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A novel deep targeted sequencing method for minimal residual disease monitoring in acute myeloid leukemia

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ABSTRACT

A high proportion of patients with acute myeloid leukemia who achieve minimal residual disease negative status ultimately relapse because a fraction of pathological clones remains undetected by standard methods. We designed and validated a high-throughput sequencing method for minimal residual disease assessment of cell clonotypes with mutations of *NPM1*, *IDH1/2* and/or *FLT3*-single nucleotide variants. For clinical validation, 106 follow-up samples from 63 patients in complete remission were studied by sequencing, evaluating the level of mutations detected at diagnosis. The predictive value of minimal residual disease status by sequencing, multiparameter flow cytometry, or quantitative polymerase chain reaction analysis was determined by survival analysis. The sequencing method achieved a sensitivity of 10^{-4} for single nucleotide variants and 10^{-5} for insertions/deletions and could be used in acute myeloid leukemia patients who carry any mutation (86% in our diagnostic data set). Sequencing–determined minimal residual disease positive status was associated with lower disease-free survival (hazard ratio 3.4, P=0.005) and lower overall survival (hazard ratio 4.2, P<0.001). Multivariate analysis showed that minimal residual disease positive status determined by sequencing was an independent factor associated with risk of death (hazard ratio 4.54, P=0.005) and the only independent factor conferring risk of relapse (hazard ratio 3.76, P=0.012). This sequencing-based method simplifies and standardizes minimal residual disease evaluation, with high applicability in acute myeloid leukemia. It is also an improvement upon flow cytometry- and quantitative polymerase chain reaction-based prediction of outcomes of patients with acute myeloid leukemia and could be incorporated in clinical settings and clinical trials.

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Introduction

Cytogenetic and molecular alterations at diagnosis and response to treatment are the most useful criteria for predicting the relative risk of relapse in acute myeloid leukemia (AML), and for guiding the choice between chemotherapy and

hematopoietic stem cell transplantation in first complete remission.[1] The definition of complete remission for AML includes criteria for the identification of patients with poor prognosis using cytomorphological methods,[2] but these studies do not have a good predictive value because most of the patients in complete remission relapse within 3 years of diagnosis.[3]

Assessment of minimal residual disease (MRD) is critical in monitoring patients in morphological remission, to inform decisions about further therapy.[1] Indeed, several studies have reported MRD status as a stronger predictor of relapse, because patients who are MRD negative have a better prognosis than those who are MRD positive.[4,5] In support of this, recent non-randomized studies from prospective multicenter trials suggested better outcomes when leukemia therapy was selected based on the results of MRD assessment.[6-8]

AML is, nevertheless, a biologically complex and heterogeneous disease, which makes MRD testing more challenging in this condition than in other hematologic neoplasms such as acute lymphoblastic leukemia or multiple myeloma. The detection of very low levels of MRD by conventional methods such as quantitative (q) polymerase chain reaction (PCR) or multiparameter flow cytometry (MFC) provides powerful independent prognostic information. Unfortunately, as described for cytomorphological complete remission, many patients who achieve MRD negative status relapse as a result of the progression of undetected leukemic cells. The most common method for MRD detection is MFC, which has intermediate applicability (70–80%) and limited sensitivity.[9,10] However, there is no consensus on multi-antibody panels with regards to inter-laboratory performance, and the technique requires a high level of expertise. The other principal method for monitoring MRD, qPCR, has good sensitivity (10^{-4}-10^{-6}), but its applicability is limited to the approximately 40% of patients who present with molecular alterations (*RUNX1-RUNX1T1*, *CBF*β-*MYH11* or *NPM1*) at diagnosis.[11]

For the above reasons, new methods with higher sensitivity, specificity, applicability and performance are needed for MRD assessment in AML. Against this background, next-generation sequencing (NGS) and digital PCR (dPCR) have recently emerged as potentially promising platforms for the assessment of MRD.[12] Here, we optimized and clinically validated a new deep targeted NGS-based method, supported with dPCR technical validation, for the detection and quantification of MRD [both small insertion/deletions (indels) and single nucleotide variants (SNV)] in AML patients, in an attempt to improve and/or complement the current techniques for MRD evaluation, and to establish its potential as a predictor of patients' outcome.

Methods

More detailed information can be found in the *Online Supplementary Data (1–6)*.

Patients and samples

One hundred and ninety patients with *de novo* or secondary non–M3 AML were included in mutational profile screening at diagnosis. We performed a new selection for retrospective MRD assessment using the following criteria: presence of the *NPM1* type A mutation, or SNV in *FLT3*, *IDH1* and/or *IDH2* at diagnosis, and availability of at least one follow-up genomic (g)-DNA sample.

The MRD approach was applied to 51 (48%) follow-up samples taken after induction therapy and 55 (52%) taken after consolidation, corresponding to 63 patients diagnosed between 2006 and 2016 (for selection criteria see *Online Supplement 6 and Supplementary Table S1*). Patients were treated according to PETHEMA (*Programa Español de Tratamientos en Hematología*) or CETLAM (*Grupo cooperativo de Estudio y Tratamiento de Leucemias Agudas y Mielodisplasias*) protocols. The study was conducted according to Spanish law 14/2007 on biomedical research, and was approved by the Research Ethics Board of each participating institution. All patients provided informed consent. The main clinical characteristics of the patients are summarized in Table 1. All patients achieved complete remission according to cytomorphological criteria after induction therapy (<5% of bone marrow blasts).

To construct calibration curves, commercial (Horizon Discovery, UK) reference standard gDNA was used for somatic SNV in *IDH1* (R132C) and *IDH2* (R172K). As a further source of gDNA, we used the OCI-AML3 cell line (ACC 582, DSMZ, Germany) with the *NPM1* type A mutation (c.863_864insCCTG) to examine indels. As OCI-AML3 cells also present a SNV in *DNMT3A* (R882C), this was included only for technical optimization.

Deep targeted sequencing workflow

The sequencing workflow included a first study at diagnosis and a second study at follow-up. Mutational profile screening at diagnosis was done with a customized NGS myeloid panel of 32 genes frequently mutated in myeloid diseases,[13] (*Online Supplementary Table S2*) and *NPM1* analysis was carried out with qPCR.[14]

The specific mutations detected at diagnosis were studied at follow-up. We first tested a variety of experimental steps to define optimal conditions (*Online Supplement 1*). We established an optimal protocol (Figure 1) that included DNA amplification, library preparation and sequencing as experimental steps (*Online Supplement 2*).

Libraries were sequenced on the Ion Proton System platform (Life Technologies, Thermo Fisher Scientific Inc.) with an estimated depth ≥1,000,000 of reads, generating .fastq files. These files were analyzed using a customized bioinformatic pipeline; which leads from the .fastq file and a .csv file that contains information about name identifier, run and barcode identifier, chromosomal position and the variant detected in the diagnosis to be evaluated in the follow-up sample. Through Ensembl Perl API,[15] the aligned mutated sequence and the aligned wild-type (wt) sequence are presented in FASTA format (sequences of 40 bp). Finally, we obtained a .csv file containing the name identifier, run and barcode identifier, chromosomal position, the variant, the specific target sequence in FASTA format (mutated forward, mutated reverse, wt forward and wt reverse), the counts of each and the ratio (mutated/wt) in absolute values.

Results

A high percentage of acute myeloid leukemia patients could benefit from deep sequencing minimal residual disease assessment

In total, 211 (80%) SNV and 46 (20%) indels were detected in the 190 patients analyzed at diagnosis using the customized NGS panel. We detected one variant (SNV or indel) in 48 (25%) cases, two or more variants in 116

(61%) cases and no variants in 26 (14%) cases. In addition, we detected the *NPM1* type A mutation in 53 (28%) patients by qPCR. Genes (*TET2*, *ASXL1*, or *DNMT3A*) with evidence of an association with clonal hematopoiesis of indeterminate potential (CHIP) were excluded from the analysis.[11] Consequently, 82% of patients in our cohort could benefit from this approach.

Based on those genes reported as potential markers for monitoring MRD,[16] and also the availability of follow-up samples, we focused on *IDH1/2* and *FLT3*-SNV. We identified at diagnosis *IDH1* mutations in 13 patients (7%), *IDH2* mutations in 27 patients (14%) and *FLT3*-SNV mutations (18%) in 34 patients.

Deep sequencing minimal residual disease assessment has a sensitivity of 10^{-4} for single nucleotide variants and 10^{-5} for insertions-deletions

To establish the limit of quantification (LOQ) of the method, we used 10-fold serial dilutions of mixed mutated and control DNA. To study prototype indels, we used gDNA from OCI-AML3 cells (*NPM1* type A) and to study prototype SNV, we used both gDNA from OCI-AML3 cells (*DNMT3A*) and commercial reference gDNA (*IDH1/IDH2*). As a control, we used a pool of gDNA from ten individuals without somatic mutations in these chromosomal regions. In all cases, initial allele frequency was 50% and a total of six dilutions were tested to construct a calibration curve, covering a theoretical dynamic range from 10^{-1} to 10^{-7}.

As shown in Figure 2A,B, MRD NGS testing of *NPM1* (indel) could quantify one mutated cell in the order of 10^{-5} normal ones and in the case of SNV (*IDH1*, *IDH2* and *DNMT3A*) the LOQ was 10^{-4}, which was reproducible for all SNV tested.

Next-generation sequencing is more sensitive than digital polymerase chain reaction analysis for minimal residual disease assessment

We compared the sensitivity of the sequencing method with that of dPCR using the same LOQ dilution protocol. Clone frequency expressed as target concentration (mutated copies/μL in wt copies/μL) gradually decreased with each dilution, reaching a LOQ of 10^{-3} for *NPM1*, *IDH1* and *IDH2* (Figure 2C,D). While both methods showed similar detection limits and good linearity, the LOQ for the sequencing method was one order of magnitude higher than that for dPCR (*IDH1* and *IDH2*), and two orders of magnitude higher for indels (*NMP1*).

Minimal residual disease status assessed by sequencing has prognosis value in acute myeloid leukemia

The median depth coverage was 401,300 aligned reads (interquartile range 195,100–825,700) for the 88 *NPM1* and 18 SNV (9 *IDH1*, 7 *IDH2*, and 2 *FLT3*) follow-up samples evaluated. We detected no mutated sequence in 13 (12%) samples, one to five mutated sequences in 19 (18%) samples, and more than ten in 74 (70%) samples. The ratio of mutated sequences to wt sequences defined MRD levels. Considering MRD levels from the 106 samples evaluated we established the optimal cutoff to classify MRD status (positive *versus* negative) by receiver operating characteristic curves (*Online Supplementary Figure S1*) at each check-point of MRD evaluation [post-induction (n=51), post-consolidation (n=55), or both together (n=106)].

Survival analysis revealed that positive MRD status (MRD levels >0.1%) after induction (n=35) was associated with a significantly lower rate of overall survival [33% *versus* 78%; hazard ratio (HR): 3.5; 95% confidence interval (CI): 1.1–10.7; *P*=0.019], but a non-significant lower rate of disease-free survival (58% *versus* 78%; HR: 2.18; 95% CI: 0.63–7.5; *P*=0.208) (Figure 3A,B). In post-consolidation samples (n=28), MRD positive status

Table 1. Main characteristics of the patients with acute myeloid leukemia included in the minimal residual disease study.

Patients (n = 63)	
Follow-up sample type	
Bone marrow	58 (92%)
Peripheral blood	5 (8%)
Sex	
Male	21 (33%)
Female	42 (67%)
Age at diagnosis, median	54 (IQR, 41.5–66.0)
Blasts at diagnosis, median count	69 (IQR, 51.0–81.0)
Leukocytes at diagnosis median count ($\times 10^9$/L)	15.7 (IQR,12.2–20.24)
Secondary AML	
No	59 (94%)
Yes	4 (6%)
Cytogenetic risk	
Favorable	25 (40%)
Intermediate	36 (57%)
Adverse	2 (3%)
FLT3-ITD	
FLT3 negative	49 (78%)
FLT3 positive	14 (22%)
FLT3-TKD	
FLT3 negative	60 (95%)
FLT3 positive	3 (5%)
NPM1	
NPM1 negative	6 (10%)
NPM1 positive	57 (90%)
Hematopoietic stem cell transplantation	
No	42 (67%)
Allogeneic	7 (11%)
Autologous	14 (22%)
Relapse	
No	42 (67%)
Yes	21 (33%)
Death	
No	40 (63%)
Yes	23 (37%)
Treatment*	
3+7 regimen	50 (80%)
Flugaza	8 (13%)
Mylotarg	2 (3%)
Panobidara	3 (4%)

AML: acute myeloid leukemia; ITD: internal tandem duplications; TKD: tyrosine kinase domain; *3+7 regimen of chemotherapy: one or two induction cycles of cytarabine and idarubicin for 7 and 3 days, respectively; and two or three consolidation cycles of high doses of cytarabine, twice a day for 3 alternate days followed by allogeneic or autologous hematopoietic stem cell transplantation. The remainder of the patients were included in other clinical trials (Mylotarg, NTC0104104; Flugaza, NCT02319135; Panobidara, NCT00840346). Clinical data were collected in the following Spanish AML epidemiological registries: NCT01700413, NCT02006004, NCT00464217, NCT02607059, NCT01041040 and NCT01296178.

(MRD levels >0.025%) was associated with both significantly shorter overall survival (33% *versus* 81%; HR: 6.0; 95% CI: 1.3–28.7; *P*<0.001) and significantly shorter disease-free survival (17% *versus* 94%; HR: 19.6; 95% CI: 2.5–155.6; *P*<0.001) (Figure 3C,D). Survival outcomes were also analyzed combining post-induction and post-consolidation (n=63) tests, in order to compare survival analysis with MFC and qPCR data sets. We observed that positive MRD status (MRD levels >0.035%) was associated with a higher risk of relapse (48% *versus* 81%; HR: 3.4; 95% CI: 1.4–8.5; *P*=0.005) and death (37% *versus* 81%; HR: 4.2; 95% CI: 1.6–10.7; *P*<0.001) (Figure 3E,F). In order to test the power of *NPM1* and SNV as independent predictive markers, we performed the analysis separately. Evaluating *NPM1* as an MRD marker (n=54), we found that MRD positive status was associated with both significantly shorter overall survival (43% *versus* 78%; HR: 3.3; 95% CI: 1.2–8.8; *P*=0.011), and shorter disease-free survival (57% *versus* 85%; HR: 2.9; 95% CI: 0.9–7.6; *P*=0.052). Similar results were found when we evaluated *IDH1*, *IDH2* or *FLT3*-SNV as MRD markers (n=11). Accordingly, MRD positive status was associated with both significantly shorter overall survival (17% *versus* 100%; HR: not applicable; *P*=0.041), and shorter disease-free survival (17% *versus* 75%; HR: 6.3; 95% CI:0.7–54; *P*=0.058).

In univariate Cox analysis (Table 2A), the risk of death was significantly higher with increasing age (HR: 1.04; *P*=0.013), in patients with *FLT3*-ITD (HR: 3.45; *P*=0.007), and in those with MRD positive status as determined by NGS (HR: 4.22; *P*=0.002). The risk of relapse was significantly higher only in those patients with MRD positive status determined by NGS (HR: 3.4; *P*=0.008). In multivariate analysis (Table 2B), the risk of death was significantly higher with increasing age (HR: 1.05; *P*=0.004), in patients with mutated *FLT3*-ITD (HR: 8.87; *P*=0.001), and

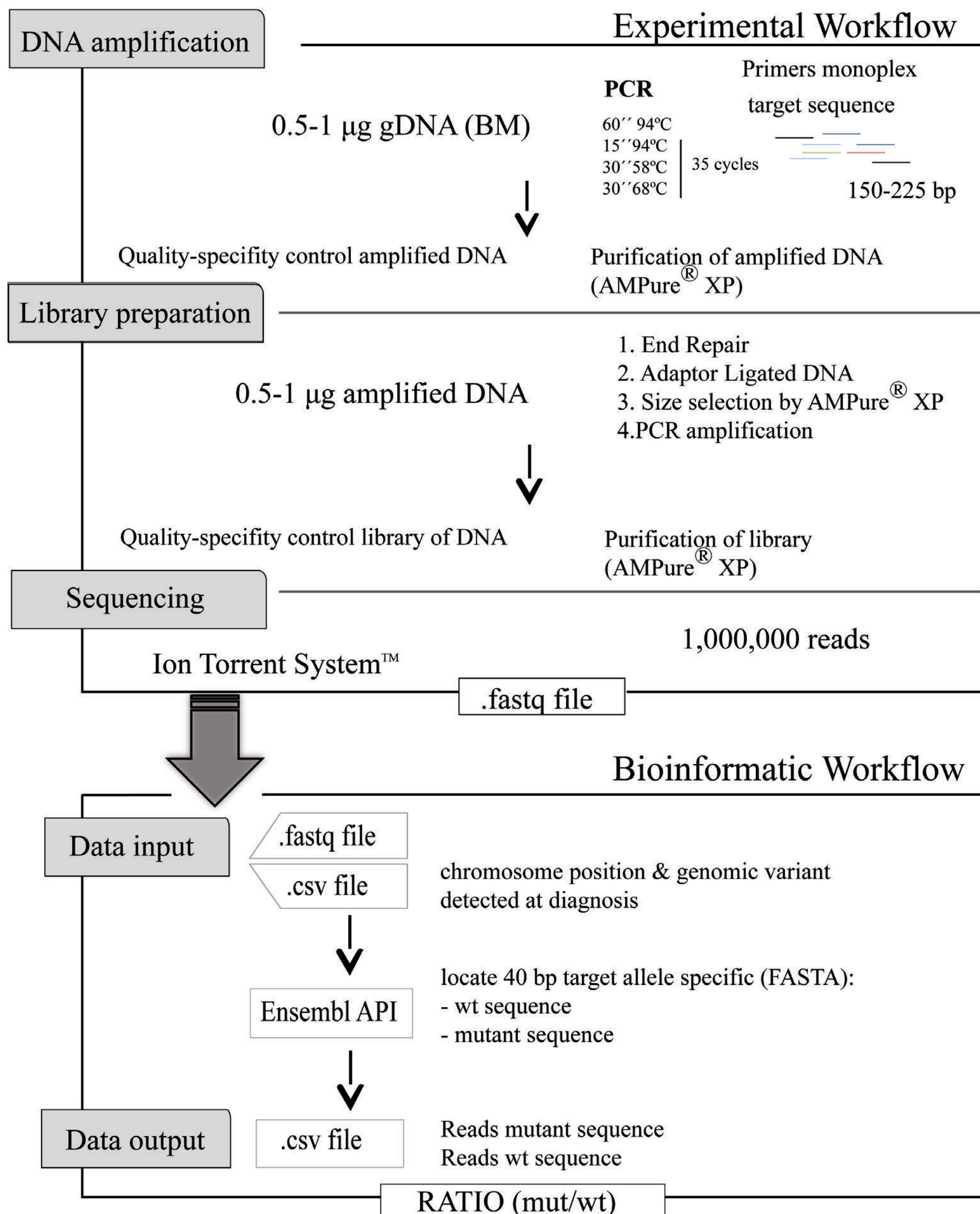

Figure 1. Workflow of the next-geneartion sequencing – minimal residual disease method. DNA amplification, library preparation and sequencing experimental workflow. Genomic DNA (gDNA) is amplified by quantitative (q) polymerase chain reaction (PCR) using specific primers. Libraries are prepared in four steps: end repair, adaptor ligation, size selection, and PCR amplification. The libraries are then sequenced. A customized bioinformatic pipeline analyzes the sequences obtained. The results are expressed as a ratio of mutated sequences (mut) among wild-type (wt) sequences.

in those with MRD positive status determined by NGS (HR: 4.54; P=0.005). The risk of relapse was higher only in patients who were MRD positive as determined by NGS (HR: 3.76; P=0.012).

Minimal residual disease assessment by sequencing predicts overall survival and disease-free survival better than multiparameter flow cytometry or quantitative polymerase chain reaction analysis

A positive correlation was found when comparing MRD assessment by NGS *versus* MFC (r=0.47, P=0.005, n=75), and NGS *versus* qPCR (r=0.62, P<0.001, n=80) (*Online Supplementary Figure S2*). There were differences between positive MRD and negative MRD groups of patients tested by MFC, but they were not significant for either overall survival (P=0.193) or disease-free survival (P=0.117) (n=46) (Figure 4A). Similarly, differences were observed between positive MRD and negative MRD groups defined by qPCR of *NPM1*, although statistical significance was not reached for either overall survival (P=0.212) or disease-free survival (P=0.086) (n=46) (Figure 4B).

Discussion

We have optimized and validated a high sensitivity NGS method for the detection and quantification of *NPM1*, *IDH1*, *IDH2* and *FLT3*-SNV mutated sequences at very low allele frequency in follow-up gDNA samples. NGS has demonstrated prognostic value for pre-treatment status in patients with AML,[17] and may also be a useful tool for detecting MRD.[18,19] We first studied the mutational profile of patients with AML using a customized NGS panel to ensure a high applicability (82% of patients). This approach is also a useful screening method for detecting all potential MRD markers and choosing those most relevant. The combination of several markers is possible and recommended to overcome limitations of MRD assessment due to sub-clonal heterogeneity of AML and CHIP.[11] Accordingly, our method has the capacity to evaluate multiple markers simultaneously and, considering that 61% of patients in our cohort had two or more genetic alterations, this approach is sufficiently robust to monitor MRD even in patients with clonal evolution.

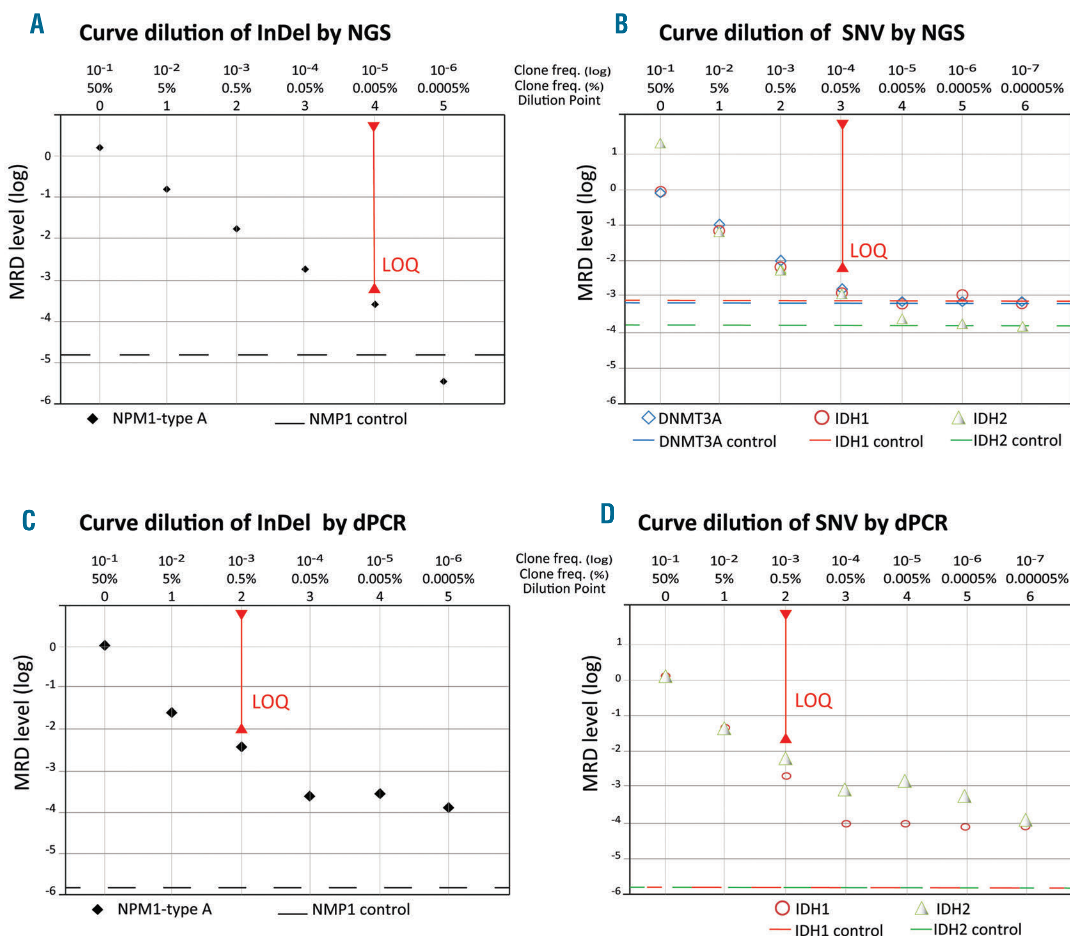

Figure 2. Calibration curve of minimal residual disease in serial dilutions. (A,B) Ten-fold dilution curves for the assessment of the sensitivity of next-generation sequencing (NGS) in (A) insertions-deletions (InDel), using OCI-AML3 gDNA with 50% *NPM1* type A mutation (R^2 = 0.98); and (B) single nucleotide variabts (SNV), using OCI-AML3 gDNA with 50% mutated *DNMT3A* (R2 = 0.98), and gDNA with 50% mutated *IDH1* or *IDH2* from a commercial standard (R^2 = 0.91 and R^2 = 0.98, respectively). (C,D) The same 10-fold dilution curves for the assessment of sensitivity of digital polymerase chain reaction (dPCR) in (C) InDel (R^2 = 0.98); and (D) SNV (R^2 = 0.91 for *IDH1* and R^2 = 0.98 for *IDH2*). The vertical red bars indicate the limit of quantification (LOQ) according to the sample. Clone frequency is expressed as target concentration as mutated copies/μL in wild-type copies/μL. Negative controls are included in the calibration curves and had levels below the corresponding LOQ values.

Reported variants associated with CHIP are frequently located in *DNMT3A*, *TET2* or *ASXL1* genes, and are detected in the preleukemic phase and during complete AML remission.[20-23] Indeed, any gene could carry both CHIP and non-CHIP variants, and these should be evaluated for each patient. Moreover, studies have shown that genes related to CHIP (*IDH1/2*) are useful for predicting prognosis because in these cases the genetic alterations have been acquired in the leukemic clone and not before.[24]

The sensitivity of this method equates to one mutated cell per 100,000 cells (LOQ 10^{-5}) for *NPM1* and one mutated cell per 10,000 cells (LOQ 10^{-4}) for *IDH1*, *IDH2* and *FLT3*-SNV. This difference in sensitivity is related to the fact that the *NPM1* type A mutation (insCCTG) is rarely

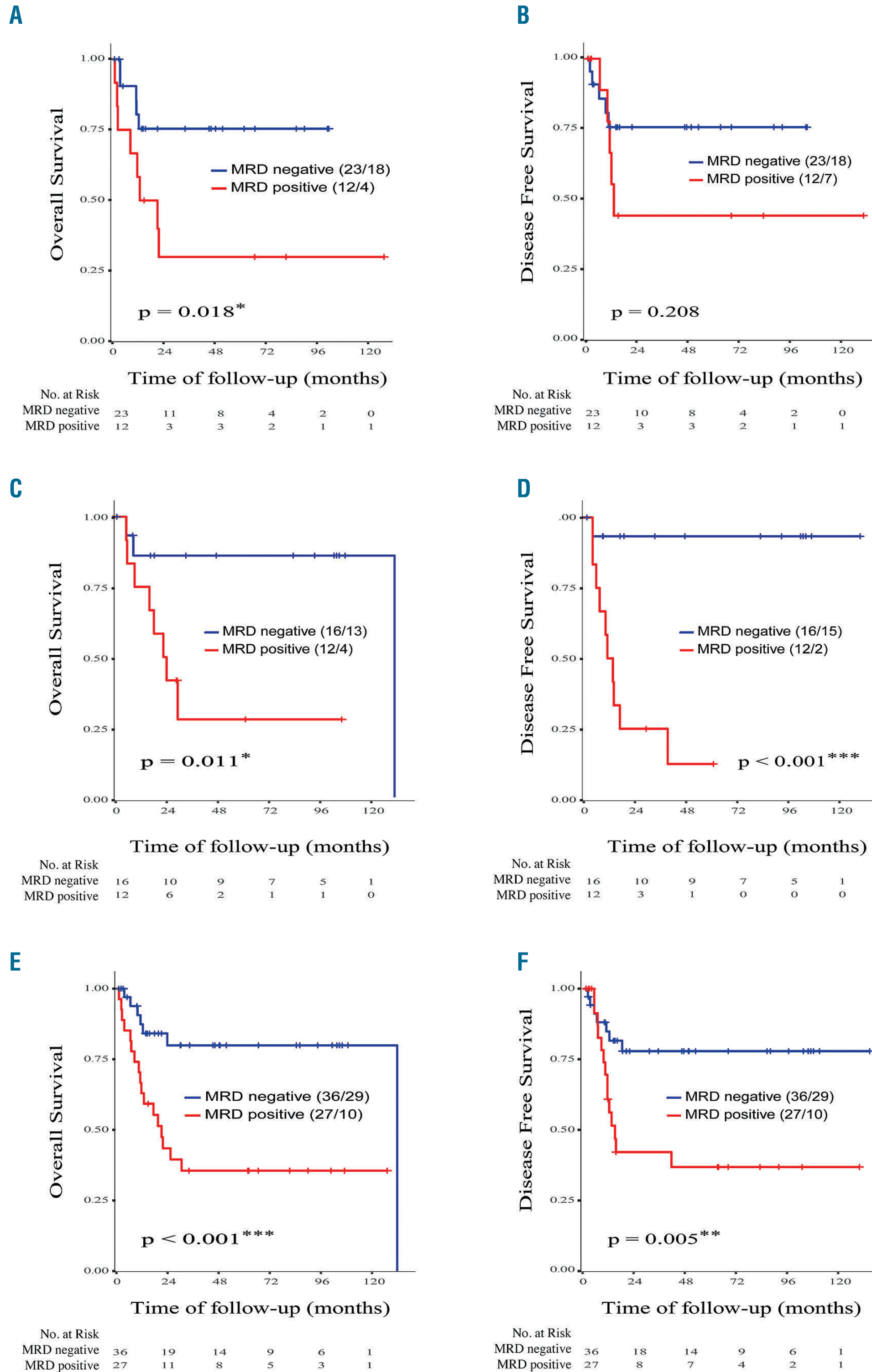

Figure 3. Analysis of overall survival and disease-free survival in patients with acute myeloid leukemia stratified according to minimal residual disease levels determined by sequencing. Analysis of overall survival for (A) the induction data set, (C) the consolidation data set, and (C) both together. Analysis of disease-free survival for (B) the induction data set, (D) the consolidation data set, and (F) both together. The cutoff used for overall and disease-free survival was 0.001 at the post–induction check-point (n=35), 0.00026 at the post-consolidation check–point (n=28) and 0.00035 for both check-points (all data set) (n=63). The numbers of censored patients with respect to the stratified groups and the numbers at risk are indicated. Statistically significant values: **P*<0.05, ***P*<0.01.

Table 2. Cox regression analyses.

A.

	Risk of death		Risk of relapse	
	HR (95%CI)	***P* value**	**HR (95%CI)**	***P* value**
Sex (female *vs.* male)	1.20 (0.50–2.83)	0.682	0.94 (0.37–2.44)	0.906
Age per year	1.04 (1.00–1.07)	0.013 *	1.03 (0.99–1.06)	0.069
Blasts at diagnosis (%)	1.00 (0.99–1.02)	0.667	1.01 (0.99–1.03)	0.532
Leukocytes at diagnosis ($\times 10^9$/L)	1.01 (0.99–1.01)	0.418	1.00 (0.99–1.01)	0.508
Favorable *vs.* adverse ELN risk	0.67 (0.08–5.43)	0.714	0.75 (0.09–6.00)	0.786
Intermediate *vs.* adverse ELN risk	1.03 (0.13–7.86)	0.976	1.02 (0.13–7.82)	0.988
Mutated *FLT3*-ITD	3.45 (1.40-8.52)	0.007 *	2.37 (0.86–6.51)	0.095
Allo-HSCT *vs.* intensive chemotherapy	1.35 (0.40–4.57)	0.634	1.78 (0.41–7.78)	0.44
Allo-HSCT *vs.* auto-HSCT	0.29 (0.05–1.74)	0.176	0.64 (0.11–3.77)	0.629
MRD^+ by MFC	2.10 (0.67–6.62)	0.203	2.40 (0.77–7.46)	0.130
MRD^+ by qPCR	2.51 (0.56–11.2)	0.228	5.01 (0.64–38.8)	0.123
MRD^+ by NGS	4.22 (1.66–10.7)	0.002 **	3.41 (1.37–8.48)	0.008 **

B.

	Risk of death		Risk of relapse	
	HR (95%CI)	***P* value**	**HR (95%CI)**	***P* value**
Age per year	1.05 (1.02–1.09)	0.004 *	1.03 (0.99–1.07)	0.061
Sex (female *vs.* male)	0.84 (0.33–2.17)	0.720	1.25 (0.44–3.52)	0.671
Leukocytes at diagnosis ($\times 10^9$/L)	1.01 (0.99–1.03)	0.219	1.07 (0.99–1.02)	0.481
Favorable *vs.* adverse ELN risk	13.75 (0.84–226.1)	0.067	7.09 (0.37–134.15)	0.192
Intermediate *vs.* adverse ELN risk	11.22(0.82–154.2)	0.071	5.86 (0.39–86.84)	0.203
Mutated *FLT3*-ITD	8.87 (2.54–30.95)	0.001 **	4.18 (1.11–15.69)	0.034*
MRD^+ by NGS	4.54 (1.58–13.03)	0.005 **	3.76 (1.34–10.54)	0.012*

Cox regression analyses of prognostic factors influencing the risk of relapse and risk of death of patients with acute myeloid leukemia. (A) Univariate Cox regression analysis of each prognostic factor. (B) Multivariate Cox regression analysis evaluating the most relevant factors detected in the univariate analyses. HR: hazard ratio; 95% CI: 95% confidence interval; ITD: internal tandem duplication; ELN: European LeukemiaNet; allo-HSCT: allogeneic hematopoietic stem cell transplantation; auto-HSCT: autologous hematopoietic stem cell transplantation; MFC, multiparametric flow cytometry; MRD, minimal residual disease; NGS, next-generation sequencing. Statistically significant values: *P<0.05, **P<0.01.

generated erroneously by NGS, and the quantification is precise.

Our method, as with any NGS method, has an intrinsic error rate that limits its sensitivity for most SNV to 1–2% of all reads. This limitation can nevertheless be overcome by virtue of the scalable nature of NGS.[16] Thus, we boosted NGS sensitivity by increasing the amount of DNA by PCR prior to sequencing, which increased the depth of coverage to one million reads. By also optimizing the bioinformatic analysis, we focused the search for the precise variant in order to eliminate random sequencing errors, enhancing the specificity of the technique and reducing the computational time. To the best of knowledge, our NGS method presents possibly the highest sensitivity reported for NGS in AML.[18,19,24-27]

dPCR is a relatively novel technique for precise and absolute quantification of nucleic acids, which is based on limiting partitions of the PCR volume and Poisson statistics.[28] It is also an extremely sensitive technique, with a high specificity due to the detection of mutant alleles.[29] However, when we compared the same standard dilutions in NGS and dPCR, NGS afforded a 2-log increment in LOQ for indels (*NPM1*) and a 1-log increment for SNV (*IDH1/2*), with the sensitivity of dPCR for indels being similar to that reported in a previously published study (10^{-2}).[30] Compared with NGS, dPCR is a faster measurement technique but, as it is focused, it requires allele-specific primers that can complicate the experimental procedure, and a high number of parallel experiments are needed to raise the sensitivity, which increases the cost of the assay. Additionally, although it is possible to multiplex dPCR, unfortunately only a few targets can be monitored simultaneously within each sample.[29] Another advantage of NGS technology is that it does not require calibration curves in each assay, and the results are reported in absolute values, facilitating its standardization.

The NGS method described here showed comparable sensitivities (10^{-4} for SNV and 10^{-5} for indels) to those of MFC methods in those cases with immunophenotyphically aberrant populations.[10,31] Although our method had a similar sensitivity to that of qPCR, it does not require oligonucleotides that hybridize specifically to a particular sequence, so all nucleotides in the amplified region can be studied. Consequently, the NGS test is capable of detecting all *NPM1* subtype mutations in the same assay.

We found positive correlations when MRD levels were evaluated by NGS *versus* MFC and *versus* qPCR, but not with the expected results. In the case of MFC, this could be explained, in part, by the fact that *NPM1* mutations are

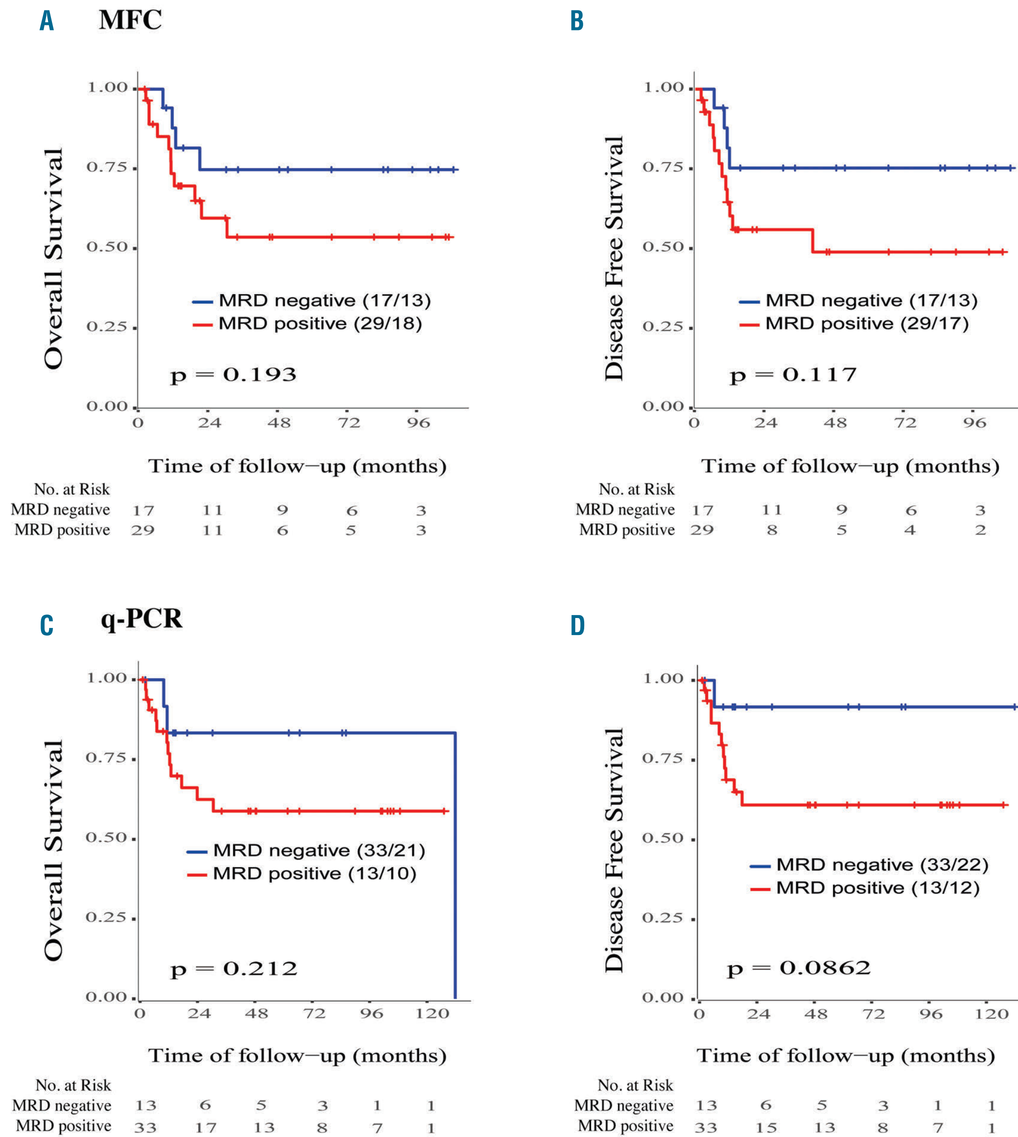

Figure 4. Analysis of overall survival and disease-free survival in patients with acute myeloid leukemia stratified according to minimal residual disease levels determined by conventional methods. Kaplan-Meier plots of (A) overall survival and (B) disease-free survival according to minimal residual disease (MRD) assessment by multiparametric flow cytometry (MRC) and (C) overall survival and (D) disease-free survival according to MRD assessment by quantitative polymerase chain reaction (qPCR) analysis. The numbers of censored patients with respect to each stratified group and numbers at risk are indicated. Statistically significant values: **P*<0.05, ***P*<0.01.

usually associated with monocytic subtype-AML, which frequently presents more difficulties for identifying MRD by MFC. Indeed, Salipante *et al.*[27] described that the level of success of MFC depends greatly on the immunophenotype of the abnormal blasts and how to discriminate them from background regenerative blasts. Moreover, due to the lack of standardization, MFC shows substantial variability across laboratories, including that of sample processing, instrument configuration, number of events, and training of pathologists.[32] The lack of a strong correlation between NGS and qPCR could be explained by the nature of the sample (sequencing uses gDNA whereas qPCR uses cDNA). Although RNA overexpression allows a higher sensitivity of detection, RNA levels do not correlate with the number of tumor cells, in contrast to mutated DNA. Accordingly, mutated DNA is more representative of the tumor burden than is overexpression of mutated RNA.[35] It should be noted that the prediction of survival and progression of AML using MRD NGS was better than that of the other methodologies employed, at least in the cohorts evaluated.

Finally, survival analysis showed that MRD positive status determined by NGS was associated with a higher risk of relapse and death and that MRD negative status in post-consolidation ssmples was associated with longer overall and disease-free survival, in accordance with recently published studies.[23] Supporting these findings, previous studies reported that an MRD check-point after consolidation could be the best moment for analysis because it afforded better prediction.[8,34-37] Cox regression multivariate analyses

confirmed that MRD positive status determined by sequencing was the only statistically significant predictor of the risk of relapse (P=0.012).

In conclusion, we have optimized a new targeted sequencing method with high sensitivity for MRD evaluation with applicability in a high percentage of AML patients, improving the capacity, over MFC or qPCR, to predict the survival outcomes of the AML patients in our cohort.

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