Identification of Fusion Gene Breakpoints is Feasible and Facilitates Accurate Sensitive Minimal Residual Disease Monitoring on Genomic Level in Patients With PML-RARA, CBFB-MYH11, and RUNX1-RUNX1T1

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Minimal residual disease (MRD) monitoring in acute myeloid leukemia (AML) plays an important role in outcome prediction, risk-stratification and therapy adjustment as well as in the early detection of impending relapse.1 The most common genetic markers utilizable for MRD monitoring in AML are fusion genes (FG). Either FG themselves or their transcripts (fusion transcripts, FT) can be monitored via quantitative PCR (qPCR). The uniform copy number per leukemic cell makes FG an ideal target for unambiguously interpretable assessment of MRD levels. However, the use of FG-based MRD monitoring has been limited by the laboriousness of PCR-based genomic fusion sequence identification2–4; thus, only the FT-based approach has been widely used so far.1,5 Although highly feasible, it has several flaws. The number of FT copies per leukemic cell is unknown and possibly inconsistent. Moreover, the diagnostic FT expression levels vary significantly among patients (>2 logs),5,6 and in the patients with lower expression levels, the sensitivity of MRD monitoring is thus reduced.

Various methods utilizing the next generation sequencing (NGS) technology have become widely available during the last few years, offering alternative tools for the identification of genomic fusions.7,8 In this study, we investigated the feasibility of genomic breakpoint identification via targeted NGS, the performance of patient-specific assays for genomic breakpoint quantification and the benefit of FG as targets for MRD monitoring in AML patients with PML-RARA/CBFB-MYH11/ RUNX1-RUNX1T1 fusions.

We performed targeted sequencing utilizing hybridization to a custom-designed probe set for target enrichment (Supplementary Fig. 1, http://links.lww.com/HS/A102). With a median sequencing output of 504 k reads mapped to target region, we successfully identified genomic fusion sequences in all 23 studied patients. In 20/23 patients we found >1 fusion sequence (Supplementary Results, Supplementary Table 1, http://links.lww.com/HS/A102), that is, >1 possible MRD target. One fusion sequence per patient was selected to design a qPCR assay for MRD monitoring. All 23 assays were successfully optimized reaching sufficiently deep quantitative range and sensitivity (10−6–10−5) without non-specific, off-target amplification. We used these in-house established assays to quantify FG and standard qPCR assays5 to quantify FT, and assessed MRD levels in 265 follow-up (FU) samples (Supplementary Table 2, http://links.lww.com/HS/A102, Fig. 1A, Supplementary Fig. 2 – 6, http://links.lww.com/HS/A102).

MRD was negative and positive by both the FG-based and FT-based approaches in 136 and 100 samples, respectively, positive only by the FG-based approach in 24 and only by the FT-based approach in 5 samples. Out of 100 “double-positive” samples, in 61 the MRD levels differed by ≤1 log (n=51) or were non-quantifiably positive (NQP) by both methods (n=10). In 32 “double-positive” samples, MRD was higher by >1 log using the FG-based compared to the FT-based approach (n=4), or was quantifiably positive only by the FG-based while NQP by the FT-based approach (n=28). Vice-versa, in 7 “double-positive” samples MRD was higher by >1 log using the FT-based compared to the FG-based approach (n=6) or NQP only by the FG-based approach (n=1).

We analyzed how discrepancy in MRD data translates into the evaluation of response to therapy (Supplementary Table 2, http://links.lww.com/HS/A102, Fig. 1B). In 8/18 patients monitored up to molecular remission (MR), the negativity of both FG and FT was achieved at the same time-point. Of the remaining 10 patients, MRD negativity was reached at an earlier time-point by the FG-based in 2 patients and by the FT-based approach in 8 patients. Besides better assessment of MR, the FG-based approach...
also improved our insight into the dynamics of MRD clearance as it provided quantitative data on MRD levels in 39 samples of 16 patients that were NQ-positive or negative by the FT-based approach. Notably, if early treatment response would be classified according to MRD levels at the end of induction treatment into categories “negative”/*positive <1E−03 (ie, <0.1%)”/*positive ≥1E−03 (ie, ≥0.1%)”, the classification would be skewed depending on the approach in 8/22 patients. Five patients were only low/NQ-positive by the FT- while ≥1E−03 by FG-quantification, 1 patient was positive ≥1E−03 by FT- and <1E−03 by FG-, and 2 patients positive by FG- were negative by FT-based approach.

Conversion from negativity to positivity was detected simultaneously by both methods in 1 patient, only by the FG-based approach in 1 patient and only by the FT-based approach in 2 patients. Altogether, the FG-based assays were more sensitive for MRD detection compared to the FT-based approach. We have analyzed in more detail the differences in target detection sensitivities in the samples with discrepant results of MRD monitoring. The FT
levels in diagnostic samples varied over >3 logs among patients (Fig. 1C, Supplementary Table 2, http://links.lww.com/HS/A102). Importantly, low diagnostic FT expression dramatically limits the sensitivity of FT-detection in FU samples (Supplementary Table 2, http://links.lww.com/HS/A102). We thus expected that the inferior sensitivity of FT-detection compared to FG-detection in the patients with low diagnostic FT levels could significantly contribute to the MRD discrepancy. Surprisingly, in a non-negligible subset of samples, the discrepancies in MRD levels were not convincingly attributable to the differences in sensitivities (Table 1).

In laboratories where NGS-based methods are well established within routine diagnostics (e.g., panel/whole-exome/whole-transcriptome sequencing, NGS-based screening of immunoreceptor gene-rearrangements in acute lymphoblastic leukemia), pooling of the FG targeted sequencing with other NGS-based experiments is fully feasible. This significantly reduces total expenses; in an optimal setting, the final costs of the genomic fusion identification could be even lower compared to the PCR-based approach.

In the subsequent part of our study we showed, that, similarly to the identification of fusion sequences, the optimization of patient-specific qPCR assays was straightforward, facilitated by the sequence uniqueness and thus the lack of non-specific amplification.

When comparing the MRD levels measured by the two approaches, we have encountered both possible types of discrepancy; in a quarter of samples (68/265), MRD levels assessed by the FG-based approach were either significantly higher or lower compared to those assessed by the FT-based approach. The first type of discrepancy (>80% discordant samples), demonstrates that MRD levels can be frequently underestimated by the FT-based approach. Primarily, this can be a result of different sensitivity; indeed, in a majority of samples, the FT approach was less sensitive than the FG-based assay. However, in some samples, the calculated FT detection sensitivity should have been sufficient to yield the same result as the FG-based approach. This data demonstrates that, unlike in the FG approach, the expression level – and thus the target to cell ratio - is inconstant,
which we consider a major pitfall of the FT-based MRD monitoring. The change in expression can go in both directions, as shown by the samples with the second type of discrepancy, where the FT-based MRD levels were significantly higher compared to FG quantification. This situation was less common and, notably, we detected such samples at the earliest treatment time-points in several patients.

Both overestimation and underestimation of MRD levels caused by expression changes and variable levels of FT per cell are certainly undesirable as they skew the evaluation of response to therapy. The only exception could be an earlier detection of molecular relapse, possibly caused in some cases by high FT levels in relapse-driving cells, resulting in an increased sensitivity of its detection. However, our data on this subject is very limited.

Multiple studies have demonstrated that levels of MRD at certain time-points during therapy are highly predictive of patients’ outcome. In the AML subtypes presented in our report, those studies mainly utilized data from FT-based MRD monitoring\(^\text{6,15}\); the predictive value of MRD levels assessed by the FG-based approach has not been evaluated yet. Our study included a limited number of patients, none of which relapsed so far. Thus, we could not compare the prognostic significance of MRD assessed by the two approaches.

In summary, our study shows that both the identification of genomic fusion sequences and the FG-based MRD monitoring are highly feasible in PML-RARA/CBFβ-MYH11/RUNX1T1-positive AML. Quantification of FG, a stable target with a constant level per cell, enables precise assessment of the proportion of positive cells and represent a technically superior tool for the evaluation of therapy response than the so far widely used FT-based monitoring. We believe that our data provides rationale for additional studies addressing the question whether such an improvement of evaluation of response to therapy could translate into an improvement of risk prediction and therapy tailoring – and, finally, of patients’ outcome.

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