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COMMENTARY

Technical Issues Behind Molecular Monitoring in Chronic Myeloid Leukemia

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Clinical papers frequently lack in-depth descriptions of analytical methods, triggering possible doubts regarding their conclusions. Through this short communication, we would like to stimulate a debate on the monitoring of chronic myeloid leukemia, a disorder that requires frequent laboratory analysis by RNA reverse transcription and real-time quantitative PCR (RQ-PCR). This is a challenging procedure influenced by a number of factors that are difficult to control [1, 2], and troubled by the need to use reference materials to standardize results against the so-called international scale (IS) [3]. The residual disease is evaluated through the amount of BCR-ABL1 transcript, and a patient's response is reported in terms of IS logarithmic reductions, up to the possible condition of apparent undetectable disease [2]. Actually, the outcome of RQ-PCR represents only an estimation of the leukemic clone based on the assumed proportionality between chimeric RNA and leukemic cells. However, it is not clear if this proportionality is still valid at very high response levels, and negative results can be interpreted in a number of ways, e.g., the true absence of leukemic cells, the presence of leukemic cells that do not transcribe chimeric RNA [4], or measurements below the detection limit. Really, an accurate determination of the detection limit is not feasible since specimen handling, RNA extraction and retro-transcription are critical pre-analytical steps (due to in vitro RNA instability), and it is practically impossible to characterize the whole process. Even positive (but barely detectable) results are difficult to interpret because of the intrinsic variability of gene expression: patients with few leukemic cells can give significantly different results, depending on small variations in the mutual transcription levels of BCR-ABL1 and the reference gene used as a normalizer (usually ABL1, BCR, or GUSB [1]). As a consequence, a clear definition of molecular response is still under elaboration, though the potential for drug discontinuation makes this subject a pressing issue [2]. A survey conducted by the College of American Pathologists pointed out that diverse laboratories testing the same sample obtained different results [1].

Pilot methods based on DNA analysis have also been introduced. These methods require the sequence of the BCR-ABL1 junction for each patient, but have the advantage, of relying on a direct and univocal relationship between cell number and breakpoints (i.e., similar to cytogenetic, but with a higher sensitivity); thus an algorithm can be used to calculate the actual percentage of leukemic cells without the need for standards and reference materials [5]. However, the need to retrieve the breakpoint of each patient affects the routine application of this practice. Recent studies [6] also argue that chronic myeloid leukemia patients (even those with undetectable levels of chimeric RNA) maintain evidence of the BCR-ABL1 DNA, implying its limitations as a prognostic marker. The procedure used to prove this claim consisted of a nested PCR on genomic DNA and a high number of replicates to enhance the chance of detecting the target sequence. This method is frequently used [7, 8], but is prone to false positive results, and a thorough technical discussion would

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be necessary. In analytical chemistry, limit of detection/limit of quantification (LOD/LOQ) and accuracy are distinct subjects [9]. In general, increasing replicates ameliorate the accuracy, but if the single result is below the LOD/LOQ, the outcome is undetectable/impossible to measure, and repeating the test has no effects. Thus, the approach to lower the LOD/LOQ by increasing the number of replicates is scientifically flawed. These limits could be lowered only by improving the PCR sensitivity or increasing the amount of template of the single reaction. For instance, assuming a PCR sensitivity up to the single breakpoint sequence, the LOD in 50 ng DNA would be $\sim 0.0001\,\%$ (1 leukemic cell/8800 cells, considering 5.7 pg DNA/cell). This limit could be lowered to 0.00001 % analyzing 500 ng DNA (if no inhibition occurs), but not analyzing 10×50 ng samples. Additionally, if replicate results are spanning the LOD (i.e., partially positive and negative), the outcome is uncertain and should be rejected. The real values are higher than the theoretical limits discussed above, and should be experimentally established in order to set acceptance criteria ahead of any clinical interpretation.

In conclusion, molecular monitoring by RNA analysis is the recommended procedure for chronic myeloid leukemia, but clinicians should be aware of the factors that affect this practice. The use of DNA measurements requires even more critical scrutiny. An in-depth understanding of the laboratory methods is necessary for the correct reading of the results, in particular, if the outcome of the residual disease monitoring is used as a prognostic indication for therapy modulation or possible drug discontinuation. We hope that a critical discussion about the abovementioned technical issues may be of value in the management of chronic myeloid leukemia patients.

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