Monitoring Minimal Residual Disease in RUNX1-Mutated Acute Myeloid Leukemia

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**Introduction**
Detection of minimal residual disease (MRD) using molecular assessment of different recurrent mutations in acute myeloid leukemia (AML) has evolved during recent years, in an effort to improve prognostication and provide a tool to optimize postremission therapy. Allogeneic hematopoietic stem cell transplant (HSCT) in AML is considered the most efficacious curative approach; however, it comes with a high cost of morbidity and mortality. Thus, the ability to discern high-risk patients according to their MRD status and regardless of their initial pretreatment prognostic markers is highly important. Moreover, monitoring of MRD postremission (prior to or after HSCT) might enable detection of molecular relapse and offer therapy to prevent full clinical relapse. While multiparametric flow cytometry-based MRD has proven validity, it is often less sensitive compared to molecular PCR-based MRD methods and requires a highly skilled laboratory [1]. To date, clinical use of PCR-based MRD detection is limited to nucleophosmin1 (NPM1) [2, 3], WT1, and the core-binding factor translocations [4]. NPM1 is the best-studied marker to date, with several large studies demonstrating that a positive MRD after consolidation treatment correlates with a significantly
shorter over-survival and a higher relapse rate [5]. Moreover, re-appearance of NPM1 after achievement of MRD negativity allows for pre-emptive treatment to prevent full clinical relapse.

RUNX1 is an essential transcription factor involved in hematopoietic differentiation [6]. In the recent 2018 WHO classification, AML with mutated RUNX1 was added, as a new provisional entity [7]. RUNX1 mutations can be detected in up to 15% of adult AML cases, with more than 30 different mutations identified so far, associated with reduced progression-free survival and overall survival [8]. Most of the mutations are clustered in the Runt homology domain and the transactivation domain and more than half are frameshift mutations [9]. RUNX1 mutations are almost mutually exclusive with core binding factor translocations and NPM1, making RUNX1 an alternative MRD marker in these patients [10].

The relatively high frequency of RUNX1 mutations and their stability along disease course and relapse makes them a good candidate for MRD monitoring. A previous study, which utilized next-generation deep sequencing of DNA for RUNX1 mutations in AML, showed that reduction in mutation burden correlates with overall survival [11]. As compared with NGS, quantitative real-time PCR (qPCR) allows higher sensitivity and does not require expensive and complex technology. Herein we report the monitoring of RUNX1 mutation by qPCR and demonstrate its correlation with disease course.

**Methods**

**Mutation Screening**

All newly diagnosed AML patients since 2018 in our institution have been screened for RUNX1 mutations, and positive cases were followed by individualized MRD monitoring. We include in this report results of all patients in whom RUNX1 mutation had been identified and who achieved morphological leukemia-free state or better.

Total WBC was extracted from BM and PB samples: red blood cells were lysed with RBC lysis buffer, WBC washed with PBS, and cell pellet subjected to DNA and RNA preparation. Maxwell® RSC Blood DNA KIT and Maxwell® RSC simply RNA Blood Kit were used for DNA and RNA extraction with Maxwell® RSC Instrument. cDNA was prepared from 2 µg RNA using High-Capacity cDNA Reverse Transcription Kit (cat#AB-4374966; Thermo Fisher Scientific). DNA was screened for mutation with Sanger sequencing or Archer® VariantPlex® myeloid panel. All found RUNX1 mutations were verified at cDNA level. Mutations with nearby sequence were amplified with individual pairs of primers; PCR product was extracted from gel, cleaned up with QIAquick Gel Extraction kit (QIAGEN), and sequenced.

RUNX1 mutant allele quantification and minimal disease monitoring were performed using allele-specific real-time PCR (ASqPCR). Individual PCR system was developed for each patient. The system includes TaqMan expression assay with mutation-specific primers and probe for ASqPCR and plasmid-containing synthetic minigene with patient mutation-specific sequence (ordered from Syntezza Bioscience) for standard curve preparation. Individual PCR system was verified at patient cDNA sample from diagnosis and then used for PCR protocol optimization and temperature gradient testing. The annealing temperature showed the best discrimination between the lowest standard curve point in serial dilution of individual plasmid, and nonspecific amplification of wild-type cDNA controls was selected for minimal disease monitoring protocol. The annealing temperature and PCR sensitivity were mutation-specific and different for each patient. Log reduction detectible range was dependent on percent of mutation in the diagnostic sample and mutated sequence. Plasmids were stored at −20 as aliquots, and a new aliquot was used in every PCR reaction. ABL1 gene expression served as a reference gene and a control of cDNA quality.

**Linearity and Limit of Detection**

The linearity of assay and limit of detection were determined with 6 points standard curve from serial dilution of individual plasmid (in triplicates, last 3 points in 5 replicas). The Ct value of the individual diagnostic sample laid within the detection range of the respective standard curve. At least 6 independent reactions (each from fresh plasmid aliquot) were performed per individual plasmid and average run parameters shown in the online supplementary Table (see www.karger.com/doi/10.1159/000526353 for all online suppl. material). In every reaction, we tested 2 wild-type controls with ABL1 gene copy number ≥32,000 copies per reaction (4 replicates/per sample) and 2 blank controls. Cut-off positivity was determined as a threshold cycle value that was one cycle above Ct value of wild-type control samples amplification at a cycling threshold of 0.05. ABL1 reference gene expression served as a control of cDNA quality was quantified with ABL1 standard curve from ABL1 ipsojen standards (Cat. No./ID: 674791). Follow-up cDNA samples were tested in 6 replicates of 45 cycles ASqPCR reaction. The reaction was performed with AmpliTaq Gold proofreading DNA polymerase in total volume of 20 µL and carried out on StepOne-Plus instrument (Applied Biosystems). RUNX1 and ABL1 reactions were performed simultaneously but separately. Samples were scored as positive when minimum of three positive replicates were detected at Ct above Ct determined as positive cut-off. PCR run includes RUNX1 mutation-specific standard curve from serial dilution of patient-specific plasmid, ABL1 standard curve from ABL1 ipsojen standards, 2 wild-type cDNA controls (6 replicates for each control sample), and 2 blank templet control. See online supplementary Table 1 for additional PCR parameters. Relative MRD change post-therapy and during follow-up was calculated by ∆∆Ct method with correction for PCR efficiency [12]. Expression ratio of RUNX1 mutant transcript (as a target gene) versus ABL1 reference gene at diagnosis served as 100% baseline value.

Detection of RUNX1 mutation was correlated with BM morphology, flow cytometry, and in patients after allogenic HSCT, with qPCR short tandem repeats (STRs) to detect donor chimerism. The retrospective data collection was reviewed and approved by the local Ethics Committee (Hadassah Medical Center, Jerusalem, Israel), approval number 009520 in accordance with the Helsinki Declaration standards. Written informed consent was exempt given the retrospective collection of the data.
Results

Correlation between Disease State and Mutated RUNX1 Transcript Levels

We present the results of the 6 patients with RUNX1-mutated AML level monitoring (Fig. 1a). Four patients received high-dose chemotherapy and proceeded to HSCT with curative intent, and two transplant-ineligible patients received treatment with hypomethylating agents, 1 patient in combination with venetoclax. Full demographic and clinical data are presented in Table 1.

RUNX1 Monitoring in Curative-Intent Treatments

Four patients received induction protocol with high-dose chemotherapy, either anthracycline, with cytarabine (7 + 3 protocol) or FLAG-Ida (fludarabine, cytarabine, and idarubicin); data are presented in Table 2 and Figure 1b. Three patients (patients 1–3) achieved complete remission (CR) by morphology and flow cytometric immunophenotyping, accompanied by significantly decreased, yet detectable levels of mutated RUNX1 (mRUNX1) transcript (1.8%, 17%, 1.4%, compared to diagnosis, patients 1–3, respectively). All patients proceeded to allogenic...
bone marrow transplant with detectable mRUNX1 levels and none achieved 4-log reduction prior to transplant. Patient 1 had mRUNX1 levels 0.33%, 3 months post-transplant. Following reduction in immunosuppression, a further decrease in mRUNX1 transcripts to undetectable levels was noted 12 months post-transplant (Fig. 1b). To date, the patient maintains a remission at a follow-up of 48 months post-transplant, with mRUNX1 levels remaining undetectable. Similarly, patient 2 showed a decrease to 0.0035% in bone marrow aspiration, 3 months post-transplant, followed by negative MRD in peripheral blood 12 months post-transplant. The patient maintains a CR at a follow-up of 25 months. Patient 3 showed a 1-log decrease of mRUNX1 levels at 1 month post-transplant (0.28–0.04%, pre- and post-transplant, respectively) and undetectable levels 3 months post-transplant and is currently in remission at a follow-up of 20 months post-transplant.

Patient 4 was refractory to 7 + 3 induction and showed a minor reduction in mRUNX1 transcript level to 43% compared to diagnosis. The patient received cytarabine (1 g/m²) with an MDM2 inhibitor. Following two cycles of treatment, a CR was achieved by morphology and flow cytometry, with a concordant reduction of mRUNX1 to 0.01% (Fig. 1b) and proceeded to a myeloablative HSCT from MUD. mRUNX1 was undetectable 2 months post-transplant. Three months later, the patient relapsed and unfortunately succumbed to neutropenic sepsis following salvage chemotherapy. mRUNX1 was readily detected by Sanger sequencing at relapse.

**RUNX1 Monitoring in Patients Who Were Ineligible for High-Dose Chemotherapy**

We present 2 patients that received hypomethylating agents with or without venetoclax. Patient 5 received azacitidine-venetoclax. After one cycle of treatment, the patient achieved CR, by BM morphology and flow cytometry, while mRUNX1 was still at 26.1%. With repeated cycles, we observed a continuous decline in mRUNX1 to 0.019% (after 4 cycles). Routine BM examination following eight cycles showed a minor increase to 0.2%, with no evidence of leukemia by morphology or immunophenotyping. A follow-up exam 3 months later demonstrated a marked increase to 9.5%, still in CR by morphology and flow. The patient developed a clinical and laboratory relapse of his leukemia within 3 months of the observed increase. Similar to patient 5, patient 6 achieved CR with a ~3.5-log mRUNX1 reduction to 0.069% after 4 cycles, a level that was maintained for over 2.5 years. Due to a reduction in blood counts, BM analysis was done, showing

| Table 1. Demographics and clinical follow-up data |
|-----------------------------------------------|
| Patient | Sex | Age | De novo | Karyotype | Other mutations | BM blast, % | WBC at dx, 10 E9/L | Induction | Outcome | Consolidation/salvage | Allo-BMT | Follow-up | OS |
|---------|-----|-----|---------|-----------|----------------|--------------|-------------------|-----------|---------|---------------------|---------|------------|----|
| 1       | F   | 18  | Y       | NK        | BCOR, K/RAS, NRAS | 40           | 2.5               | 7+3a      | CR      | HIDAC               | MUD     | 45         | NR |
| 2       | M   | 6   | MDS     | 20-mosaic | None           | 25           | 7                 | FLAG      | CR      | –                   | MUD     | 30         | NR |
| 3       | F   | 59  | Y       | Inv3      | ASXL1, SFB17    | 40           | 4                 | 7+3a      | CR      | –                   | MUD     | 30         | NR |
| 4       | M   | 24  | MDS     | Complex, involving NPM1 | 5.7, 17 | 35 | 4.4 | 7+3a | Refractory | Cytarabine (1 g/m²) ± idasanutlin | MUD | 13 | 10 m | 13 |
| 5       | M   | 74  | Y       | NK        | BCOR, SFB2, TET2 | 80           | 50                | 1         | Decitabine | CR              | –       | –          | –  |
| 6       | M   | 76  | Y       | iso14     | BCOR, SFB2, TET2 | 80           | 50                | 1         | Decitabine | CR              | –       | –          | –  |

FLT3 and NPM1 mutations were assessed for all patients and were not detected. MUD, matched unrelated donor; NK, normal karyotype; CR, complete remission; NR, not reached; HIDAC, high-dose Ara-c; N/A, no available sample for analysis by NGS; OS, overall survival; PFS, progression-free survival; 7+3 – patient 1: cytarabine 100 mg/m² and idarubicin 12 mg/m²; HAM protocol: high-dose cytarabine with mitoxantrone. Patient 2–3: cytarabine 100 mg/m² and daunorubicin 90 mg/m² FLAG.
hypocellularity with no excess blasts by flow. mRUNX1 levels were detected at 20%. Prolonged follow-up demonstrated fluctuating positive mRUNX1 levels for 12 months before the appearance of overt leukemia. The patient received azacitidine and venetoclax and achieved CR by immunophenotyping and normal blood counts, with a 3.3-log reduction in mRUNX1 level.

**Sensitivity and Quantifiable Range of Detection**

In order to have a consistent and reliable standard curve for accurate quantification, we generated plasmids harboring each individual patient-specific RUNX1 mutation. This contributed to an accurate transcript level quantification along the clinical follow-up. The initial amount of material at diagnosis was quite high as evidenced by ABL1 detection (Ct values of 20–21) in most patients. The Ct values of RUNX1 in the diagnostic sample varied (Ct values of 20–26). Sensitivity of individual mutation PCR was $10^{-4}$ and higher in all but 1 patient (Table 3) [13]. The quantifiable detection range (defined as the quantifiable log reduction relative to the diagnosis and measured by RT-PCR of the serially diluted diagnostic samples) was ≥3 (Table 3). Ct value at which negative control samples show signal detection (i.e., false positivity) is affected by the choice of the primers used for individual mutation detection (Table 4). This determines the lowest level at which patient’s sample can be reliably defined as true positive, which influences both the sensitivity and the quantifiable detection range. For additional PCR-related values, see online supplementary Table 1.

To evaluate the possibility of residual RUNX1-mutated clone with no expression, undetectable by RNA analysis, we analyzed parallel DNA-RNA samples by qRT-PCR. The presence of the RUNX1 mutations was detected at diagnosis in all paired DNA-RNA samples. There were no cases of RNA-negative DNA-positive samples, either at diagnosis or during follow-up, suggesting there are no identifiable nonexpressing RUNX1 mutated cells, by the available methods. Of note, the RNA-based technique showed a higher sensitivity of detection in the follow-up samples (online suppl. Table 2).

**Discussion**

**RUNX1 mutation is considered a poor prognostic marker in AML, and according to the ELN guidelines transplant-eligible patients should proceed to allogeneic**
### Table 3. Primers and PCR sensitivity for RNA-based analysis

| Patient | Mutation location | Protein change | Primers for cDNA AS-qPCR: | Probe for cDNA | PCR sensitivity |
|---------|-------------------|----------------|----------------------------|----------------|-----------------|
| #1      | chr21:36252866G>C  | R166G          | Mutation-specific Reverse  | 6-FAM-5′-GCTGAGGCTGAAAT GCTACCGC-3′-MGB | 10^(-4.8) |
|         | exon 4             |                | 5′-GCTTTTCCCTCTTCC ACTaCC -3′ Forward |                      |                 |
|         | c.496C>G           |                | 5′-CTCTGGTCACT GTGATGGCTG-3′ Reverse |                      |                 |
| #2      | chr21:36231792C>T  | D198N          | Mutation-specific Forward  | 6-FAM-5′-CTGCGGCCAGCACGC CATGAGGTTC-3′-MGB | 10^(-4.5) |
|         | exon 5             |                | 5′-ACAGAGCCCATCAAAT TCACAGaGA -3′ Forward |                      |                 |
|         | c.592G>A           |                | 5′-AGGCACGAGGGTTGGA GGTG-3′ Reverse |                      |                 |
| #3      | 1.chr21:36252854 c.507_508dup | G170E          | Forward                      | 6-FAM-5′-GCTGAGGCTGAAAT GCTAC-3′-MGB | 10^(-3.0) |
|         | 2. chr21:36231871 exon5 c.513A>T | K171N          | Mutant-specific Reverse       |                      |                 |
|         |                   |                | 5′-TCAGAGTGAGCTATTCCCTTCTC-3′  |                      |                 |
| #4      | chr21:36206893G>A  | R207W          | Mutation-specific Forward    | 6-FAM-5′-CTGCGGCCGCAGCACGC ATGAGGTTC-3′-MGB | 10^(-4.5) |
|         | exon 7             |                | 5′-CCTGAGATGCGTGG CAATTG-3′ Forward |                      |                 |
|         | c.619C>T           |                | 5′-AGGCGACAGGGTTGGA GGTG-3′ Reverse |                      |                 |
| #5      | chr21:36259278_36259279insA | R72Es*66      | Mutation-specific Forward    | 6-FAM-5′-TGCTTACCGACTGGC GCTGCAAC-3′-MGB | 10^(-5.6) |
|         | exon 3             |                | 5′-CTGCGACATCGACGCA GACAAT-3′ Forward |                      |                 |
|         | c.212_213insT      |                | 5′-GCCATCGGAACATCC CCTAG-3′ Reverse |                      |                 |
| #6      | chr21:36164845_36164846insGA | Original stop-codon lost, results in prolonged protein | Mutation-specific Forward     | 6-FAM-5′-CATCGGCCATGGCAT GTCGCCATG-3′-MGB | 10^(-4.0) |
|         | Exon 8             |                | 5′-GGCGGCTGCCCTCAT CTCTC-3′ Forward |                      |                 |
|         | c.1029_1030 ins.TC |                | 5′-GGCGGCTGCCCTCAT CTCTC-3′ Reverse |                      |                 |

PCR runs included RUNX1 mutation-specific standard curve from serial dilution of patient-specific plasmid (except for patient #2 Aby Layla, which had standard curve prepared from cDNA diagnostic sample) to determine PCR sensitivity. Small letters in primer sequence: noncomplementary nucleotides were included in the sequence to increase discrimination facilities between mutated and wild-type sequence of the primer.

### Table 4. Calculation of quantifiable log reduction

| Patient | ABL1 quantification | RUNX1 quantification | Wild-type control-detected Ct | Quantifiable log reduction relative to the diagnostic sample |
|---------|---------------------|----------------------|-------------------------------|----------------------------------------------------------|
|         | Ct value of detection of diagnostic sample | Ct value of detection of diagnostic sample | Ct value of the highest quantifiable dilution of the diagnostic sample |                            |
| #1      | 21.3                | 24                   | 39                            | 41 5                                                      |
| #2      | 20.5                | 25                   | 36                            | 40 4                                                      |
| #3      | 21.0                | 24                   | 35                            | 35 3                                                      |
| #4      | 21.02               | 25                   | 37                            | 39 4                                                      |
| #5      | 21.7                | 20                   | 35                            | 42 5                                                      |
| #6      | 21.7                | 20                   | 35                            | 42 5                                                      |
HSCT in first CR. Our data support the possible use of mRUNX1 expression as an MRD marker. While the small number of patients precludes full assessment of the prognostic value of mRUNX1 MRD, our findings provide several important insights. The level of mRUNX1 level correlated with disease status in all of our patients and over the range of therapeutic interventions (chemotherapy, HSCT, immunosuppression tapering), with significantly higher sensitivity than could be achieved with standard flow cytometry and STR analysis. Our data regarding follow-up and early detection are limited due to the small number of patients, the variabilities in proliferation between leukemias, and the variability in mutation detection between the patients. While in 1 patient, we were able to detect an increase in mRUNX1 level that preceded clinical relapse by 5 months (patient 5), patient 4 relapsed within 3 months of the last test which was negative for mRUNX1. This might suggest high-risk patients require a closer follow-up, possibly accomplished by monthly assessment of mRUNX1 from peripheral blood, as was similarly reported with core binding factor (CBF) AML [14]. In addition, in patient 6, we observed a discordancy between mRUNX1 levels which rose to 20% with no evidence of leukemia detected in the BM by flow and morphology over 12 months follow-up. Detection of mRUNX1 might indicate that the patients’ cytopenia was due to residual disease on active treatment or pre-existing MDS, and argues against drug toxicity, which was considered initially. Indeed, the patient relapsed 12 months following the re-detection of mRUNX1. The patient was retreated with hypomethylating agent (azacitidine) in combination with venetoclax and achieved CR after cycle one, concomitantly with a marked reduction of mRUNX1 levels, again demonstrating mRUNX1 levels are in correlation with disease status.

To reduce variability, we generated a plasmid that bears the specific mutated transcript for each patient. This allowed us to generate a reproducible dilution curve among different time points and more accurately determine the detection sensitivity of each marker. The method we used is based on analysis of expression level (RNA) of RUNX1 rather than on the allelic frequency at the DNA level as used in amplicon or whole exome NGS. This RNA-based methodology potentially allows for identification of MRD at lower level of sensitivity without imposing additional technical difficulties, as RNA extraction from blood and bone marrow samples is routinely performed in AML patients. Indeed, the sensitivity and quantifiable range were generally high and above what is reported for NGS. Nonetheless, certain mutation-specific PCR reactions carry a lower sensitivity, as observed in patient 3, and should be considered when interpreting the results.

The main limitation of mRUNX1 as an MRD marker compared to NPM1 or CBF is the need to design mutation-specific primers for each patient at diagnosis, due to the high variety of mutations. However, this type of limitation is manageable as it is widely used for patient-specific MRD assessment in acute lymphoblastic leukemia, based on individually sequenced immunoglobulin or T-cell receptor rearrangements [15].

Taken together, our preliminary data show a novel use of mRUNX1 MRD for clinical decision-making in the postremission period. In the post-transplant setting, it may supplement STR data for guiding immunosuppression reduction, as well as guiding prophylactic interventions such as DLI in high-risk patients. In transplant-ineligible patients, it may also guide intensity. Several key questions regarding RUNX1 as an MRD marker await larger, prospective studies. These include the prognostic value of achieving negative RUNX1 MRD prior to transplant, especially in high-risk patients, the relapse-predicting value of post-transplant increase in mRUNX1 level, and the use of various therapeutic interventions for targeting post-transplant MRD positivity.

Statement of Ethics
The retrospective data collection was reviewed and approved by the local Ethics Committee (Hadassah Medical Center, Jerusalem, Israel), approval number 009520 in accordance with the Helsinki Declaration standards. Written informed consent was exempt given the retrospective collection of the data.

Conflict of Interest Statement
No conflicts of interest to disclose.

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Author Contributions
Boaz Nachmias – designed the research, analyzed the data, and wrote the manuscript. Svetlana Krichevsky – designed the research, performed the research, and reviewed the manuscript. Dvora Filon – performed the research and reviewed the manuscript. Ehud Even-Or, Revital Saban, Batia Avni, Sigal Grisariu.
and Shlomzion Aumann – collected the data and reviewed the manuscript. Moshe Gatt – reviewed the manuscript. Vladimir Vainstein – designed the research and wrote the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

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