various whole cancer genomes, whole exomes and transcriptomes have brought new insights in diagnostic, prognostic, preventive and personalized medicine. The three most commonly used NGS platforms are Illumina, Life SOLiD, and Roche 454. NGS technology has demonstrated effective, accurate and robust performance for sequence detection and enumeration.

In the era of NGS, whole cancer genome sequencing has revealed novel candidate genes that may contribute to leukemic pathogenesis. In T-cell acute lymphoblastic leukemia (T-ALL), which is predominant in male, the X-linked *PHF6* gene was reported to be a tumor suppressor gene and the mutational loss of *PHF6* is significantly associated with its pathogenesis [40,41]. Moreover, the diagnosis of CN-AML has been difficult due to cryptic manifestation. NGS studies have revealed a number of driver mutations such as *NPM1* and *CEBPA* mutations in AML [42,43]. Other newly discovered potential markers include *TP53* mutations in MDS [44] and *NOTCH1*, *SF3B1* and *BIRC3* mutations in CLL [45,46].

NGS could provide a diagnostic setting where multiple genetic markers and patient genomes are sequenced in a massively parallel manner. Godley [47] suggested having a diagnosis setting that includes Q-PCR, microarray analysis, and NGS for hematological malignancies. NGS technology has not only contributed to clinical and therapeutic research but also to clinical service and prenatal diagnosis. NGS may be introduced into diagnostic services in the foreseeable future.

Conclusions

Conventional diagnostics methods are solely based on morphological analysis, special phenotypic tests and cytogenetic analysis. However, there are many cases that conventional methods cannot come up with a conclusion. The invention of molecular technologies and the understanding of genetics have introduced new insights to clinical hematology. In the past decades, molecular diagnostics have become increasingly important for diagnosis and treatment monitoring. Next generation sequencing is another powerful tool for identifying disease associated genetic changes in a genome-wide fashion. There is no doubt that molecular diagnostics will take a significant role in near future.

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