Molecular diagnostics and reporting in lymphoid malignancies: Current status and beyond

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1 INTRODUCTION

Thanks to the advent of next-generation sequencing (NGS) technologies more than 10 years ago, the genomic landscape of most larger cancer types has been unraveled. Hematological and lymphoid malignancies were among the first cancer types to be sequenced, probably as they are more easily accessible. Based on these studies, the number of clinically relevant genetic aberrations with diagnostic, prognostic, and/or predictive impact has increased rapidly. This increases demands on molecular diagnostics to ensure that different types of genetic alterations can be readily detected in a diagnostic setting and within a reasonable time frame.

For hematological malignancies, genetics have been an integral part of diagnostics since decades, including methods such as cytogenetics, fluorescence in situ hybridization (FISH) and targeted mutational analysis, and/or Sanger sequencing (Figure 1). With the more powerful NGS technologies, targeted gene panels have been added to the arsenal of methods in the diagnostic laboratory. Regardless of the type of diagnostic test, it is essential to harmonize methodology according to international standards and guidelines. To ensure comparable results, especially in multicenter studies, it is equally important that the interpretation of the results and the clinical reporting follow established guidelines. This is particularly relevant as genetic test results are becoming more complex with different NGS assays covering a range of different genetic aberrations.

This paper gives an overview of different state-of-the-art molecular technologies that are applied in clinical diagnostics of lymphoid malignancies, their advantages, and limitations. It also discusses aspects that need to be considered to harmonize clinical interpretation and reporting of NGS data.

2 TECHNOLOGIES APPLIED FOR DIAGNOSTICS OF LYMPHOID MALIGNANCIES

2.1 Molecular cytogenetics

Cytogenetics, or chromosome banding analysis, has long been instrumental to define recurrent chromosomal aberrations observed in different lymphoid malignancies. While cytogenetics has remained central in leukemias (i.e., acute myeloid leukemia [AML], acute lymphoblastic leukemia [ALL], and chronic myeloid leukemia), molecular cytogenetics or FISH is preferred for mature lymphoid malignancies. The introduction of the FISH technology enabled analysis not only on metaphase chromosomes, but also on interphase chromosomes (i.e., nondividing cells). FISH is therefore more rapid than cytogenetics and can be used to screen for recurrent diagnostic or risk stratifying genomic aberrations. Moreover, FISH can be applied to various tissue types, such as blood smears, imprints as well as formalin-fixed, paraffin-embedded (FFPE) tissue. Robust, commercial FISH probes are available for most recurrent aberrations, although they are relatively expensive. It is commonly used to detect translocations in lymphomas; for example, t(11;14) in mantle cell lymphoma and MYC and BCL2 translocations in diffuse large B-cell lymphoma (DLBCL). In chronic lymphocytic leukemia (CLL), a panel of probes is usually applied to detect risk-stratifying aberrations, that is, 11q-deletion, 13q-deletion, 17p-deletion, and trisomy 12, whereas...
in multiple myeloma, probes are used to identify t(4;14), t(11;14), t(14;16), 1q gain, and 17p deletion. However, while FISH on interphase cells can give a quick answer as to whether a specific genetic aberration is present or not, it is not a particularly sensitive technique; the cutoff level to detect an aberration has to be determined by each laboratory and is usually around 5%.

2.2 Targeted mutation analysis

Before the NGS era, most clinical laboratories applied Sanger sequencing for targeted mutation analysis. Sanger sequencing has been the gold standard since the 1980s and is a very robust technology. However, it rapidly becomes expensive and time consuming if several genes or large genes are investigated. Another limitation with Sanger sequencing is its sensitivity which is usually between 10% and 20%.

In CLL, TP53 mutations are linked to poor response to chemotheraphy and poor overall survival, and TP53 gene screening is nowadays mandatory before start of any line of treatment. TP53 gene analysis (encompassing exons 2-11) has typically been performed by Sanger sequencing, although more recently many laboratories have shifted to NGS.

Another sequenced-based molecular test is the immunoglobulin heavy variable (IGHV) gene mutational status that defines CLL with unmutated IGHV genes and an inferior outcome, and CLL with mutated IGHV genes and an expected favorable prognosis. This analysis is mainly performed by polymerase chain reaction (PCR) amplification of the clonal IGH rearrangement followed by Sanger sequencing, although new NGS protocols have been developed.

For both TP53 analysis and IGHV gene analysis, the European Research Initiative on CLL (ERIC) has provided technical recommendations and guidance for interpretation. It has also implemented dedicated certification systems that enable clinical laboratories to regularly certify their method (Sanger sequencing or NGS) against standard operation procedures.

For hotspot mutation detection, such as BRAF (V600E) in hairy cell leukemia and MYD88 mutations (L265P) in Waldenström’s macroglobulinemia, specific assays have been established. These use allele-specific PCR or quantitative PCR to detect the mutant allele. More recently, digital droplet PCR has been developed that enables a very sensitive detection (down to 0.01%) and can be applied to detect recurrent mutations or to follow patients over time. It is, for instance, used to detect BTK/PLCG2 mutations in CLL patients progressing on ibrutinib treatment.

2.3 Next-generation sequencing

Since more than 10 years, we have had access to new types of sequencing instruments that provide massive parallel sequencing or NGS. Using this technology, we can analyze the entire genome (whole-genome sequencing [WGS]), the exome (whole-exome sequencing), or selected regions of particular interest, that is, targeted NGS or gene panels. Depending on the detail of sequencing, the number of obtained sequence reads differs. Using WGS, the recommendation for tumor samples is to sequence to a sequence depth of 90× (and 30× for the matched normal sample). For targeted gene panels, depending on the size of the panel, one aims for a high sequence depth of at least 500× but preferably above 1000×.

Until recently, the majority of gene panels used within clinical diagnostics were amplicon-based, meaning they use PCR to amplify selected amplicons covering the genes/exons of interest. These amplicon-based panels can be implemented relatively easily using either a panel that is designed in-house, or commercially available panels. However, the limitations with amplicon-based technology are potential biases in amplification, particularly in difficult to sequence regions (i.e., GC rich and/or repetitive regions), that can cause drop-out of amplicons, as well as an increased risk of low-frequency, false-positive mutations; this can happen in particular when FFPE material is used as source of input.

In an attempt to study the robustness of amplicon-based gene panels, we within ERIC recently conducted a multicenter study using three different technologies (Multiplicom, HaloPlex, and TruSeq), targeting 11 genes recurrently mutated in CLL. All six centers analyzed the same 48 CLL samples, and each technique was analyzed...
by two centers. A very high concordance was achieved between technologies and centers for gene mutations with a variant allele frequency (VAF) above 5%, while discrepancies started to appear for variants with a VAF <5%. Hence, we conclude that amplicon-based sequencing can be safely adopted for somatic mutation detection with VAFs >5%.

We also tested a high-sensitivity assay containing unique molecular identifiers (UMIs) which could confirm subclonal mutations with a VAF <5%. In this approach, duplicate reads are removed which improved sensitivity. Therefore, inclusion of UMIs should be considered when new panels are designed.

More recently, capture-based enrichment panels, using baits (probes) to hybridize to the region of interest, have been developed. These panels are usually larger in size (hundreds of genes) and produce more even sequence reads. This means that more “problematic” regions can be sequenced. Another advantage is that they enable simultaneous detection of different types of genomic aberrations (as they are larger in size). For instance, in addition to enable simultaneous detection of different types of genomic aberrations variants (SNVs) and insertions/deletions (indels), it is also possible to analyze copy-number changes (i.e., deletions and amplifications) and structural variants (e.g., translocations). He et al.8 combined a DNA and RNA-based broad, capture-based gene panel for diagnostics of a large number of hematological malignancies (n = 3696) including different lymphoid malignancies. Using either bone marrow or FFPE samples, they could detect all aforementioned types of genomic aberrations with high accuracy and reproducibility.

Gene panels have rapidly been introduced into routine diagnostics of myeloid malignancies. In Sweden, we recently shifted from a 54 gene amplicon-based gene panel to a national capture-based myeloid panel including 195 genes. In lymphoid malignancies, smaller amplicon-based gene panels are currently in clinical use. As mentioned, in CLL, we test for TP53 mutations before start of treatment and many centers have switched to amplicon-based NGS-gene panels. There are also additional genes of diagnostic, prognostic, and predictive impact in lymphomas that can be captured with these smaller panels. For instance, detection of certain mutations has diagnostic utility, for example, MYD88 mutations in Waldenström’s macroglobulinemia, BRAF mutations in hairy cell leukemia, KLF2 mutations in splenic marginal zone lymphomas, and STAT3/STAT5B mutations in T-cell lymphomas, while other gene mutations are associated with response to treatment (e.g., MYD88/ CXCR4 mutations in Waldenström’s macroglobulinemia patients treated with ibrutinib) or treatment resistance (e.g., BTK/PLCG2 mutations in ibrutinib-treated CLL).2

Considering that a broad spectrum of genetic aberrations is involved in ontology and evolution of lymphoid malignancies,1 the introduction of capture-based panels will be particularly useful in this patient group to capture not only SNV/indels, but also copy-number aberrations (CNAs) and translocations as well as more complex markers such as Ig/T-cell receptor gene analysis. In Sweden, we have recently developed a (national) capture-based lymphoid panel including 252 genes that is currently under validation and will soon be implemented into routine diagnostics.

### 3 Reporting and Interpretation of Results

For all types of molecular reports, it is important to include a number of key parameters so that the results can be easily understood by other laboratories. The report should include general information, such as personal id, referring doctor, and more specific information, such as tissue type investigated, method applied, and genetic aberrations assessed (Table 1). For FISH analysis, it is important to state the probes used and number of cells investigated as well as the cutoff applied. For more targeted analyses, such as hotspot mutation detection, the technology, and sensitivity should also be provided.

For NGS-based analysis, it is important to state gene coverage and sequence depth as well as cutoffs for variant calling. The sequence variants should be listed and include variant description at cDNA/protein level, following the HGVS nomenclature, the number of variant reads versus total number of sequence reads and the VAF (Table 1). It should also be noted if the variant has been deemed as pathogenic using locus-specific databases or international guidelines (e.g., following the American College of Medical Genetics (ACMG) criteria). Furthermore, in the concluding remark of the report, it should be stated if a somatic variant has been detected before in this disease entity that has diagnostic, prognostic, or predictive impact, based on the WHO classification, clinical consensus guidelines, or available published literature. More complex data that includes CNAs and/or structural aberrations will require continued development of bioinformatics tools to visualize the reported data, for example, in the format of copy-number plots or circus plots.

As we enter the precision medicine era, more and more targeted therapies have become available in oncology. Some academic centers and commercial companies have developed new support systems for clinical decision-making that assist in defining if a mutation is considered “actionable” or not. Based on large databases, these tools can, for a certain variant, provide an alert if a clinically relevant finding has been made and provide links to available potential targeted drugs and/or clinical trials (e.g., FDA-approved drugs, ongoing clinical trials, or if a drug has been used for another malignancy). Thus far, these support systems have been mainly used for solid cancer and less frequently in hematological malignancies.

### 4 Concluding Remarks

Genetic diagnostics within hematological malignancies has over the years evolved dramatically from cytogenetics to targeted NGS strategies (Figure 1). For lymphoid malignancies, we apply different FISH analysis as well as targeted sequencing/NGS for diagnostic and risk-stratifying purposes. However, with the ever-increasing
number of clinically relevant genetic variants that are detected in lymphoid malignancies, we need to continue developing "dynamic" NGS-based strategies, such as capture-based sequencing, that include large numbers of genes and types of genetic aberrations. In this regard, a recent study identified seven novel distinct subgroups of DLBCL based on broad genetic characterization. This illustrates the need to develop more comprehensive analyses for diagnostic purposes, also to carry out future precision medicine studies. Furthermore, it is important to develop decision support tools that can inform clinicians on "actionability" for the different genetic events detected.

Within rare disease diagnostics, WGS has increasingly replaced multigene testing. Could this be a way forward also for hematological malignancies where we usually perform multiple testing? Some national and/or regional programs are now testing if WGS combined with RNA-sequencing could replace the "old" technologies in acute leukemia (ALL and AML). In a proof-of-concept study, Klintman et al. demonstrated that WGS and targeted NGS had a high concordance for SNVs/indels in CLL, while the concordance between FISH and WGS was lower. Hence, before we can start to use whole-genome techniques, we have to be certain that all mandatory genomic aberrations can be detected and provided within a reasonable time frame.

Another rapidly evolving area of interest are liquid biopsies and the detection of circulating tumor DNA, which have the potential to enable sensitive NGS-based follow-up of lymphoma patients and can detect genetic aberrations in cases where it is difficult to take a biopsy. These analyses have not yet entered clinical use in lymphoid malignancies, but they are foreseen to be an important part of future diagnostics.

Finally, although there are a few ongoing precision medicine studies in lymphoid malignancies world-wide, it will be very important to initiate future clinical trials based on targeted drugs/immune therapy to realize the full potential of precision medicine.

**CONFLICT OF INTEREST**
Richard Rosenquist has received honoraria from Abbvie, AstraZeneca, Illumina, Janssen and Roche.

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