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Droplet Digital PCR Is a Reliable Tool for Monitoring Minimal Residual Disease in Acute Promyelocytic Leukemia

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Nested PCR (nPCR) and real-time quantitative PCR (qPCR) are well-established methods for monitoring minimal residual disease (MRD) in acute promyelocytic leukemia (APL). Despite their remarkable sensitivity and specificity, both methods have inherent limitations, such as qualitative MRD evaluation and relative quantification. Herein, we used droplet digital PCR (ddPCR) to monitor MRD in 21 APL patients and compared its performance with nPCR and qPCR. After assessing the limit of detection (LOD) for each technique on serial dilutions of *PML-RARA* bcr1 and bcr3 transcripts, a total of 48 follow-up samples were analyzed and the results compared. ddPCR showed good linearity and efficiency and reached an LOD comparable or even superior to nPCR and qPCR. When tested on primary samples, ddPCR exhibited a sensitivity and specificity of ≥95% and ≥91% for bcr1 and bcr3 transcripts and displayed a significant concordance with both techniques, particularly with nPCR. The peculiar advantage of ddPCR-based monitoring of MRD is represented by absolute quantification, which provides crucial information for the management of patients whose MRD fluctuates under the LOD of qPCR and is detectable, but not quantifiable, by nPCR. Our findings highlight ddPCR as a reliable complementary approach to monitor MRD in APL, and suggest its advantageous application, particularly for the molecular follow-up of patients at high risk of relapse. *(J Mol Diagn 2017, ■: 1–8; http://dx.doi.org/10.1016/j.jmoldx.2017.01.004)*

Q8 Acute promyelocytic leukemia (APL) is a rare hematological malignancy commonly associated with the chromosomal translocation t(15;17)(q24;q21), which involves the promyelocytic leukemia (*PML*) and the retinoic acid receptor-α (*RARA*) genes, resulting in the oncogenic fusion transcript *PML-RARA*.[1] Although the breakpoints on chromosome 17 are localized within a 17-kb fragment of the *RARA* intron 2, up to three regions of the *PML* locus may be involved in the translocation: intron 6, exon 6, and intron 3, accounting for 55%, 5%, and 40% of cases, respectively. The different breakpoints lead to three possible *PML-RARA* isoforms, referred to as long (L or bcr1), variant (V or bcr2), and short (S or bcr3).[2] Notably, the bcr3 isoform is associated with Q9 two well-established adverse prognostic factors (ie, higher white cell counts and the M3 variant morphology).[3]

Current treatment is highly successful, leading to long-term remission and possibly the cure for approximately 70% of newly diagnosed patients.[4–8] However, a small group of patients are at particular risk of relapse, which is not predictable on the basis of clinical parameters, and may potentially benefit from an early assessment of the minimal residual disease (MRD).[2,3,9–13] For this reason, the detection of the *PML-RARA* transcript, performed at the post-consolidation phase, provides an independent prognostic factor in APL.[14,15]

PML-RARA amplification by qualitative RT-PCR is the method most commonly used to confirm the morphological

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diagnosis of APL and is essential for defining the *PML* breakpoint location and establishing the target for reliable molecular monitoring.[16] Nested RT-PCR (nPCR) was widely used for MRD evaluation, despite the disadvantage of providing unreliable results (ie, MRD positivity even in long-term remission patients who never experience a further hematological relapse).[17] This limitation, together with the need for a precise quantification of the transcript, led to the introduction of real-time quantitative PCR (qPCR), which is now the method generally used to monitor MRD in APL.[18] qPCR offers several advantages compared to nPCR, such as a higher sensitivity, reduced risk of contamination,[19] and the possibility of monitoring the quality of samples (by the amplification of a housekeeping gene) and following the disease kinetics.[6,20] The major limitation of qPCR is represented by relative quantification, and most important, an inadequate quantification of samples that have a tumor burden between the sensitivity threshold and the quantitative range of the technique.[21]

Nanoliter-sized droplet technology paired with digital PCR (ddPCR) is a direct method for the precise and absolute quantification of nucleic acids, based on limiting partition of the PCR volume and on Poisson statistics.[22,23] Being independent of a reference standard curve and allowing for high-sensitive absolute quantification of the target, ddPCR could have a high potential in monitoring MRD.

Herein, we investigate whether ddPCR could overcome some of the above-mentioned limitations of nPCR and qPCR. We, therefore, compared the performances of these three techniques both on reference dilutions and primary samples, to seek for the most appropriate and reliable technology for the molecular monitoring of MRD in APL.

Materials and Methods

Patients

The APL patients included in this report were treated according to the AIDA2000 GIMEMA group protocol.[24] The diagnosis was initially morphological and was confirmed by detection of the *PML-RARA* fusion gene, as reported.[16] ddPCR was compared head-to-head with nPCR and qPCR in 21 patients, 11 bearing the bcr1 transcript and 10 bearing the bcr3 transcript, for a total of 48 follow-up samples. Patients with at least 12 months of follow-up from the end of consolidation therapy were selected, and grouped on the basis of their relapse risk score (Sanz score) and disease course. The bcr1-positive patients were assigned either to the complete hematological remission (CHR; $n = 5$) or to the molecular or hematological relapse/relapse-risk group (R/RR; $n = 6$). Similarly, four and six bcr3-positive patients were assigned to the CHR and the relapse (R) group, respectively.

The study protocol was approved by the local ethics committee, and all patients provided written informed consent to take part in the study.

RNA Extraction

Technique performances were evaluated on serial dilutions of cell lines and patients' bone marrow RNA. NB4 and HL60 cell lines (DSMZ, Braunschweig, Germany) were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% antibiotics (penicillin/streptomycin; 100 U/mL) (all from EuroClone S.p.A., Milan, Italy) in a 5% CO_2-enriched atmosphere at 37°C. RNA was extracted with the Qiacube automated extraction system using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified by a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). To generate proper standard curves, 1 μg of RNA from NB4 cells, which have the *PML* breakpoint in the bcr1 region, or 1 μg of RNA from a primary sample, with the *PML* breakpoint in the bcr3 region, was serially 10-fold diluted in RNA from HL-60 cells (negative for both the transcripts). Each sample was reverse-transcribed using the QuantiTect reverse transcription kit (Qiagen) in triplicates, and triplicates were pooled before molecular analyses. To minimize possible biases related to sampling, all PCR experiments were performed on the same cDNA, after pooling the reverse-transcribed triplicates.

nPCR, qPCR, and ddPCR

nPCR was performed according to the BIOMED-1 concerted action report.[16] Only samples showing amplification of the housekeeping gene β-actin (*ACTB*) were further investigated for the presence of the transcript of interest. The first round of nPCR was performed on 100 ng of cDNA, whereas the second round was conducted on 1 μL of the first round reaction, in triplicates. Samples displaying nPCR positivity were further analyzed by Sanger sequencing of the amplification product, to confirm the presence of the rearrangement.

qPCR was performed on a LightCycler II 480 system (Roche Diagnostics, Monza, Italy), with the Ipsogen *PML-RARA* bcr1 and bcr3 IVD kits (Qiagen). MRD estimation was based on five plasmid 10-fold standard dilutions for the bcr1 and bcr3 transcripts, and on three plasmid standard dilutions for the ABL proto-oncogene 1 *(ABL1)* control gene. MRD analysis was conducted starting from 100 ng of cDNA in duplicates, and results were interpreted according to the manufacturer's instructions. In particular, samples with an ABL1 copy number <1318 were classified as not analyzable and excluded from further analyses.

ddPCR for both *PML-RARA* bcr1 and bcr3 isoforms was performed with primers and probes, as previously described.[25] Briefly, glucuronidase β (*GUSB*) was used to assess the quality of cDNA samples; in addition, because of the need to test a large amount of material, *PML-RARA* and GUSB transcripts were tested in separate reactions. *PML-RARA* primers and probes were used at the final concentrations of 900 and 250 nmol/L, respectively, and 200 ng of cDNA template was used in a final volume of

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