New Clinical Use of Next-Generation Sequencing

In this issue of the Journal, Schumacher and colleagues\(^1\) describe important principles to consider when applying next-generation sequencing (NGS) for the identification of clonal sequences to support the diagnosis of T-cell neoplasms. NGS has been shown to be clinically useful in identifying germline mutations in hereditary conditions and somatic mutations in numerous cancers. In 2009, one of the first reports of the use of NGS methods to describe the repertoire of TRB gene rearrangements was published.\(^2\) Since then, it has been used to characterize the T-cell repertoire in patients with various diseases. The next application described was the identification of unique sequences of minimal residual disease in acute lymphoblastic leukemia.\(^3,4\) It was a logical step to go from defining a T-cell repertoire to the application of NGS in clonality detection, whether at diagnosis\(^3\) or in follow-up.\(^3,4\)

Technical Issues for NGS of TRG Gene Rearrangements

One must aim for certain goals in preparing unbiased libraries based on polymerase chain reaction (PCR) amplified products of any gene targets. The first desirable goal is to use primers that give proportional representation of the variable (V) and joining (J) region genes that are used in the TRG gene rearrangements. Second, the primers selected and the products produced must have equal amplification efficiencies. Proportional representation for each of the V and J gene groups is largely achieved by the primers selected, but it will be less than 100%, since specific primers for each of the individual group I variable genes were not designed in the set of primers that the authors chose to use.\(^5,6\) This is most evident in the V\(_\gamma\) group 1 genes, which includes variable region genes 2 to 8. To make certain that the V\(_\gamma\)3 was amplified in our original capillary electrophoresis protocol, two primers were designed to cover all genes in group 1. Each gene has variable alignment to these primers. The primers for V\(_\gamma\)3, V\(_\gamma\)9, V\(_\gamma\)10, and V\(_\gamma\)11 are exactly aligned to their own specific TRG sequences. However, the V\(_\gamma\)2 and V\(_\gamma\)3 primers, which will amplify the V\(_\gamma\)2, V\(_\gamma\)4, V\(_\gamma\)5, and V\(_\gamma\)6 genes, do not align exactly with each TRG gene. There are one to four mismatches with these subunits of the known TRG sequence,\(^7\) and the optimal annealing temperature for each primer may be slightly different. This may be reflected in the bias of amplification of biallelic rearrangements in some of the NGS tumor results in Schumacher et al.\(^1\) There is an approximately twofold difference in the percent reads for one gene rearrangement compared with the second rearranged allele, as seen in the following clonal samples: clonal 6, V\(_\gamma\)2 is greater than V\(_\gamma\)5; clonal 11, V\(_\gamma\)10 is greater than V\(_\gamma\)5; clonal 17, V\(_\gamma\)3 is greater than V\(_\gamma\)5; and clonal 19, V\(_\gamma\)3 is greater than V\(_\gamma\)4.

A second reason for amplification bias may be illustrated in the cell line data where the V\(_\gamma\)11 sequence reads exceed the V\(_\gamma\)8 reads. Why is there a twofold difference when both primers align with TRG sequences exactly? Since V\(_\gamma\)11 sequences are rare sequences that occur among polyclonal T cells, there may be a competitive advantage for the V\(_\gamma\)11 primer when amplifying the clonal V\(_\gamma\)11 gene rearrangement. In contrast, the V\(_\gamma\)2 and V\(_\gamma\)3 primers must amplify all the gene rearrangements from the group 1 variable genes (V\(_\gamma\)2-V\(_\gamma\)8), which include V\(_\gamma\)8 among its members.
Understanding the Difference in Result Output Between Capillary Electrophoresis and NGS

Why might there be a difference in the ratio of the peaks compared with the polyclonal background between the capillary electrophoresis and the NGS data in Figure 2 in Schumacher et al? In many examples, the ratio differs by a factor of 2. The authors point out that capillary electrophoresis is based on separation of length; therefore, different products of the same length with a mixture of unique sequences (eg, 191 base pairs) will stack up on each other. In contrast, the same unique TRG sequences are separated into hundreds to thousands of subclusters in NGS, thereby flattening the background. This then yields a higher fold difference between the NGS peak in question and its polyclonal background.

Capillary electrophoresis was actually a step backward from more elegant electrophoresis methods such as denaturing gradient gel electrophoresis, single-strand conformation polymorphism analysis, and temperature gradient gel electrophoresis. Each of those methods separates PCR products by the unique sequences in each rearrangement and not just by the length of the rearrangement alone. However, the reason that capillary electrophoresis has been the main technique in the past decade has been the rapidity of the analysis, since no complex gels need to be poured and the data can be collected electronically. NGS resolves that sequence separation weakness of capillary electrophoresis. It also allows one to develop quantitative rules for analysis that go beyond the contribution of capillary electrophoresis protocols excluded a J\textsubscript{P} primer. In preliminary studies on peripheral blood lymphocytes using another NGS method for TRG gene rearrangements, I have observed that canonical V\textsubscript{9}-J\textsubscript{P} rearrangements can be seen in up to 4.4% of reads in normal peripheral blood, which was 2.3-fold higher than the next sequence. Clinical laboratories should be aware that, depending on the NGS method used and the clinical situation, this canonical V\textsubscript{9}-J\textsubscript{P} rearrangement could be seen in a high enough percentage that may exceed the rule set for a positive NGS clonal result. It would be valuable to the laboratory community if the authors could deposit in GenBank the specific clonal TRG and the high-frequency normal sequences obtained or publish them as supplemental data to aid the community in learning how frequent this canonical V\textsubscript{9}-J\textsubscript{P} rearrangement occurs.

Using NGS Will Not Eliminate All Interpretation Problems

Now lest one think that NGS is a panacea method, medical judgment calls will still occur with the analysis of NGS TRG data. Correlation of the NGS results with the morphology is still needed to define what is a clinically relevant clonal expansion. Thinking will still be necessary in some situations that hinder capillary electrophoresis, such as in the setting of few T cells. Therefore, skin specimens, cerebrospinal fluid specimens, and reconstituting bone marrow, which all often have a relatively limited number of T cells, may produce limited diversity in the NGS data. These types of samples will have to be studied in greater detail to determine the intelligent rule sets necessary to avoid a false-positive diagnosis.

Caution in Defining a Clonal NGS Result

An interesting observation is the relatively frequent finding of a V\textsubscript{9}-J\textsubscript{P} rearrangement at 191 nucleotides (sequence not published) in the 10 clusters seen in eight of the normal patient samples. The displayed NGS sequence output shows quantitatively that no single TRG sequence is significantly greater (defined as >4.5-fold) than any other sequence in the normal samples. However, clonal sample 2 has a V\textsubscript{9}-J\textsubscript{P} sequence containing a read length of 191 nucleotides that has a 4.6-fold ratio with the polyclonal background. It is important to remember that a V\textsubscript{9}-J\textsubscript{P} canonical rearranged sequence (with no insertion or deletion) is seen most frequently in peripheral blood. This can be problematic in capillary electrophoresis assays when V\textsubscript{9} and/or J\textsubscript{P} is amplified in separate amplifications and displayed in separate electropherogram distributions, since it will lead to nonspecific false-positive results. To avoid an interpretation problem, some previous capillary electrophoresis protocols excluded a J\textsubscript{P} primer. In preliminary studies on peripheral blood lymphocytes using another NGS method for TRG gene rearrangements, I have observed that canonical V\textsubscript{9}-J\textsubscript{P} rearrangements can be seen in up to 4.4% of reads in normal peripheral blood, which was 2.3-fold higher than the next sequence. Clinical laboratories should be aware that, depending on the NGS method used and the clinical situation, this canonical V\textsubscript{9}-J\textsubscript{P} rearrangement could be seen in a high enough percentage that may exceed the rule set for a positive NGS clonal result. It would be valuable to the laboratory community if the authors could deposit in GenBank the specific clonal TRG and the high-frequency normal sequences obtained or publish them as supplemental data to aid the community in learning how frequent this canonical V\textsubscript{9}-J\textsubscript{P} rearrangement occurs.

Conclusion

Schumacher et al have provided an important contribution in adopting an NGS method in characterizing clonal TRG sequences for the diagnosis of T-cell neoplasms.

References


