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Monitoring relapse in post-transplant acute myeloid leukemia requires integrated assessment, with flow cytometry showing the highest efficacy

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Running heads: Flow Cytometry Detects Early AML Relapse Post-SCT

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Disease monitoring after allogeneic hematopoietic stem cell transplant (allo-HSCT) in patients with acute myeloid leukemia (AML) is critical in guiding clinical management and prognostication.[1] Various diagnostic modalities are currently employed to assess disease status post allo-HSCT, including bone marrow morphologic evaluation, multiparameter flow cytometry (MFC) immunophenotyping for minimal residual disease (MRD), polymerase chain reaction (PCR), next-generation sequencing (NGS) analysis, conventional karyotyping, fluorescence in situ hybridization (FISH), and chimerism analysis.[1-5] Each provides valuable information but differs in detection sensitivity. Here, we sought to assess the diagnostic performance of these assays for detecting relapse in AML patients following HSCT.

Briefly, MFC was performed using our previously characterized two-tube, 12-color panel designed to evaluate MRD.[6] NGS analysis was performed using an 81-gene panel covering genes frequently mutated in myeloid neoplasms.[7] Semi-quantitative PCR to detect *FLT3* mutations and qRT-PCR analysis to detect *RUNX1::RUNX1T1* and *CBFB::MYH11* fusion transcripts were described previously.[8,9] For the *NPM1*-targeted PCR-NGS MRD assay detecting the exon 12 hotspot insertion mutations, molecular barcode-tagged primers were utilized to perform PCR amplification, followed by bidirectional paired-end NGS of the PCR products. Conventional karyotyping and FISH analysis was conducted as described previously.[10] Assessment of stem cell engraftment (chimerism) was performed from sorted T-lymphocytes and myeloid cells.[6] The criteria for defining disease relapse and the sensitivities of each assay are provided in **supplementary Table 1**. This study was approved by the Institutional Review Board of our institution.

We reviewed 220 allo-HSCT procedures performed in AML patients with detailed clinicopathologic follow-up information and screened them for disease relapse. A total of 48 patients experienced relapse, including 7 who underwent two allo-HSCT procedures and relapsed after both. This yielded 55 relapse cases that were included for further analysis. The 48 patients included 31 (64.6%) men and 17 (35.4%) women with a median age of 53 years (range, 2-76) at the time of relapse. The median time to relapse following allo-HSCT was 146 days (range, 32-2,809 days).

First, we focused on the specific time point at which disease relapse was detected and summarized the performance of various assays in detecting relapse (**Fig. 1)**. Among these 55 cases, disease relapse was detected by MFC-MRD in 52 cases, NGS in 32 cases, cytogenetic studies in 28 cases, chimerism analysis in 19 cases, PCR in 11 cases, and morphology (≥5% blasts) in 24 cases. In detail, MFC MRD was performed in 54 cases at the time of relapse, of which 52 (96.3%) (cases #1-52, **Fig. 1**) showed aberrant myeloblast populations consistent with relapse. The median percentage of aberrant blasts detected by MFC-MRD was 2.05% of total nucleated cells (range, 0.01% – 83.5%). Of the two cases (#53 and #54) missed by MFC-MRD, relapse was detected by RT-PCR at a disease level of 0.01% in case #53 (AML with *RUNX1::RUNX1T1*) and by targeted NGS-based *NPM1*-MRD testing at a disease level of <0.005% in case #54 (AML with *NPM1* mutation). MFC-MRD was not performed in case #55 at the time of relapse, and the disease relapse was detected by cytogenetics and morphology (**Fig. 1**). NGS was performed in 45 cases at the time of relapse, and among them, 32 (71.1%) showed mutations consistent with relapsed disease (**Fig. 1**). Only 12 (21.8%) cases harbored PCR-detectable genetic abnormalities: *FLT3- ITD* (n=5; #3, #21, #23, #43, and #47) and *FLT3*-(*TKD*) (n=3; #28, #33, and #49) by semiquantitative PCR, *NPM1* (n=1; #54) by targeted PCR-NGS MRD assay, and *CBFB::MYH11* (n=2; #38 and #52) and *RUNX1::RUNX1T1* (n=1; #53) by qPCR. Among those 12 cases, 11 (91.7%) were positive. Cytogenetic analysis was performed in 49 cases, with 28 (57.1%) positive for cytogenetic abnormalities consistent with disease relapse. Chimerism data were available in 39 patients, with 19 (48.7%) demonstrating recipient-derived cells, consistent with disease relapse. All 55 patients were assessed by morphologic examination, and 24 (43.6%) had morphologic evidence of relapse with ≥5% blasts. Considering all diagnostic studies together, disease relapse was detected exclusively by MFC-MRD in 10 cases (#12-17, #19, #35, #36, and #48), accounting for 18.5% (10/54) of total relapsed cases evaluated by MFC-MRD (**Fig. 1**). The relapse in these 10 cases was later confirmed by

other diagnostic methods after a median of 65 days (range, 8-274 days) (**Fig. 2**). A representative case of early relapse detected solely by MFC-MRD is illustrated in **Fig. 3**.

Next, we evaluated the status of the aforementioned diagnostic assays during follow-up. The median follow-up was 5.5 months (range, 0.4 – 90 months). Here, we focused on cases that either had negative results or were not tested by the specific assay at the time of relapse (**Fig. 2**). For MFC-MRD, among the two cases (#53, #54) that were negative at relapse, case #53 became positive after 93 days, while case #54 remained negative after 83 days. Case #55 was not tested at relapse but was positive 18 days later. For NGS, 13 cases were negative, and 10 were not tested at relapse. Of the 13 negative cases, 10 became positive after a median of 59 days (range, 8–272 days). Of the 10 not tested at relapse, 7 were subsequently retested, and 6 became positive. Excluding 3 cases without follow-up NGS, 4 of 52 (7.7%) remained negative throughout follow-up. Three of these had mutations at diagnosis but were consistently negative after relapse; one had no diagnostic mutation with AML driven by a balanced translocation, precluding NGS monitoring. For PCR analysis, among the 12 cases with genetic targets available for PCR-based monitoring, one case (#38) was PCR-negative at the time of relapse. This case was an AML with a *CBFB::MYH11* fusion at initial diagnosis. At relapse, aberrant myeloblasts (0.94% of total nucleated cells) were detected by flow cytometry and demonstrated an immunophenotypic shift compared with the original leukemic blasts. *CBFB* rearrangement was not detected at relapse or in five subsequent follow-up specimens. Instead, a *GATA2* mutation (VAF, 33%) was identified (**Fig. 2**), which was not present at the time of initial diagnosis. These findings illustrate clonal evolution in AML from a *CBFB* rearrangement to a *GATA2* mutation. For cytogenetics, 21 cases were negative and 6 were not tested at relapse. During follow-up, 8 of the 21 negative cases became positive after a median of 82 days (range, 15–274 days), and 2 of 6 untested cases were positive. Overall, 17 cases (30.9%) remained negative throughout follow-up. Of these, 9 had a normal karyotype and 8 had an abnormal karyotype at initial diagnosis. For chimerism, 20 cases were negative, and 16 were not tested at relapse. During follow-up, 7 of 20 negative cases became positive after a median of 84 days (range, 30–274). Of the 16 cases not evaluated at the time of relapse, 10 had chimerism testing during follow-up, and 7 were positive for the detection of recipient-derived cells. Excluding 6 cases without follow-up testing, 16 of 49 (32.7%) remained negative throughout, mostly associated with low blast counts. For morphology, 31 cases showed no increase in blasts at relapse; 12 later developed ≥5% blasts after a median of 68 days (range, 5–876 days). Thus, 19 of 55 (34.5%) showed no morphologic relapse during the entire follow-up period.

Finally, we explored treatment and outcome. Treatment regimens after disease relapse varied considerably among patients (**Supplementary Table 2)**. Two patients (#8, #19) did not receive any treatment due to death shortly after relapse. One (#52) was managed with tapering of immunosuppressive therapy only, and the remaining patients received various interventions, including chemotherapy, targeted therapy, and 14 with a second allo-HSCT. During follow-up (median 5.5 months; range, 0.4 – 90 months), 31 patients died. We compared the overall survival (OS) of patients whose initial relapse was only detected by MFC-MRD to the remaining patients, and there was no statistically significant difference between these two groups (median: 11.6 vs. 9.4 months; p=0.28; **Supplementary Fig. 1A**). This underscores the clinical relevance of MFC-detected relapse and highlights its value as a sensitive and meaningful tool for post-transplant disease monitoring. We also compared the OS of relapses detected by morphology (blasts ≥5%, n = 24) versus those by ancillary tests only (blasts <5%, n = 31), the difference was not statistically significant (median, 7.1 vs 13.4 months; p=0.18) (**Supplementary Fig. 1B).** This finding suggests that, in the post-transplant setting, patient outcome is primarily influenced by the detection of leukemic cells, and the leukemic burden at the time of detection plays a less important role.

In summary, our study demonstrated that the MFC-MRD assay outperformed other techniques by providing reliable and earliest identification of relapse post-allo-HSCT. At relapse (**Fig. 1**), MFC-MRD demonstrated the highest

positivity of 96.3%, followed by PCR assays at 91.7%, NGS at 71.1%, cytogenetics at 57.1%, chimerism analysis at 48.7%, and morphologic evaluation at 43.6%. Notably, 18.5% of relapses were first detected by MFC-MRD while other methods remained negative, underscoring its overall superior sensitivity in detecting early disease recurrence. In every such case, AML relapse was subsequently confirmed by other diagnostic modalities during follow-up (after a median interval of 65 days), affirming the clinical validity of MFC-MRD. These findings underscore the preeminent role of MFC in early relapse surveillance, enabling detection of relapsing disease well before overt relapse in a significant subset of cases. MFC has gradually become an integral tool in routine clinical practice for detecting relapsed disease in AML.[2,3,6,11] Multiple studies have demonstrated that post-SCT AML MRD detection by MFC carries significant prognostic value.[3,12-14]

Molecular MRD assays, especially qPCR methods, often provide higher analytical sensitivity than MFC-MRD. In our cohort, two cases (#53, 54) negative by MFC-MRD were found to have disease relapse by molecular MRD testing at levels of 0.01% and <0.005% respectively. These results align with the validated sensitivity of our MFC assay (~0.1 to 0.01%) and illustrate that highly sensitive molecular target-specific assays can detect relapse at levels below the threshold of MFC.[6] However, the applicability of molecular MRD testing is inherently restricted to AML subsets with established molecular targets such as *RUNX1::RUNX1T1*, *CBFB::MYH11* rearrangements, and *NPM1* mutations,[1,2] which accounted for only 21.8% cases in our cohort. Moreover, molecular MRD testing is incapable of detecting relapse in cases where clonal evolution leads to the loss of the original molecular targets (such as #38 in our cohort). Conventional NGS, cytogenetic, and chimerism studies generally lack the sensitivity required for early MRD detection.[1,5] Additionally, some AML cases do not harbor cytogenetic abnormalities or somatic mutations that can be reliably used for relapse monitoring. Collectively, the limitations of these assays underscore the indispensable role of MFC, which can be applied broadly across all cases, irrespective of morphologic features, immunophenotypic profiles, mutational status, or cytogenetic abnormalities. It is a more universally applicable approach to post-transplant relapse surveillance. Another advantage of MFC-MRD is its rapid turnaround time (Supplementary Table 1). Among all monitoring methodologies evaluated in this study, MFC-MRD has the shortest turnaround time.

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Figure Legends

Fig. 1: The result of various diagnostic tests at the time of disease relapse. In the 'Blasts%' column, red indicates a blast count ≥5%, while grey represents <5%. In all other columns, red denotes a positive result, grey indicates a negative result, and white indicates that the test was not performed. **MFC**, multiparameter flow cytometry; **NGS**, next-generation sequencing; **PCR**, polymerase chain reaction. The criteria for defining disease relapse and the sensitivity of each assay are provided in Supplementary Table 1. In this study, we separated the highly sensitive PCR-NGS based *NPM1* MRD assay from the regular NGS assay and categorized it under PCR due to its high sensitivity.

Fig. 2: The result of various diagnostic tests during the follow-up after disease relapse. X axis: days after allo-HSCT. Y axis: case #. Of note, only odd-numbered cases are labelled due to space limitations. MFC, multiparameter flow cytometry; NGS, next-generation sequencing; PCR, polymerase chain reaction; FISH, fluorescence in situ hybridization. Each row represents an individual patient, and the length of the horizontal line corresponds to the duration of follow-up after the first detected relapse. In a few cases (#11, #23, #50, #51, and #52), the follow-up duration exceeded 1,000 days, fully illustrated in the figure. Notably, an abnormal result from any single assay routinely triggers additional evaluation using other assays if they have not already been performed. Once early disease relapse is established, patients are closely monitored with regular follow-up or initiate treatment as clinically indicated.

Fig. 3: Dynamic monitoring of leukemia cell burden using multiple ancillary tests in a patient with AML before and after allo-HSCT

A. This corresponds to case #12 in Figs. 1 and 2. At initial AML diagnosis, flow cytometry identified 47% aberrant blasts. Next-generation sequencing (NGS) revealed mutations in *JAK2* and *NRAS*, and conventional karyotyping showed del(16). Morphologic evaluation showed 51% blasts.

B. One month post-transplant, all assays showed normal results.

C. Three months post-transplant, flow cytometry detected a small aberrant blast population (red), representing 0.15% of total cells. This population corresponded to a minor subset of blasts (circled in A) present at initial diagnosis, consistent with early relapse. In addition to the aberrant expression of CD7, CD34-positive myeloblasts also showed multiple other immunophenotypic abnormalities, including decreased CD38 expression and increased expression of CD34, CD54, and CD123 (not shown). The results from all other assays remained normal.

D. Four months post-transplant, the patient developed a full relapse, with all assays showing abnormal findings. In addition to del(16), conventional karyotyping revealed additional abnormalities, indicating clonal evolution.

Note: on flow cytometry plots, red indicates aberrant blasts, and black indicates normal myeloid precursors.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55
MFC
NGS
PCR
Cytogenetics
Chimerism
Blasts%

MFC
NGS
PCR
Conventional karyotype
FISH
Chimerism
Morphology
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19
17
15
13
11
9
7
5
3
1
0
1
10
30
50
70
90
100
400
700
Detected at the time of relapse
Emerged during the follow-up (days)

Flow Cytometry	47% aberrant blasts	0.1% normal blasts	0.15% aberrant blasts	15.4% abnormal blasts
NGS (*JAK2* and *NRAS*)	Positive	Negative	Negative	Positive
Karyotype	46,XY,del(16)(q21q24)[11] /46,XY[9]	Normal	Normal	46,XY,+1,dic(1;14)(p13;q32)del(16)(q21q24)[13]/46,XY,+1,dic(1;2)(p13;q37),del(16)(q21q24)[1]/46,XY[6]
Chimerism	NA	100% donor	100% donor	99% donor
Morphology	51% blasts	2% blasts	2% blasts	22% blasts

Supplementary Table 1. The summary of diagnostic assays used in this study

Assays		Criteria for relapse	Sensitivity
MFC		1) Detection of aberrant myeloblasts with an immunophenotype similar to that of the original blasts. 2) Detection of aberrant myeloblasts with an immunophenotype different from original blasts, with underlying clonal hematopoiesis, preleukemic cells, or dysplastic clones excluded.	0.1-0.01%
NGS		The detection of the same mutations in the pre-SCT myeloid neoplasms, or the emergence of any new mutations, excluding those typical of clonal hematopoiesis of indeterminate potential (CHIP).	2-5%
PCR	Semi-PCR	Detection of *FLT3* internal tandem duplication (ITD) mutation and mutations involving codons 835 and 836 in the tyrosine kinase domain (TKD). Positive results indicate relapse.	1%
	qRT-PCR	Detection of *RUNX1::RUNX1T1* and *CBFB::MYH11* fusion transcripts. Positive results indicate relapse.	0.01-0.001%
	NPM1 PCR-NGS	Detection of *NPM1* exon 12 hotspot insertion mutations. Positive results indicate relapse.	0.005%
Cytogenetics		1) Detection of cytogenetic abnormalities that were present at initial diagnosis. 2) Emergence of the recipient's sex chromosome in sex-mismatched SCT. 3) Emergence of new clonal karyotypic abnormalities after excluding donor-derived clonal cytogenetic abnormalities of unknown clinical significance.	5% (Karyotyping) 0.5% (FISH)
Chimerism		Detection of recipient DNA(≥ 1%) in addition to donor-derived DNA (mixed chimerism) using sorted T lymphocytes and myeloid cells from BM or PB aspirate samples.	5%

Note: MFC: multiparameter flow cytometry (MFC); NGS: next-generation sequencing; PCR: polymerase chain reaction; MRD: minimal residual disease; SCT: Stem cell transplant; FISH: fluorescence in situ hybridization; BM: bone marrow; PB: peripheral blood.
The approximate turnaround time for the assays used in this study were as follows: MFC, ~12-24 hours; PCR-based assays, ~48- 72 hours; NGS, ~72 hours; conventional karyotyping, ~5 days; FISH, ~24-72 hours; chimerism analysis, ~48-72 hours; and morphologic evaluation, ~24-72 hours.

Supplementary Table 2. Treatment after post-transplant disease relapse

No.	HMA/VEN-based	Intensive chemotherapy	Targeted therapy	Immunotherapy	DLI	IST	Second allo-HSCT	No treatment	Therapy in detail
1	√								1 cycle of Venetoclax + Azacitidine
2		√	√						Cytarabine; Revumenib; 1 cycle of Fludarabine + Cytarabine; Ziftomenib; Venetoclax; Camibirstat
3	√		√						1 cycle of Decitabine + Venetoclax + Quizartinib
4				√					1 cycle of Nivolumab + Ipilimumab
5	√								1 cycle of Azacitidine + Venetoclax
6				√			√		4 cycles of Vibecotamab; allo-HSCT
7	√								1 cycle of Cladribine + low-dose cytarabine + Venetoclax
8								√	No treatment
9	√						√		Decitabine; allo-HSCT
10	√			√	√				7 cycles of low-dose Azacitidine +Venetoclax; DLI × 2; 1 cycle of Hu8F4; Azacitidine
11			√						74 cycles of Venetoclax + Ivosidenib
12	√								7 cycles of Azacitidine + Venetoclax + Ruxolitinib; 1 cycle of Cladribine + low-dose Cytarabine + Venetoclax
13		√	√						1 cycle of Venetoclax+ Cladribine + Cytarabine + Idarubicin + Gemtuzumab ozogamicin
14				√					2 cycles of Ipilimumab + Nivolumab
15			√						2 cycles of decitabine + Ivosidenib
16	√								1 cycle of Cladribine + low-dose Cytarabine alternating with Decitabine + Venetoclax
17							√		Allo-HSCT
18	√	√	√						1 cycle of Revumenib + Cobicistat; 1 cycle of Venetoclax + Azacitidine; 1 cycle of Vyxeos
19								√	No treatment
20	√	√	√		√				5 cycles of Azacitidine; DLI × 2; 1 cycle of Emavusertib monotherapy; 1 cycle of Cladribine + Idarubicin + Cytarabine + Venetoclax; 1 cycle of IRAK4 inhibitor
21		√	√	√					1 cycle of Fludarabine + Idarubicin + Gilteritinib + Blinatumomab

22	√		√			1cycle of Cytarabine + Decitabine + Venetoclax + Revumenib
23					√	Allo-HSCT
24	√				√	1 cycle of Cladribine + Cytarabine + Venetoclax; allo-HSCT
25	√		√			1 cycle of Cladribine + low-dose Cytarabine + Venetoclax + Enasidenib
26			√			VIP943 (targeting CD123)
27		√				1 cycle of Cladribine+ low-dose cytarabine
28	√					1 cycle of Azacitidine + Venetoclax + Magrolimab; 1 cycle of Cladribine + low-dose Cytarabine alternating with Decitabine
29	√		√		√	5 cycle of Decitabine + Venetoclax + Revumenib; allo-HSCT
30	√					1 cycle of Azacitidine + Magrolimab + Venetoclax
31	√	√				1 cycle of Azacitidine + Venetoclax; 1 cycle of Cladribine + Idarubicin + Cytarabine + Venetoclax
32		√	√		√	1 cycle of Cladribine + Idarubicin + Cytarabine +Dexrazoxane + Venetoclax; 2 cycles of Revumenib + Venetoclax; allo-HSCT
33	√		√	√		1 cycle of Azacitidine + Venetoclax + Gilteritinib; DLI × 2; 1 cycle of Momelotinib + Gilteritinib
34		√			√	1 cycle of Cladribine + Idarubicin + Cytarabine + Venetoclax; allo-HSCT
35	√					1 cycle of Cladribine + low-dose Cytarabine + Venetoclax
36	√		√			4 cycles of Decitabine; 2 cycles of Azacitidine + Venetoclax+HC-7366 (CDK9 inhibitor)
37					√	Allo-HSCT
38	√					4 cycle of Venetoclax + Decitabine; 1 cycle of Gemtuzumab ozogamicin
39			√			Sorafenib
40	√		√		√	1 cycle of Venetoclax+ Decitabin-cedazuridine+ Enasidenib; allo-HSCT
41			√		√	1 cycle of IMGN632; allo-HSCT
42					√	Allo-HSCT
43	√		√			1 cycle of Azacitidine + Venetoclax + Enasidenib
44	√		√		√	1 cycle of Decitabine + Venetoclax + Enasidenib; allo-HSCT

45	√	√	√				1 cycle of Omacetaxine + venetoclax; 1 cycle of Azacitidine + Venetoclax + ADI–PEG; 1 cycle of Fludarabine + high-dose Cytarabine + Filgrastim + Venetoclax; 1 cycle of Revumenib
46		√					1 cycle of Cyclophosphomide + Cytarabine + Topotecan + Go
47		√	√				2 cycles of Cladribine + Idarubicin + Cytarabine + Quizartinib
48	√						1 cycle of Decitabine-cedazuridine; 1 cycle of Venetoclax + Azacitidine
49	√		√			√	1 cycle of Decitabine + Venetoclax + Gilteritinib; allo-HSCT
50			√				Decitabine; Bortezomib
51	√						1 cycle of Venetoclax + Azacitidine,
52					√		Immunosuppressor tapering
53	√		√				1 cycle of Revumenib; 1 cycle of Clad + low-dose Cytarabine + Venetoclax
54	√		√				1 cycle of Cladribine + low-dose Cytarabine + Venetoclax + Revumenib
55	√						1 cycle of Venetoclax + Azacitidine

HMA/VEN: hypomethylating agent/Venetoclax; DLI: donor lymphocyte infusion; IST: immunosuppressor taper; allo-HSCT: allogeneic-hematopoietic stem cell transplantation.

Supplementary Figure 1: Overall survival following disease relapse after hematopoietic stem cell transplantation (HSCT).

Supplementary Figure 1 legend:

A. Comparison of overall survival (OS) between patients with relapse detected exclusively by MFC (n=10) and those with relapse identified by others, including MFC (n=45).
B. Comparison of OS between cases with morphologic relapse (blasts ≥5%, n=24) and patients with MRD relapse (blasts <5%, n=31).

For each patient, the date of disease relapse was designated as day 1, with all subsequent time points calculated from this reference date. OS was defined as the time from disease relapse to death or the last follow-up. For the seven patients who relapsed after both their first and second allo-HSCT, survival following the first transplant was censored at the time of the second transplant, and OS following the second transplant was calculated from the date of relapse after the second transplant to the time of death or last follow-up.