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Precision and prognostic value of clone-specific minimal residual disease in acute myeloid leukemia

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ABSTRACT

The genetic landscape of adult acute myeloid leukemias (AML) has been recently unraveled. However, due to their genetic heterogeneity, only a handful of markers are currently used for the evaluation of minimal residual disease (MRD). Recent studies using multi-target strategies indicate that detection of residual mutations in less than 5% of cells in complete remission is associated with a better survival. Here, in a series of 69 AMLs with known clonal architecture, we design a clone-specific strategy based on fluorescent *in situ* hybridization and high-sensitivity next generation sequencing to detect chromosomal aberrations and mutations, respectively, in follow-up samples. The combination of these techniques allows tracking chromosomal and genomic lesions down to 0.5-0.4% of the cell population in remission samples. By testing all lesions in follow-up samples from 65 of 69 evaluable patients, we find that initiating events often persist and appear to be, on their own, inappropriate markers to predict short-term relapse. In contrast, the persistence of two or more lesions in more than 0.4% of the cells from remission samples is strongly associated with lower leukemia-free and overall survivals in univariate and multivariate analyses. Although larger prospective studies are needed to extend these results, our data show that a personalized, clone-specific, MRD follow-up strategy is feasible in the vast majority of AML cases.

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Introduction

Acute myeloid leukemias (AMLs) are heterogeneous diseases which occur after accumulation of various chromosomal and genomic lesions in hematopoietic stem or progenitor cells.[1-5] Some of these events, including *DNMT3A*, *TET2* and *ASXL1* mutations, also occur with aging, leading to clonal hematopoiesis of indeterminate potential (CHIP).[6-9] High throughput and single cell derived analyses of AML clonal architecture have shown that CHIP lesions are founding events in the leukemic clone.[2-5] Moreover, some mutations, such as those affecting *DNMT3A* or *TET2*, can still be detected after treatment, whereas others, such as *NPM1* mutations, are mostly not detected.[2,3,10] This suggests that most relapses emerge from a resistant pre-leukemic clone that behaves as a disease reservoir.

Despite this greater understanding of leukemogenesis mechanisms, AML is still associated with poor prognosis.[11] The initial level of response to treatment has been

Table 1. Comparison of patients' characteristics according to clonal clearance.

Variable	All patients‡ (n=68)	Analysis of all target clearance (n=59) Clearance (VCF<3.33%) (n=33)	No clearance (VCF≥3.33%) (n=26)	P	Analysis of persisting markers in CR (n=58) 0 or 1 marker (n=31)	Two markers or more (n=27)	P
Sex M/F: n (%)	37 (55) / 31 (45)	18 (55) / 15 (45)	15 (58) / 11 (42)	NS	19 (61) / 12 (39)	13 (48) / 14 (52)	NS
Age (years): median (range)	58 (18-84)	53 (18-84)	63 (37-80)	0.0176	54 (19-84)	62 (18-80)	NS
FAB: n (%)				NS			NS
M0-M2	39 (57)	18 (55)	13 (50)		20 (65)	11 (41)	
M4-M5	26 (38)	15 (45)	11 (42)		11 (35)	15 (55)	
M6-M7	3 (4)	0 (0)	2 (8)		0 (0)	1 (4)	
Cytogenetic group*				0.0532			NS
Good: n (%)	7 (11)	5 (16)	0 (0)		5 (16)	0 (0)	
Intermediate: n (%)	43 (65)	18 (56)	22 (88)		19 (63)	21 (81)	
Poor: n (%)	16 (24)	9 (28)	3 (12)		6 (20)	5 (19)	
FLT3-ITD : n (%)	15 (22)	7 (21)	7 (26)	NS	8 (25)	6 (22)	NS
NPM1 mut: n (%)	23 (34)	11 (33)	10 (38)	NS	11 (35)	10 (37)	NS
Initial WBC (x10⁹/L): Median (range)	14.2 (0.5-350)	12.2 (0.5-350)	15.1 (0.82-117)	NS	14.6 (0.5-350)	13.9 (0.82-117)	NS
Day of CR1 evaluation: median (range)	47 (28-194)	45 (29-194)	47 (28-139)	NS	47 (29-194)	43 (28-139)	NS

‡One patient had no target (normal karyotype and no mutation) and was excluded from the analyses. *Not available (NA) in 2 patients (failure). CR: complete remission; VCF: variant cell fractions; M: male; F: female; NS: not significant; mutated; CR1: first complete remission.FAB: French American and British classification; WBC: white blood cell count; mut: mutated.

identified as a major prognostic factor in adults. Many tools have been developed to evaluate the minimal residual disease (MRD) in complete remission (CR). Cytogenetic[12-14] and flow cytometry[15-20] follow-up strategies can provide prognostic information, but their use is limited by either a poor sensitivity or a lack of specific assessable markers. Molecular MRD tools have been well validated in AMLs with recurrent gene fusions, mostly those with core-binding factor translocations[18,21-23] or with *NPM1* mutations.[24-26] These markers cover up to 50% of all AMLs.[11] However, with the recent understanding of comprehensive genetic landscapes of AMLs,[27,28] nearly all patients could, in theory, be assessed for MRD by specific molecular markers. A few studies have investigated alternative MRD markers. Most of these studies used strategies focused on one or two genes. Among them, *RUNX1* mutation evaluation seems to be of particular interest[29] due to its strong prognostic value and a mutual exclusion with *NPM1* mutated cases. *IDH1* and *IDH2* mutations and *FLT3*-ITD detection could also be useful tools,[30-32] but these are frequently either associated with *NPM1* mutations or lost at relapse.[31,33] Finally, *DNMT3A* mutations seem to be of little interest because they frequently persist at a high level in CR,[2,3,10,30] even in long-term responders.[34]

One recent study analyzed the clearance of all variants found in 50 AMLs.[10] Such clearance after one course, defined as variant allele frequency (VAF) below 2.5%, was associated with both better event-free survival and overall survival (OS) probabilities. In the present study, we asked whether an architecture-based clone-specific MRD strategy could provide more powerful prognostic information than evaluating the clearance of all variants. A combination of highly sensitive NGS (HS-NGS) assay and chromosomal analyses was used to monitor MRD. We found only a trend towards association of a residual clonal disease below 3.33% with better leukemia-free survival (LFS). Strikingly, the earliest event of the clonal architecture was frequently detected, even in long term-responders. In contrast, our data revealed that persistence of the two or three first lesions of the clone was strongly associated with a poor prognosis, and was predictive for relapse at one year.

Methods

Patients' samples

Bone marrow (BM) and blood samples were collected from AML patients after informed consent. CD3⁺ cells were isolated from CR blood samples.[3] The study was conducted with the approval of the MyPAC clinical research group according to French law and the Declaration of Helsinki. Forty-five AMLs were prospectively included in the study between 2013 and 2015. Thirteen patients who experienced relapse between 2013 and 2015, and 11 other patients diagnosed before 2013, were retrospectively included (Table 1 and Figure 1). Of these 69 patients, 46 have already been reported.[3] Patients received an initial anthracycline- and cytarabine-based induction treatment, and a cytarabine-based post-induction treatment. Twenty-two patients received hematopoietic stem cell transplantation (HSCT) in first CR (*Online Supplementary Table S1*).

Targeted resequencing

Sequencing was performed using a 122 gene panel (HaloPlex Target Enrichment System®, Agilent Technologies) on a MiSeq® sequencer (Illumina[INC]). Whole exome sequencing was performed in 4 patients with *KMT2A* translocations. Detailed protocols have been reported previously[3] (*Online Supplementary Tables S1* and *S2*). After alignment, described variants were called using an Ensembl database. Non-described variants of potential interest were also sequenced using the Sanger method in the CD3⁺ fraction or in the CR samples.

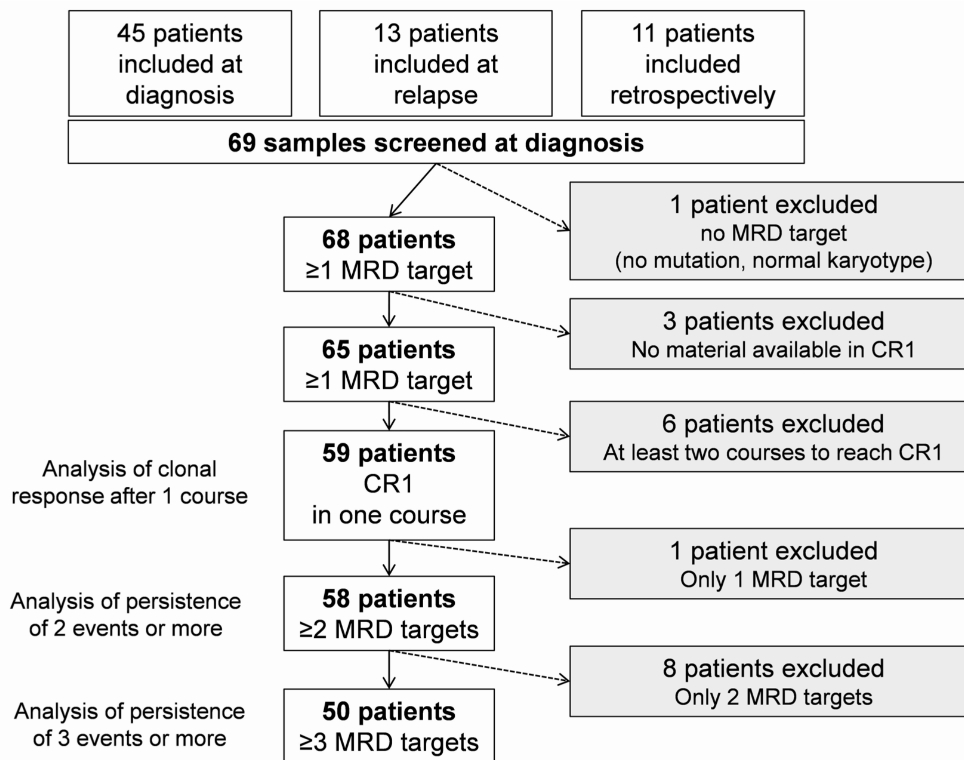

Figure 1. Flow-chart of inclusion in survival analyses.

FLT3-ITD, NPM1 and *CEBPA* mutation detection

Mutation detection was performed with standard routine techniques.[3]

Cytogenetic and fluorescent *in situ* hybridization analyses

Cytogenetic analysis was performed on R-banding metaphases after 24-hour culture using standard procedures. Chromosomal rearrangements were confirmed by fluorescent *in situ* hybridization (FISH) analysis on 200 interphase nuclei, as described.[3] FISH was performed in CR samples (200-400 nuclei) to evaluate the clearance of chromosomal abnormalities with a theoretical detection threshold of 0.5%.

Variant cell fraction determination

Variant cell fractions (VCFs) were determined from frequencies of nuclei harboring chromosomal aberrations, variant allele frequency (VAFs) for single nucleotide variants (SNVs), indels, and single nucleotide polymorphisms (SNPs) in sequenced regions with loss of heterozygosity, peak height ratios of high-resolution sizing of fluorescent dye-labeled PCR amplification for *FLT3*-ITDs. VCF and VCF confidence intervals were used to determine the clonal architecture, as already described.[3]

High-sensitivity targeted sequencing

Four different targeted-resequencing panels using HaloPlex HS Target Enrichment System® (Agilent Technologies) were designed, each covering lesions from 10-25 distinct patients. CR samples from each patient were analyzed with appropriate panels as described.[3] With this assay, single DNA fragments are tagged with unique random 10-nucleotide indexes before the first PCR amplification step of library preparation. After the PCR step, each tagged DNA fragment is amplified, generating an amplicon family. When libraries are sequenced, raw reads are generated from amplicons, and a family read corresponds to the group of reads harboring the same random index generated from a unique amplicon family. This improves discrimination of variant nucleotides from background sequencing errors, and allows a more confident detection of low frequency variants.

Digital droplet PCR

Digital droplet PCR (ddPCR) experiments were performed using a ddPCR droplet generator system (Biorad), according to the manufacturer's protocol, and 40 PCR cycles (Biorad iCycler). Droplet reading was performed with QX200 droplet reader (Biorad). Quantasoft software v.1.7.4 (Biorad) was used for result interpretation. Primers and probes are described in *Online Supplementary Table S3*.

Statistical analyses

Associations between patients' characteristics were analyzed using Fisher, Mann-Whitney or χ^2 tests. The Spearman test was used to assess the correlation between ddPCR and HS-NGS results. Standard definitions were used for LFS, OS and CR. Probabilities of survival were calculated using the Kaplan-Meier method. Differences between survival distributions were evaluated by the log-rank test. Cox models were constructed for multivariate analyses, including all variables of interest. Survival analyses were performed with and without data censoring at allo-HSCT. The median follow-up time for surviving patients was 24.2 months. $P<0.05$ was considered significant. We used StatView software (v.5.0) for statistical analyses (SAS Institute Inc., San Diego, CA, USA).

Results

Definition of MRD target panels using AML clonal architecture at diagnosis

To set up a multitarget MRD follow-up strategy, we first established the clonal composition of AMLs by combining cytogenetic, standard molecular and NGS data, which led to the detection of 63 chromosomal abnormalities and 235 mutations. In total, a median of 4 (range 0-10) chromosomal or genetic events were identified per patient, with a median of 3 (0-10) mutations (*Online Supplementary Tables S1* and *S2*). The clonal architecture inferred from VCFs (Figure 2A) indicated that most patients had a mixture of founding lesions (i.e. VCFs >0.5) and subclonal lesions (i.e. VCFs <0.5) (Figure 2B). Overall, in 1 of 69 patients no target was found (normal karyotype and no mutation). In 3 additional cases, no material was available at the time of first CR (CR1). These 4 patients were excluded from further analyses.

Detection of low variant allele fractions with the high-sensitivity NGS assay

To determine the threshold of detection of the high-sensitivity NGS (HS-NGS) assay, we performed serial dilutions (1/20, 1/100, 1/400) of 4 samples with 29 known variants (VAFs ranging from 1.6% to 48.1%) in non-mutated control genomic DNA. With the HS-NGS assay, confident detection of variants relies on the presence of mutant family

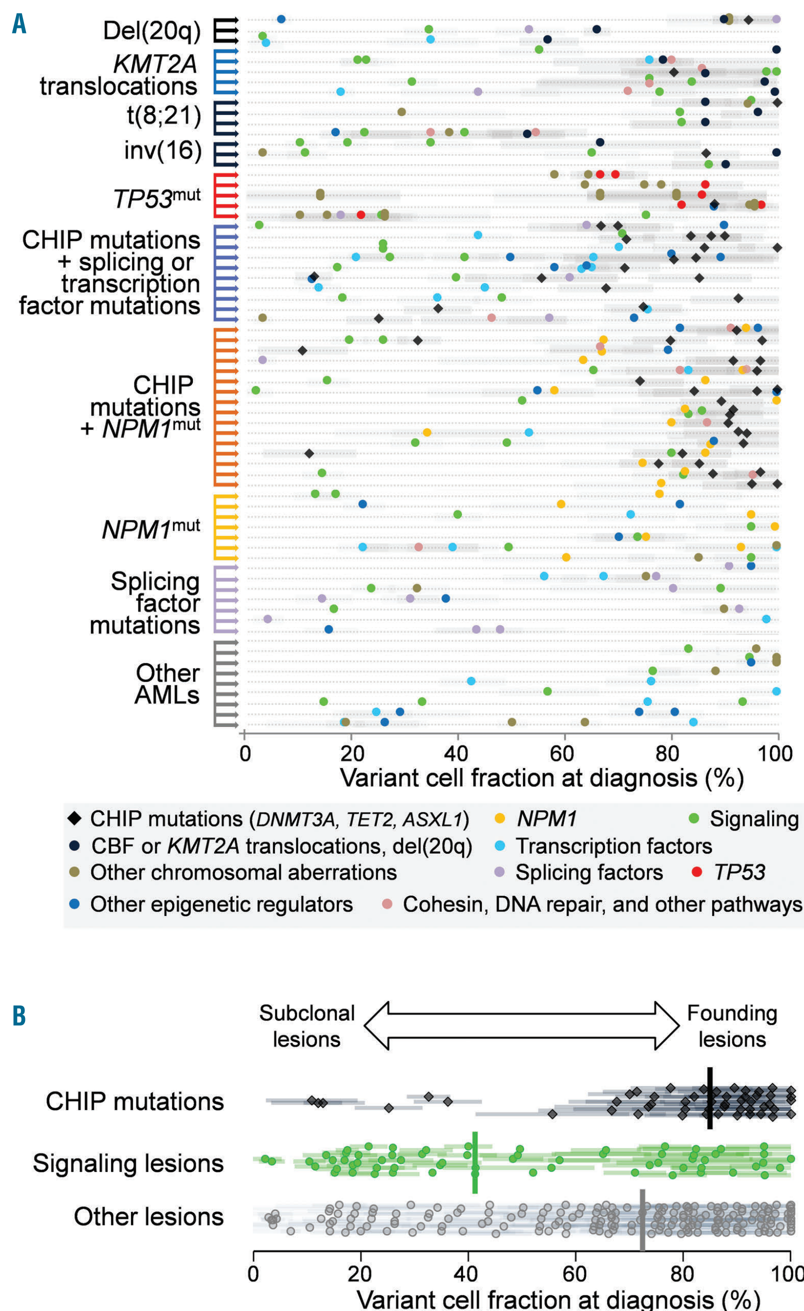

Figure 2. Clonal composition of acute myeloid leukemias (AMLs) at diagnosis. (A) Distribution of variant cell fractions (VCFs) of individual diagnosis samples: colored dots indicating the fraction of cells bearing genetic or chromosomal lesion are distributed along one line per patient. Error bars represent VCF confidence intervals. Distinct categories of clonal composition are listed in the left legend and indicated by individual colored arrows. (B) VCFs of CHIP mutations, signaling lesions, and all other lesions. Error bars indicate VCF confidence intervals. Vertical bars indicate median VCFs.

reads with unique molecular indexes. When paired end sequencing is performed, a unique molecular index yields two family reads. We therefore considered as positive specimen samples with more than two mutant and distinct family reads, meaning that more than one variant DNA molecule was tagged by unique indexes in the sample. By this approach, we detected all diluted variants with theoretical VAFs above 0.12%. Moreover, we found that all variants with VAFs measured over 0.18% were supported by 6 family reads or more (Figure 3A).

To address the issue of false positive detection, we screened all samples expected to be wild-type for 31 given SNVs. For each variant, we calculated the threshold of detection as the median percentage of positive family reads in expected negative samples + 2 standard deviations (SD) (Figure 3B). For example, 45 expected negative samples were screened for the *DNMT3A* p.R882H mutation. In these samples, with a median of 5106 (1782-45974) family reads per sample, variants were detected in 0.06±0.059% of family reads (median±SD). The detection threshold for this variant was consequently calculated at 0.18%. Detection thresholds of 30 other SNV targets ranged from 0 to 0.19%.

We next evaluated the threshold of detection of indel variants. We first focused on a CUX1 indel (p.E221fs. chr7:101758539 AG>A). Out of 34 expected negative samples, we only found one mutant read family in one sample. We then analyzed *NPM1* type A mutation. In 83 expected negative samples, we detected mutants in 4 cases, with a maximum level of 0.17% of read families. In these 4 cases, bulk AML samples with high *NPM1* type A mutation ratios (VAF >20%) were processed in the same library preparation experiment. This suggests inter-sample cross contamination during the process that could probably be avoided.

For further analyses, we consequently set a consensus SNV and indel detection threshold at 0.2% VAF (i.e. VCF of 0.4% for heterozygous lesions). In further experiments, all variants with VAF less than 0.2% were considered as not detected. DdPCR experiments were performed to test 17 remission samples for targets detected with VAF less than 2% using HS-NGS. VAFs obtained using ddPCR were correlated to those obtained using HS-NGS (r^2=0.92, *P*=0.001) (Figure 3C and D).

Residual genomic landscape in post-treatment samples

To evaluate the persistence of both chromosomal and genomic lesions at the time of CR1, we performed a combination of karyotypic, FISH, and HS-NGS analyses in the 65 patients with available material and with at least one lesion identified at diagnosis (Figures 4 and 5 and *Online Supplementary Table S1*). Among the 281 lesions found in these patients, three chromosomal abnormalities were not evaluated due to karyotype failure in one patient (2015-035), and five subclonal lesions were not included in the follow-up NGS panels (Figure 4B). Of the 273 remaining lesions, 83 were still detected with VCFs over 2%, which we arbitrarily considered at high level. Those events included mutations in *DNMT3A* (19 of 21 mutations at diagnosis), *TET2* (14 of 23), *ASXL1* (4 of 5), *EZH2* (3 of 3), *IDH1* (4 of 7), *TP53* (5 of 6), *SRSF2* (4 of 7), and *U2AF1* (2 of 3). The earliest event of the clonal architecture (Figure 2) was detected at high level in CR1 samples from 30 of 65 patients. Two lesions were still detectable at high level (>2%) in CR1 in 20 of 65 patients. Detection of one or more events at high level in CR did not correlate with blast count evaluated by morphological examination in CR BM samples. Persistence of a high *DNMT3A, U2AF1, TET2,* or *SRSF2* mutation burden, despite multiple chemotherapy courses, was observed in 8 patients who did not experience relapse (Figure 5B and E). Clearance of these events was observed after BM transplantation in 6 patients (UPN 2013-003, 2014-008, 2014-010, 2014-016, 2014-020, and 2015-036), including one who finally relapsed with a mutational profile similar to that at diagnosis (2014-016) (Figure 5C).

One hundred and fifty-eight out of 273 evaluable events were not detected in CR. These cleared events included most mutations in *FLT3* (23 of 25), *NRAS* (11 of 12), *KIT* (3 of 3), *NPM1* (18 of 22), *CEBPA* (5 of 6), *WT1* (6 of 8), IDH2 (2 of 3) and *BCOR* (4 of 5). Among these mutations, some were missing at relapse, indicating clonal evolution, including mutations in *FLT3, NRAS*, and *NPM1* (*Online Supplementary Table S1*).

In 3 patients, we detected the rise of minor subclones independent of the initial leukemic clones. This was observed after the first induction course in 2 patients and at relapse in the third (Figure 5D). In all 3 patients, the

Table 2. Results from multivariate analyses for leukemia-free survival and overall survival in the 58 evaluable patients with two or more minimal residual disease targets.

	All patients (n=58)						Intermediate cytogenetics (n=40)					
	LFS			OS			LFS			OS		
Variable	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
Age (continuous)	0.986	0.951-1.023	0.46	0.986	0.935-1.04	0.60	0.962	0.915-1.012	0.13	0.936	0.868-1.009	0.084
Cytogenetic (poor *vs.* other)	1.553	0.370-6.528	0.54	0.421	0.09-1.962	0.27		NOT INCLUDED			NOT INCLUDED	
0-1 marker *vs.* 2 or more markers in CR1	0.109	0.031-0.390	0.0006	0.071	0.01-0.480	0.006	0.075	0.016-0.342	0.0008	0.028	0.002-0.434	0.010
NPM1 status (WT *vs.* MUT)		NOT INCLUDED			NOT INCLUDED		1.316	0.424-4.087	0.63	2.088	0.337-12.9	0.42
FLT3 (no ITD *vs.* ITD)		NOT INCLUDED			NOT INCLUDED		0.336	0.094-1.195	0.092	0.118	0.14-0.966	0.046

LFS: leukemia-free survival; OS: overall survival; HR: Hazard Ratio; CI: Confidence Interval; CR1: first complete remission; WT: wild-type; MUT: mutant; ITD: internal tandem duplication.

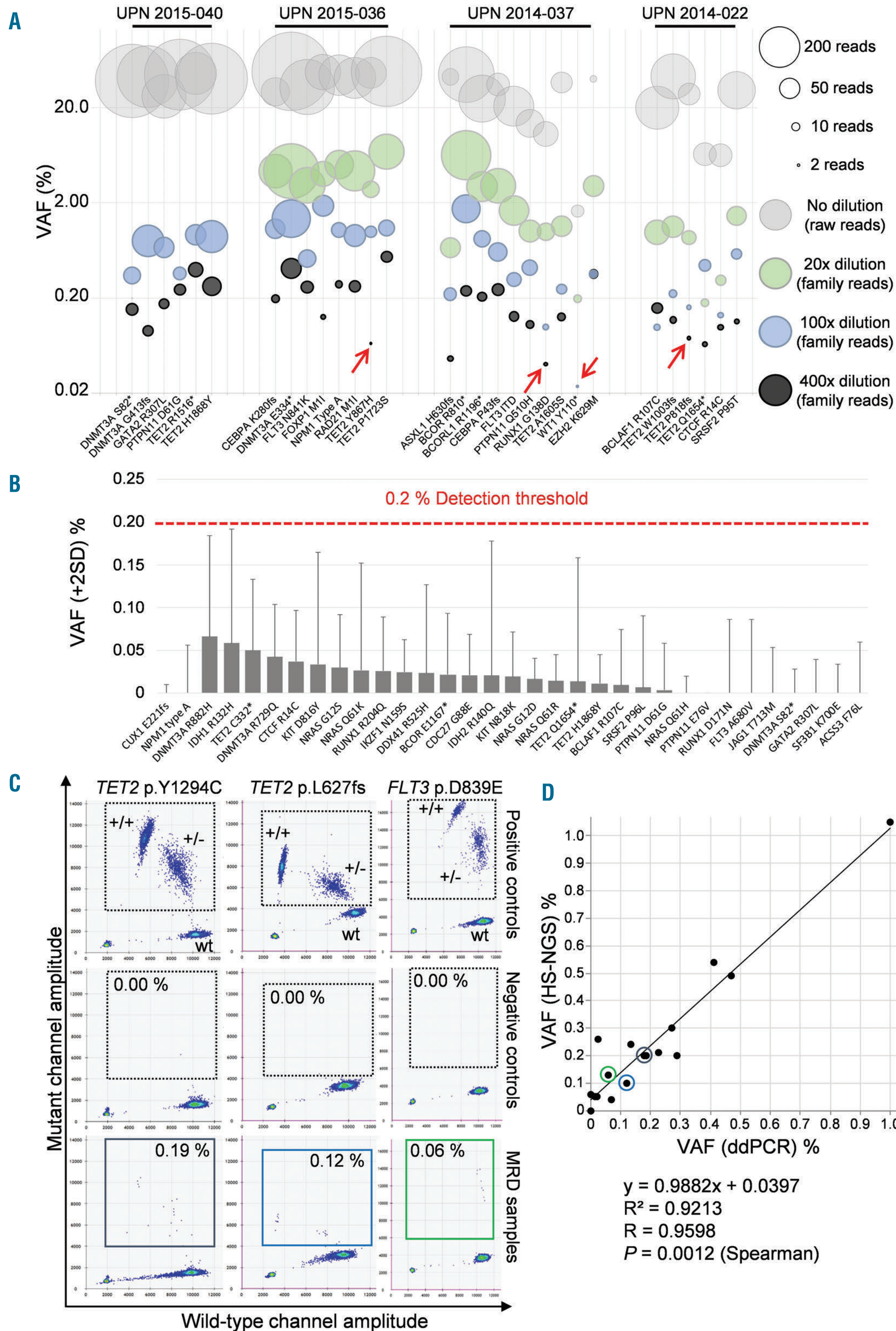

Figure 3. Determination of the threshold of detection of the high sensitivity next-generation sequencing (NGS) assay. (A) Sensitivity assay. Four acute myeloid leukemia (AML) DNA samples were diluted into control DNA (20x, 100x, 400x dilutions). Circles indicate variant allele frequencies (VAFs) determined using the HS-NGS assay. Circle size is proportional to the number of reads supporting each variant as indicated in the right key. Arrows indicate variants which were considered as not confidently detected because they were supported by less than 3 family reads. (B) Threshold of detection of two indel variants and 31 single nucleotide variants (SNVs). Histograms represent the median VAF of each variant in multiple expected negative samples. Error bars indicate two standard deviations. The subsequent consensus 0.2% detection threshold of the highly sensitive NGS (HS-NGS) assay is represented by the red dotted line. (C) Representative ddPCR dotplot analyses of three mutations. Positive controls, negative controls, and complete remission samples are shown. Squares indicate the areas of positive droplets (+/+: mutant, ±: mutant + wild-type, wt: wild-type). VAFs are noted. (D) Analysis of 17 mutations by ddPCR and HS-NGS. Colored circles correspond to the minimal residual disease samples shown in (B).

clone involved *DNMT3A* variants (p.R882 and p.R729 mutations) suggesting a selection of a pre-existent CHIP by treatment pressure.

Prognostic value of clonal response in CR

We first tried to determine if the response level of all targets after one course was associated with a prognostic value in patients who reached cytological CR. Nine patients were excluded from these analyses (6 needed two courses or more to reach CR and in 3 no material was available) (Figure 1). The 59 remaining patients were then distributed into two groups. To discriminate good from

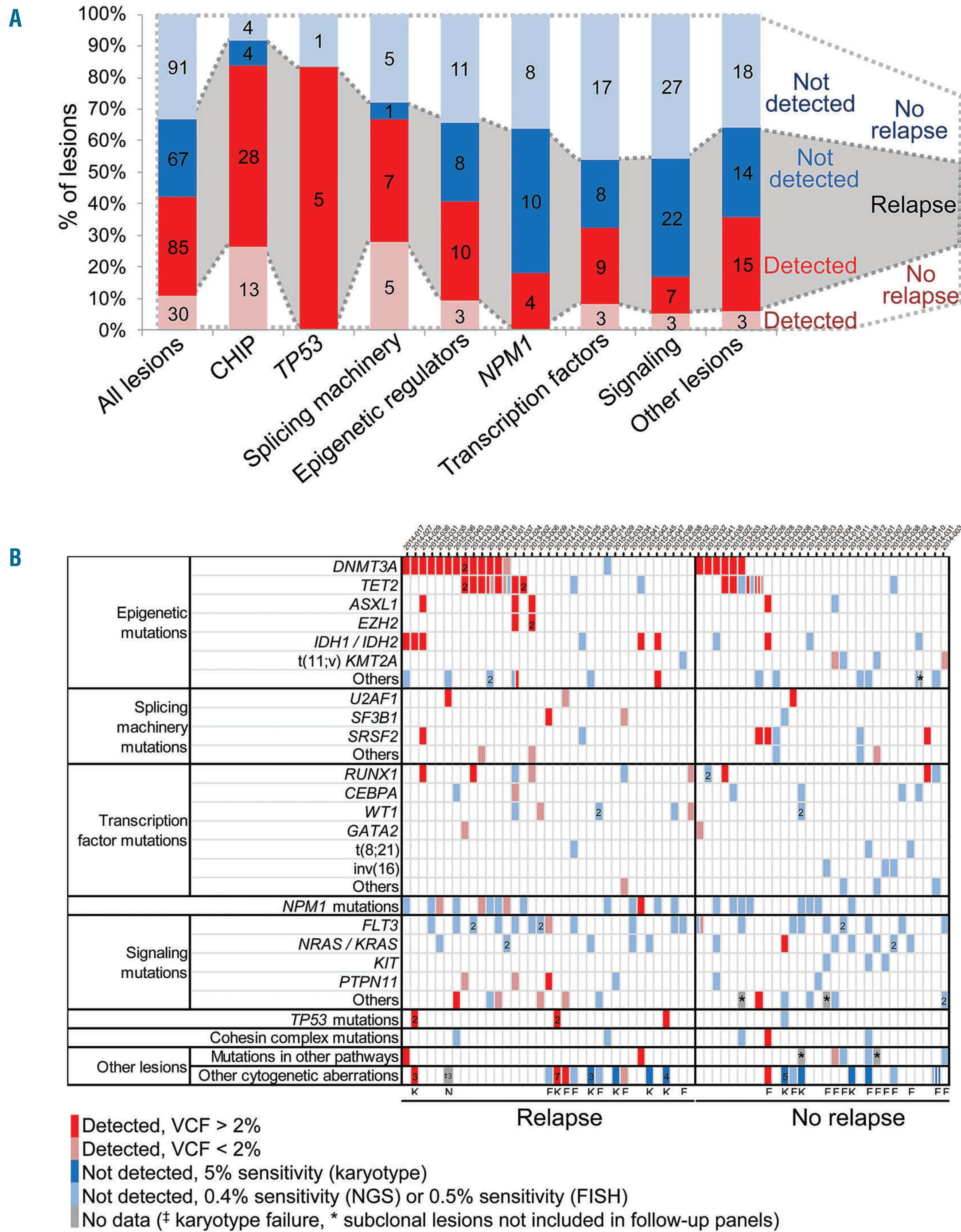

Figure 4. Residual genomic landscape of 65 acute myeloid leukemia (AML) patients after treatment. (A) Percentages of lesions above or below the threshold of detection, i.e. 0.4% variant cell fraction (VCF) for high-sensitivity next-generation sequencing (HS-NGS) assay, 0.5% VCF for fluorescent *in situ* hybridization (FISH) assay, or 5% VCF for karyotype evaluation, in first complete remission (CR1) samples according to the occurrence of further relapse or not. The shaded area represents lesions from patients who went into relapse. (B) Co-mutation table showing the detection, as indicated in the bottom key, of initial lesions at the time of CR1 in the 65 patients. Data from patients who further experienced relapse (left) and data from those who did not (right). Numbers in boxes indicate multiple lesions in a gene or pathway. Letters below the table indicate the method used for the detection of cytogenetic aberrations. K: karyotype; N: not done.

poor responders, we used a VCF of 3.33% as threshold. This value corresponds to the median VCF of the first lesion, determined by HS-NGS quantification in the CR1 samples. Patients with VCF less than 3.33% were considered as good responders whereas patients with VCF 3.33% or more were considered as poor responders. The latter were significantly older than good responders (63 *vs.* 53 years; *P*=0.01), and tended to more frequently harbor intermediate risk cytogenetics (*P*=0.0532) (Table 1). There was a trend to lower LFS probability in poor responders (31.7±9.9% *vs.* 51.7±9.8% at 2 years; *P*=0.08) with no translation in OS (Figure 6A). The difference in LFS became significant when censoring data after allo-HSCT (20.1+11.3% *vs.* 63.6+11%; *P*=0.01) (*Online Supplementary Figure S1*). When focusing on the 40 patients with intermediate cytogenetics, good and poor responders had similar LFS (Figure 6A) and OS probabilities. Similar results were observed when using 2% and 5% VCF thresholds to separate the two groups (*data not shown*).

The earliest events of the clonal architecture retrieved in poor responder patients (i.e. VCF>3.33%) were mutations in *DNMT3A* (8 of 10 patients with *DNMT3A* mutation as earliest event), *TET2* (n=8 of 10), components of the splice machinery (n=3 of 3), *TP53* (n=2 of 2) or *ASXL1* (n=1 of 1). Conversely, all 10 patients with t(8;21), inv(16) or *KMT2A* translocations as earliest events were good responders (Figures 4 and 5 and *Online Supplementary Table S1*). This suggests a frequent resistance of the pre-leukemic clone when the initial lesion is one of the major CHIP lesions.

Prognostic value of the persistence of multiple markers in CR

The earliest lesion of the clone was frequently detected in CR samples, which may blur the prognostic value of persisting events. We therefore performed a second analysis to evaluate if the detection of two or more markers had a prognostic impact. The 58 patients with more than one evaluable event and who reached CR in one course were separated into two groups (Table 1): 1) patients with 0 or 1 marker (responders) above the detection threshold after treatment (i.e. VCF ≥0.4%, n=31); and 2) patients with 2 or more detectable lesions (non-responders, n=27). LFS at two years was 64.9±9.3% in responders and 19.8±8.7% in non-responders (*P*=0.001). The OS probability at two years was higher in responders (84±6.6% *vs.* 57.1±10.5%; *P*=0.023) (Figure 6B). When focusing on the 40 patients with intermediate cytogenetics, non-responders had lower LFS at two years

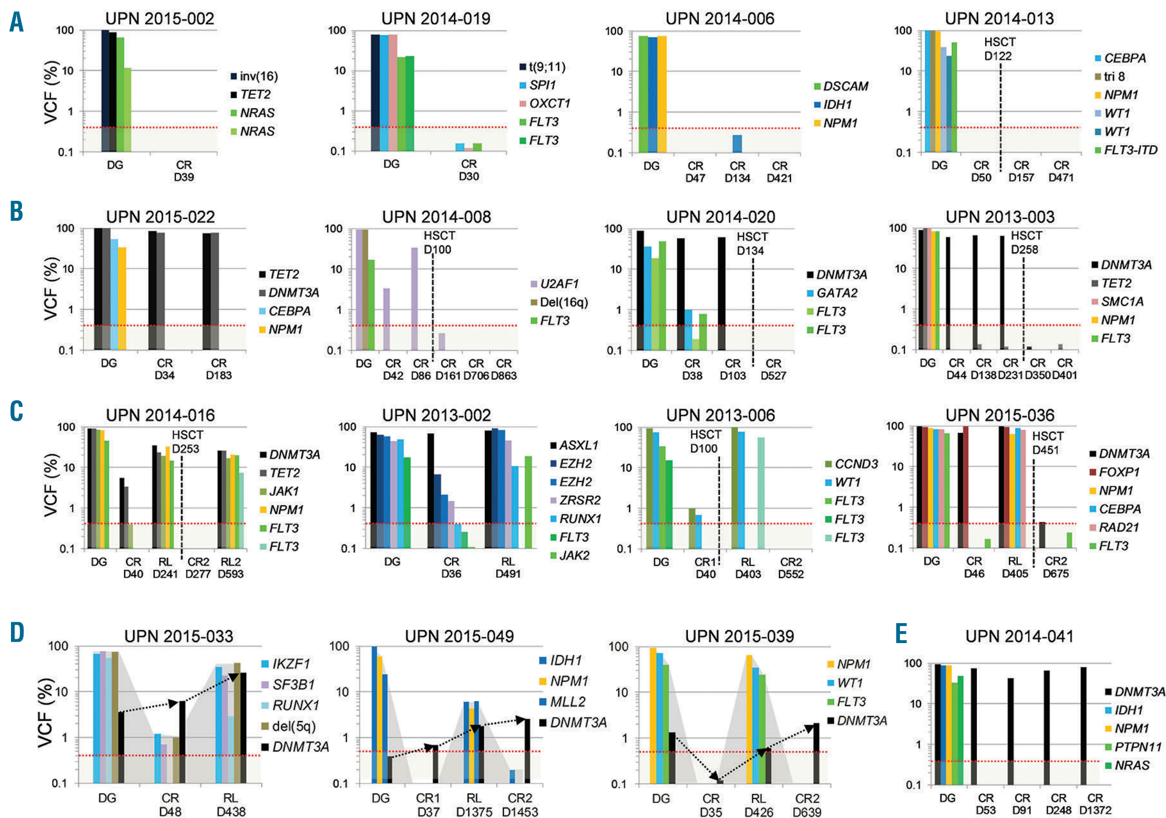

Figure 5. Multi-target monitoring of minimal residual disease (MRD) in 16 representative acute myeloid leukemia (AML) patients. (A-C) Histograms represent sequential analyses of variant cell fractions (VCF) in patients with early clearance of all targets (A), with one or more targets persisting at a high level in complete remission (CR) and who did not experience relapse (B), with one or more targets persisting at high level in CR and who experienced relapse (C). Red dotted lines indicate the 0.4% threshold of detection of the next-generation sequencing (NGS) assay. Black dotted lines indicate hematopoietic stem cell transplantation (HSCT). D: day of sampling or HSCT; DG: diagnosis sample; RL: relapse sample. (D) Emergence of *DNMT3A* mutant clones in 3 treated AML patients. Histograms are as in (A-C). Shaded areas show the global evolution of the initial AML clone. Arrows indicate the progression of independent *DNMT3A* mutant clones. (E) Persistence of an isolated major *DNMT3A* mutant clone in a patient with long-term (46 months) CR.

(57±11.8% *vs.* 19.4±10.5; *P*=0.0048) and a trend to lower OS (85±8% *vs.* 61±11.9%; *P*=0.07) (Figure 6B). Similar results were observed when restricting the analyses to the 42 prospectively included patients with two or more MRD targets (LFS at 2 years 73±10% *vs.* 24±10%, *P*=0.0026, and OS at 2 years 90.2±6.6% *vs.* 62.8±11.5%, *P*=0.036) (*data not shown*). Results were the same when censoring observations after allo-HSCT (*Online Supplementary Figure S1*).

Multivariate Cox models were constructed including variables of clinical interest and censoring data after allo-HSCT. In all analyses, persistence of 2 or more lesions was an independent variable for LFS and OS (Table 2).

Finally, we analyzed the 50 patients with 3 or more lesions. The persistence of 3 or more markers after one course was associated with a very high risk of relapse (LFS 23.5±10.3% *vs.* 75.8±7.5% at one year, *P*<0.0001; median LFS at 7 months in the non-responder group and not reached after 2 years in the responder group), and a lower OS probability (45.2±13.5% *vs.* 84.8±6.2 at 2 years, *P*=0.026) with similar results when focusing on the 36 analyzable intermediate-risk cytogenetics patients (Figure 6C) and when censoring data after allo-HSCT (*Online Supplementary Figure S1*).

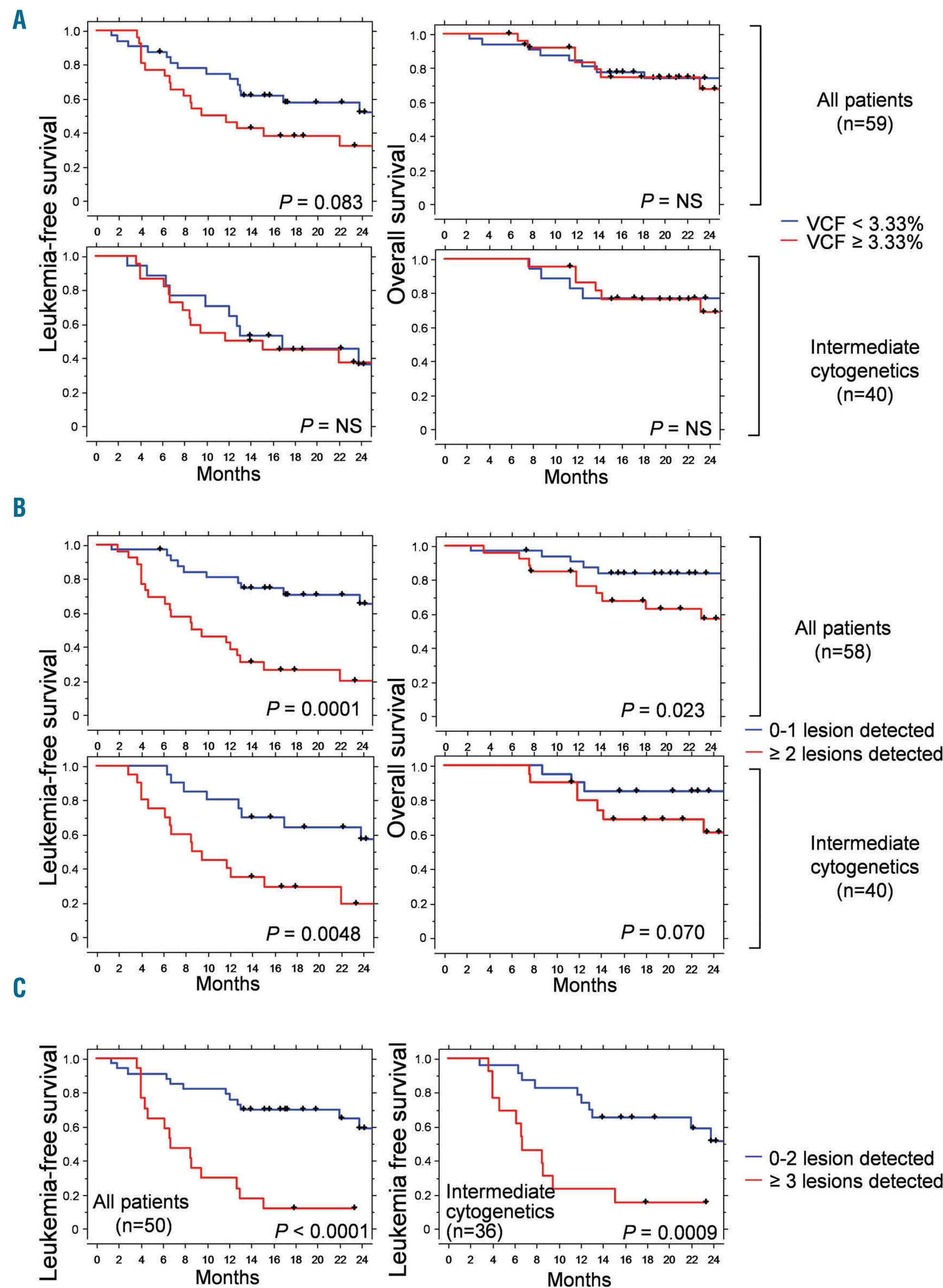

Figure 6. Persistence of multiple genetic lesions in first complete remission (CR1) is associated with poor prognosis. (A) Leukemia-free survival and overall survival according to the clonal response. Variant cell fraction (VCF) less than 3.33% indicates good responders (i.e. all lesions were found below the 3.33% VCF value). VCF 3.33% or more indicates poor responders (i.e. at least one lesion was found above the threshold VCF of 3.33%). Data from the 59 evaluable patients (top) and from patients with intermediate-risk cytogenetics (bottom) are shown. (B) Leukemia-free survival and overall survival according to the detection of 0-1 lesions or 2 or more lesions above the 0.4% VCF detection threshold. Data from the 58 evaluable patients (top) and from patients with intermediate-risk cytogenetics (bottom) are shown. (C) Leukemia-free survival according to the detection of 0-2 lesions or 3 or more lesions above the 0.4% VCF detection threshold. Data from the 50 evaluable patients (left) and from patients with intermediate-risk cytogenetics (right) are shown.

Discussion

In the present work, we used a multi-target strategy to monitor MRD in a series of 69 patients. In most cases, the clonal compositions, as determined by cytogenetic and NGS techniques, allowed personalized follow-up panels to be set up using a combination of FISH and HS-NGS. The persistence of two or more lesions in CR1 is associated with a high risk of relapse in patients with all cytogenetic profiles, and in patients with intermediate-risk cytogenetics. Our results highlight the benefits of a high-sensitivity multi-target evaluation of MRD in AML patients, based on the follow up of the two or three first lesions of the clonal architecture.

In our series, the clearance of all genetic events only tended to be associated with LFS, in contrast to a previous retrospective study.[10] This difference could be explained in part by patient selection, treatment diversity, and the small numbers of cases included. However, we found similar results when restricting the analysis to prospectively included patients. Moreover, setting the threshold of 5% used in the Klco study[10] to discriminate good from poor responders did not alter our conclusions. The difference became significant when censoring data after allo-HSCT, with LFS probabilities lower in poor responders but showing an increase in good responders. This suggests that allo-HSCT could, in part, minimize the prognostic impact of clearing all targets after one course. Patients with favorable cytogenetics were always good responders in our study, and when we focused on patients with an intermediate-risk karyotype, clearance of all events lost all prognostic impact. The analysis of the genetic landscape in CR indicated that this was mainly due to the frequent persistence of pre-leukemic hematopoiesis after treatment (persistence of the main CHIP lesions), which had no prognostic impact at two years of follow up. CHIP seems to be resistant to AML treatment, especially in the case of *TET2* or *DNMT3A* mutations, which account for long-term remissions with persistent clonal hematopoiesis.[3,30,34] Furthermore, we identified 3 cases with the emergence of *DNMT3A* mutant cells distinct from the initial AML clone after treatment pressure, as previously described.[35] In several patients, repeated chemotherapy courses did not impact CHIP, and only HSCT led to the exhaustion of the mutant clone. Thus, MRD evaluation focused on CHIP lesions may be of potential interest to monitor post-graft response and the risk of long-term recurrence, as late relapse can occur after the re-evolution of persistent pre-leukemic clones.[3]

The persistence of the two or three first events of the clone above the threshold of 0.4% after one course was predictive of poor outcome in our patients. This result had not been reported in previous studies using thresholds around 2%.[10] Larger prospective cohorts should, however, be investigated to validate these results. Indeed, in our series, the number of patients included is a limitation to the multivariate analysis conclusions. The impact of treatment strategies according to MRD levels and, in particular, the value of HSCT in good responders, according to the different genotype/karyotype subgroups could also not be properly evaluated.

Interestingly, in 5 patients with available material, molecular re-evolution preceded cytological relapse by 1-9 months. This suggests that multi-target MRD monitoring could also be a useful tool for early therapeutic intervention.

Our study indicates that a personalized MRD strategy could easily be used in daily practice. The 122 gene panel represents a suitable and affordable diagnostic tool, and could probably be refined by reducing the number of targets to the 50-60 most frequently mutated genes in AML.[20,28,29] With the combination of this panel and simple cytogenetic analyses, nearly all AML patients may have two or more evaluable lesions, and could be eligible for MRD monitoring. However, in our study, CBF patients were all good responders. Several studies have shown that CBF translocations are the earliest events in the clone,[3,36] and CBF transcripts monitoring has been proven to be associated with prognosis. Consequently, our multi-target strategy appears not to be useful in these patients.

In the current study, MRD analysis was performed using patient-specific HS-NGS assays. To reduce the delays involved in MRD analysis, ddPCR could be an alternative strategy. Indeed, personalized ddPCR probes can be designed soon after the diagnostic NGS screening, and can be made available by the end of the first treatment course. As results from ddPCR and HS-NGS assays are correlated, this clone-specific ddPCR-based strategy should be evaluated in large prospective studies.

In conclusion, our study shows the prognostic value of a personalized clone-specific MRD evaluation that can be used in most AML patients. Detection of two or more events in more than 0.4% of the cells after one course is strongly associated with lower survival, in particular in patients with intermediate-risk cytogenetics. Forty-five consecutive patients were prospectively investigated, but larger studies are needed to confirm the results and to evaluate whether similar ddPCR and FISH based strategies could be useful to guide treatment decisions.

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