Minimal residual disease monitoring in acute myeloid leukemia with non-A/B/D *NPM1* mutations by digital polymerase chain reaction: feasibility and clinical use

In patients with acute myeloid leukemia (AML), treatment stratification is primarily based on pre-therapeutic factors, including cytogenetic and molecular aberrations and measurable/minimal residual disease (MRD) during treatment.[1] Sequential MRD monitoring allows for assessment of the response to chemotherapy and early detection of relapses, possibly identifying patients who need pre-emptive or more intensive therapy.[2] In clinical practice, MRD monitoring is based on molecular real-time quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) and/or flow cytometry.[3] Currently available molecular markers are basically represented by fusion transcripts (especially *CBFB-MYH11, RUNX1-RUNX1T1* and *PML-RARA*)[4,5] or mutations, mainly *NPM1* mutations.[6] However, 60 to 70% of AML patients lack these leukemia-specific MRD targets and their samples are not informative for MRD detection by RT-qPCR.[3] Additionally, RT-qPCR assays require the generation of standard curves covering the cycling threshold range of patients' samples to ensure the linearity of the assay at the measured MRD level. This implies the maintenance of plasmid standards for each molecular target, limiting widespread use of this technique for rare markers in clinical practice. In this context, digital polymerase chain reaction (dPCR) is a promising approach to validate new MRD markers in AML patients. dPCR provides absolute quantification of nucleic acid target sequences with high sensitivity. Notably, it avoids the absolute quantification of plasmid standards and the pitfalls associated with variations in reaction efficiencies (e.g., number of technical replicates performed, effect of the volume transferred throughout the dilution series).[7] This makes dPCR more convenient for quantifying rare molecular markers and an accurate alternative method for monitoring MRD. Briefly, the sample is divided into thousands of partitions (wells or droplets depending on the technology) containing amplification reagents in which the targets are randomly distributed. Each partition is analyzed and classified in a positive or negative category depending on the initial presence of the target. The absolute quantification is then estimated by modeling the measured number of positive fractions as a Poisson distribution model that estimates how many compartments contained one, two or more targets before amplification.

NPM1 mutations are one of the most frequent genetic abnormalities in adult AML, being detected in approximately 35% of all patients with AML and in 50 to 60% of those with cytogenetically normal AML, in whom they are a major prognostic factor.[1,8] Since their discovery in 2005, more than 50 different mutations located in exon 11 of *NPM1* have been identified.[9] Type A (c.860_863dupTCTG), B (c.863_864insCATG) and D (c.863_864insCCTG) mutations predominate in approximately 90% of *NPM1*-mutated AML patients.[10] While RT-qPCR could be effectively used to monitor all *NPM1*-mutated transcripts,[11,12] in clinical practice, RT-qPCR analysis is mostly restricted to type A, B and D mutations for which commercial plasmid standards are available. Recently, in a study focused on *NPM1*-type A, B or D mutation quantification by RT-qPCR, the Acute Leukemia French Association (ALFA) group supported the strong prognostic significance of post-induction *NPM1*-based MRD on outcome, independently of additional molecular or cytogenetic aberrations.[6] Patients who did not achieve a 4-log reduction (poor responders) in *NPM1*-based MRD in peripheral blood were shown to have a higher cumulative incidence of relapse and shorter survival. Additionally, *NPM1*-based MRD was shown to be a good predictive factor for the indication of allogeneic stem cell transplantation in poor responders.

The purpose of the present study was to define the

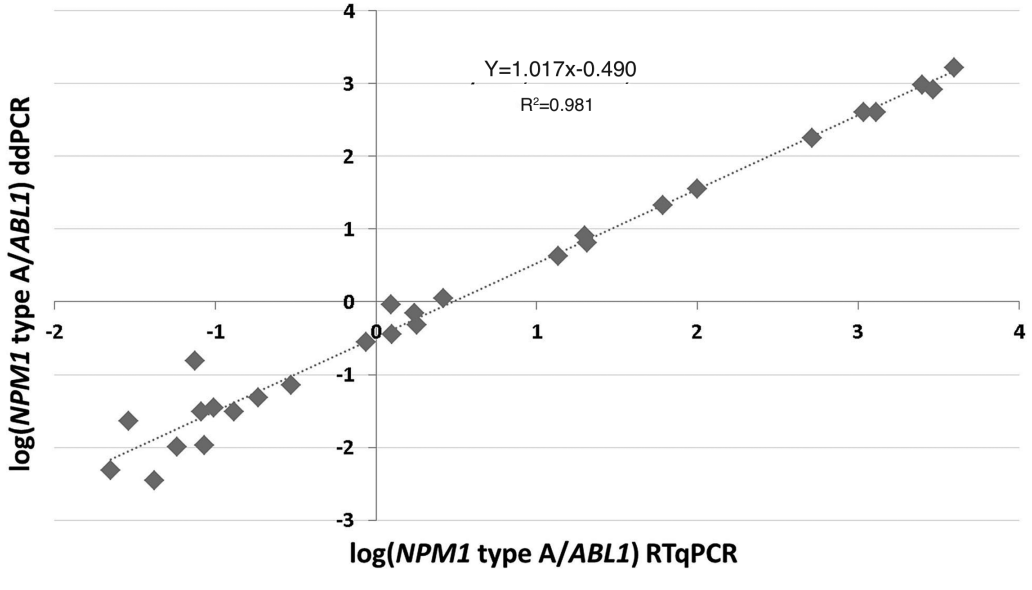

Figure 1. Correlation between the quantification of levels of *NPM1* type A mutation transcripts determined by real-time quantitative reverse transcriptase-polymerase chain reaction and droplet digital polymerase chain reaction. *NPM1* type A mutation transcript levels were quantified in samples from 28 patients with acute myeloid leukemia using both real-time quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) with a TaqMan chemistry assay and a droplet digital polymerase chain reaction (ddPCR) assay. The correlation between the ddPCR and RT-qPCR results was assessed using least squares regression after logarithmic transformation.

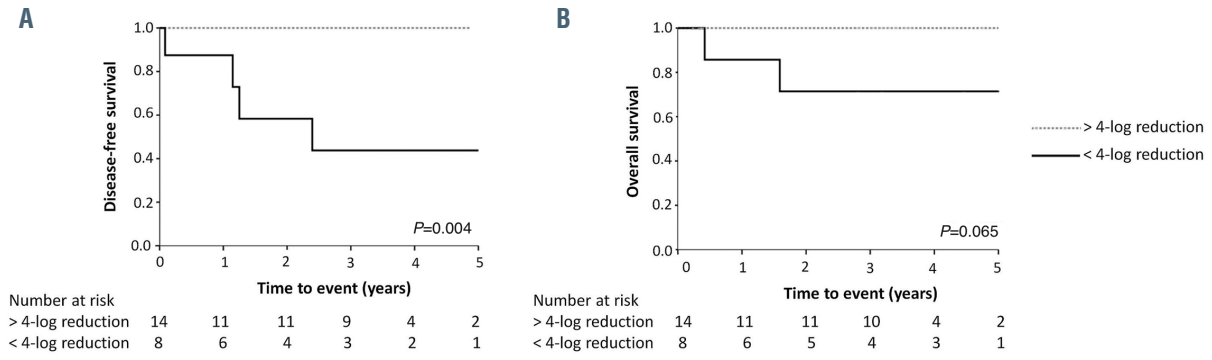

Figure 2. Outcomes according to *NPM1*-based minimal residual disease. (A) Disease-free survival and (B) overall survival in acute myeloid leukemia patients with rare *NPM1* mutations according to post-induction log reduction in minimal residual disease in peripheral blood (<4-log reduction or >4-log reduction). Disease-free and overall survival were censored at allogeneic stem cell transplantation.

ability of droplet dPCR to quantify non-A/B/D *NPM1* mutations and to retrospectively evaluate the prognostic impact of post-induction *NPM1*-based MRD in AML patients with a "rare" *NPM1* mutation (i.e. non-A/B/D) enrolled in the ALFA-0702 trial.

From March 2009 to September 2013, 713 patients aged 18–59 years old with *de novo* AML were included in the phase II randomized multicenter ALFA-0702 trial (Eudra-CT, 2008-000668-18; ClinicalTrials.gov, NCT00932412).[13] The study was approved in December 2008 by the Institutional Review Board of the French Regulatory Agency and the Ethics Committee Sud-Est IV, France. All patients gave informed consent for both treatment and genetic analyses, according to the Declaration of Helsinki. *NPM1* mutations and *FLT3*-internal tandem duplications (ITD) were determined by fragment analysis and Sanger sequencing as part of the patients' care.[13] Overall, *NPM1*-mutated AML accounted for 36% of the cases of AML (234 patients), of which 13% (31 patients) had non-A/B/D mutations. Among them, 22 patients with available RNA-complementary DNA (cDNA) extracted from peripheral blood after induction therapy were selected for this study. Additionally, 28 AML patients with a *NPM1*-type A mutation were selected to compare the performance of droplet dPCR *versus* standard RT-qPCR performed as previously described.[6]

Peripheral blood samples were collected at diagnosis and after induction (MRD1) for patients in complete remission. Droplet dPCR was performed on cDNA using the Bio-Rad QX200™ droplet dPCR system with FAM- and HEX-labeled probes (*Online Supplementary Figure S1, Online Supplementary Table S1*). *NPM1* mutations and *ABL1* transcripts were quantified in multiplex. Each sample was partitioned into 20,000 uniform droplets allowing a random distribution of the target cDNA. End-point PCR amplification of the nucleic acid target was carried out within each droplet using the high-performance T100™ Thermal Cycler (Bio-Rad). PCR products were then subjected to the QX200 Droplet Reader (Bio-Rad), which measures the fluorescence of each droplet using a two-color detection system. Raw data were analyzed using QuantaSoft™ software (Bio-Rad). Data were shown as a one-dimension plot with each droplet from a sample plotted on the graph of fluorescence intensity *versus* droplet number. The fraction of positive droplets was then estimated using a Poisson distribution model. Assays performed to optimize and validate the quantification of *NPM1*-mutated transcript levels are described in the *Online Supplementary Appendix* (*Online Supplementary Methods, Online Supplementary Figures S2-S7, Online Supplementary Tables S2-S4*). For statistical analyses, overall survival and disease-free survival were estimated by the Kaplan-Meier method and compared by cause-specific hazard Cox models. Overall survival was measured from the date of diagnosis until death from any cause. Disease-free survival was measured from the date of complete remission until the date of relapse. Patients were censored at the time of allogeneic stem cell transplantation in first remission. A *P*-value <0.05 was considered statistically significant.

NPM1-type A mutation transcript levels in 28 AML samples were quantified using both RT-qPCR and droplet dPCR and produced concordant results, showing that dPCR could be considered as an alternative for monitoring type A mutations[3] (Figure 1). Subsequently, 22 AML patients enrolled in the ALFA-0702 trial who achieved complete remission and harbored 16 different rare *NPM1* mutations were studied by droplet dPCR (*Online Supplementary Table S5*). Although the number of subjects was very small, AML patients with rare *NPM1* mutations who did not achieve a 4-log reduction of *NPM1*-based MRD in peripheral blood had a significantly shorter disease-free survival (3-year disease-free survival: 43.8% *vs.* 100%; *P*=0.004) (Figure 2A) as described for classical *NPM1*-type A, B and D mutations.[6] The difference did not reach statistical significance for overall survival (3-year overall survival: 71.4% *vs.* 100%; *P*=0.065) (Figure 2B), perhaps due to low numbers. *FLT3*-ITD was found in one poor responder (ratio 0.95) and three good responders (ratios 0.4, 0.4 and 1.0). Interestingly, some studies have found that rare *NPM1* mutations (i.e., non-A/B/D) have different clinical or biological behaviors compared to classical *NPM1* mutations (i.e., type A, B or D).[10,14] This could result from different amino-acid substitutions or accompanying alterations in commonly mutated genes such as *FLT3, DNMT3A* or *IDH1/IDH2*. In current practice, it can be assumed that the ability to monitor classical *NPM1* mutations by RT-qPCR in most laboratories could lead to earlier detection of relapses, better selection of patients with an indication for allogeneic stem cell transplantation, and easier administration of pre-emptive therapy. Although focused on a small subgroup of patients, our results extend those previously published by the ALFA group[6] and suggest that a post-induction

NPM1-based MRD log reduction in peripheral blood greater than 4-logs defines a group of patients with a very low risk of relapse when treated with chemotherapy alone whatever the type of *NPM1* mutation. Additionally, this study highlights the robustness and accuracy of dPCR for detecting MRD in patients for whom standard MRD markers are not available.[15] The dPCR assay reliably detected five copies of mutated *NPM1* transcript (the limit-of-detection assay was performed with type A, B and D transcripts). The detection limit was therefore 0.01% for a sample containing 50,000 copies of the housekeeping *ABL1* gene, which is equivalent to the RT-qPCR assay used in most laboratories. Thus, dPCR could be informative for the early detection of relapses and be used for MRD follow-up in patients. Considering its sensitivity and ease of use (especially the absolute quantification without the need for standard curves), dPCR may represent an alternative method equivalent to RT-qPCR for MRD monitoring of classical *NPM1*-type A, B and D mutations.

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doi:10.3324/haematol.2020.260133

Received: May 20, 2020.

Accepted: November 16, 2020.

Pre-published: December 10, 2020.

Disclosures: no conflicts of interest to disclose.

Contributions: AL, ON, LF, EF, CPr and ND performed the molecular analyses; AL and ON designed the experiments; XT was the principal investigator of the ALFA-0702 trial; KCL ensured data management; XT, NB, SdB, ER, CR, JL, CB, AP, RI, PT, CPa, JV and HD provided samples and clinical data. AL and ND wrote the manuscript which was approved by all the authors.

Acknowledgments: the authors thank all Acute Leukemia French Association (ALFA) investigators and French Innovative Leukemia Organization (FILO) investigators who participated in the ALFA-0702 trial. The authors also thank Christophe Roumier and the tumor bank of Lille University Hospital (certification NF 96900-2014/65453-1) for handling, conditioning, and storing patients' samples. The work of all clinical research assistants is also acknowledged here.

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