Multicolor flow cytometry and multi-gene next generation sequencing are complementary and highly predictive for relapse in Acute Myeloid Leukemia following Allogeneic Transplant

Bartlomiej M. Getta, MBBS[1], **Sean M. Devlin, PhD**[2], **Ross L. Levine, MD**[3,4], **Maria E. Arcila, MD**[5], **Abhinita S. Mohanty**[5], **Ahmet Zehir, PhD**[5], **Martin S. Tallman, MD**[3], **Sergio A. Giralt, MD**[1], **Mikhail Roshal, MD PhD**[6]

[1]Memorial Sloan Kettering Cancer Center, Bone Marrow Transplant Service,

[2]Memorial Sloan Kettering Cancer Center, Department of Epidemiology and Biostatistics,

[3]Memorial Sloan Kettering Cancer Center, Leukemia Service,

[4]Memorial Sloan Kettering Center for Hematologic Malignancies,

[5]Memorial Sloan Kettering Cancer Center, Diagnostic Molecular Pathology Laboratory,

[6]Memorial Sloan Kettering Cancer Center, Department of Laboratory Medicine

Abstract

Minimal Residual disease (MRD) in acute myeloid leukemia (AML) is typically measured using multi-parameter flow cytometry (MFC). Detection of leukemia mutations using multi-gene next generation sequencing (NGS) can potentially be used to measure residual disease. We used a targeted 28-gene NGS panel to detect mutations and different-from-normal 10-colour MFC to measure MRD in AML patients before allogeneic hematopoietic stem cell transplant (HCT). Residual disease was defined when any abnormal blast population was detected using MFC and when any leukemia allele was detected with a variant allele frequency (VAF) 5% using NGS. We tracked the clearance of leukemia alleles between AML diagnosis and immediately before HCT and found that mutations in *DNMT3A*, *TET2* and *JAK2* were less likely to be cleared than *NPM1*, *IDH 1/2* and *FLT3-ITD*. Despite varying sensitivities, the concordance rate of residual disease detection before HCT using the two assays was 44/62 (71%) evaluable cases. Discordance could be explained by residual mutations in *DNMT3A* and *TET2* that were not detected by MFC and presence of residual leukemia mutations with VAF below the established thresholds for mutation calling. Presence of flow MRD and residual mutations immediately before HCT using the two assays was associated with relapse risk (MFC: HR=4.62 95%CI 1.32–16.09, p=0.016 and NGS: HR=4.35 95%CI 1.63–11.6, p=0.003) and survival (MFC: HR=2.44 95%CI 1–5.97, p=0.05 and NGS: HR=2.1 95%CI 0.97–4.55, p=0.059) after HCT. Residual disease detected concurrently by

Corresponding author: Name: Bartlomiej Getta, gettab@mskcc.org and b.getta@gmail.com, Address: 1275 York Avenue New York, NY 10065 USA.

MFC and NGS conferred the highest relapse risk compared to patients who were either negative by both assays or had discordant status (overall, p=0.008). While MFC is universally applicable, a multi-gene NGS approach to measuring residual disease in AML provides additional information on differential clearance of disease alleles and can assess clonal architecture before transplant.

Keywords

minimal residual disease; flow cytometry; next generation sequencing; Acute myeloid leukemia

Introduction:

Next generation sequencing (NGS) has shown that acute myeloid leukemia (AML) is a genetically heterogeneous disease composed of multiple clones each characterized by combinations of somatic molecular mutations. Sequencing at various time points highlights a specific order in which mutations are acquired. Founder mutations typically occur in genes involved in epigenetic regulation (*DNMT3A, TET2, ASXL1*). While not enough to cause leukemia on their own, these mutations confer replicative advantage to affected hematopoietic stem cells, a state referred to as clonal hematopoiesis of indeterminate potential (CHIP)[1]. Secondary genetic events typically arising in genes involved in signal transduction (eg. *FLT3, RAS*) have the capacity to transform these cells into AML[1]. Tracking these leukemia alleles can be used as a method of monitoring treatment response.

Studies that track disease specific mutations as markers of AML treatment response have shown differences in the probability of clearing clones characterized by specific mutations[2,3]. In these studies, mutations in *DNMT3A* and *TET2* had a lower probability of clearance, while *NPM1, FLT3* and *IDH1/2* mutations were more likely to be cleared. Despite this, detection of any residual mutation following induction chemotherapy was predictive of relapse and survival[4]. The differential clearance of leukemia alleles results in a potential bias when residual disease is measured by tracking single mutations like *NPM1*[5]. PCR based assays, which track single alleles are not applicable to all patients given that leukemia mutations vary in incidence and can arise in various regions of affected genes making it difficult to design universally applicable PCR assays. Multi-gene NGS has the potential to overcome the limitations of single-gene PCR techniques. Detection of residual mutations at the time of HCT using multi-gene NGS has never been correlated with outcome in AML patients undergoing HCT.

Multi-parameter flow cytometry (MFC) leverages aberrant antigen expression on leukemic blasts to detect residual disease and has emerged as a powerful predictor of outcome among patients with AML undergoing HCT[6,7]. A different-from-normal approach may be universally applicable; however, demands analytical and technical expertise[7,8]. This method is imperfect with approximately 20% of MFC negative patients relapsing and a similar percentage of positive patients remaining in remission after transplant[7]. Detection of residual disease by MFC has never been correlated with detection of residual mutations by multi-gene NGS.

Methods:

Study cohort:

104 AML patients who underwent allogeneic transplant at our center between 2014 and 2015 were included. Patients were included if NGS was performed at AML diagnosis (n=58), or if NGS (n=83) or MFC (n=77) was performed immediately before HCT. Not all patients had available samples at all time points (table 1). AML was defined according to WHO AML classification and disease status was defined per the international working group criteria[9]. Relapse was defined as presence of >5% blasts on marrow aspirate. One transplant event per patient was allowed with preference given to events that had residual disease assessment. The median time between the pre-HCT assessment time-point and transplantation was 22 days with no intervening therapy administered between these times. Tandem assessment of residual disease by multi-gene NGS and MFC was performed on the same marrow aspirate in 62 patients who had available samples. Concordant assessment was defined by detection of an abnormal blast population and presence of leukemia mutation(s) with VAF ≥ 5% (F+M+) or absence of abnormal blasts and absence of mutations (F−M−). Discordant results occurred when one assay was positive and the other was negative (F+M− or F−M+). Disease risk index[10], HCT-CI comorbidity score[11] and conditioning intensity[12,13] were defined according to published guidelines. The institutional review board approved the study and consent was obtained from all participants.

Multi-gene NGS

A 28-gene amplicon, capture based NGS assay, designed to identify mutations seen in myeloid neoplasms, was used. Sequenced gene hot-spots are described in the supplementary table 1. Details of this assay and validation have been published[14] and are outlined below.

Sample processing: DNA was extracted from bone marrow aspirates using Qiagen DNAeasy blood kit (Quiagen, Venlo, Netherlands). DNA (500ng) was fragmented using an E-series DNA Sonicator (Covaris, Woburn, MA) and purified using Quiagen MinElute Clean-up (Qiagen). The extracted template DNA and custom primers were merged using the RainDance Thunderstorm instrument to form droplets for amplification (RainDance Technologies, Billerica, MA). Emulsion PCR was performed (RainDance Technologies) using RainDance Droplet Stabilizer and Platinum High Fidelity Taq (Life Technologies; following vendor protocol). Adaptor tails were added to the 5' ends of the sequence-specific regions of amplicon primers to facilitate secondary PCR. The emulsion was broken and PCR products were recovered using RainDance Droplet Destabilizer (RainDance Technologies) and Qiagen MinElute Clean-up. 10ng of this amplified library was sampled (Agilent Technologies, Santa Clara, CA) for secondary PCR. Custom designed reverse primers (Integrated DNA Technologies, Coralville, IA) with integrated unique Illumina Truseq barcodes and universal forward primers were used for secondary PCR. The product was recovered using Qiagen MinElute Clean-up and 15ng of this library product was sampled using Agilent BioAnalyzer system Agilent. The library pool was quantified using a Qubit dsDNA Broad Range Assay kit, diluted and loaded into a MiSeq 300 cycle reagent kit (Illumina, San Diego, CA) for sequencing. Custom sequencing primers from Integrated DNA Technologies were loaded into the MiSeq cartridge for forward and turn-around read

for 150-base pair paired-end sequencing. Sequencing was performed using a Illumina MiSeq v.2 instrument (Illumina) with Illumina MiSeq Library QC sequencing workflow.

Variant Calling: A Hapmap sample was used as a normal control. Variant calling was performed on target regions that had bidirectional coverage. Variants had to be present on at least 4 reads in each direction. Variants were annotated with Annovar and checked against external databases (COSMIC, Single Nucleotide Polymorphism database and 1000 Genomes). Variant calls were filtered for their annotation as non-synonymous, exonic variants with clear impact on protein function. Variants annotated as 'synonymous' or 'silent' were removed, along with variants annotated as being located in introns, UTRs, ncRNA or intergenic regions. Coding variants including missense, nonsense, splice-site and frameshift mutations or in-frame indels were catalogued. Know polymorphisms were removed after correlation with the dbSNP database and 1000 genomes. Final mutation calls were reviewed by a molecular pathologist. A minimum threshold variant frequency of 5% was used to call mutations at diagnosis and to identify residual mutations on pre-HCT samples. The catalogued mutations from diagnostic and pre-HCT samples are included in supplementary table 2.

Qualitative testing for *FLT3* ITD and TKD were performed separately using a sizing assay with a sensitivity of 5% (performed locally or InVivoscribe).

Flow cytometry MRD

Ten-color multi-parameter flow cytometry (MFC) was performed on marrow aspirates before and after HCT. An abnormal population was identified by visual assessment of populations with antigen expression that was 'different-from-normal' as described in prior publications[6,7]. Antibody panels are included in supplementary table 3. Briefly, up to 1.5 million cells in 100–200 microliters of freshly drawn aspirate sample were stained with three panels, washed with phosphate-buffered saline containing 0.1% bovine albumin and lysed and fixed with ammonium chloride and 0.1% formalin solution. Following the last wash, samples were acquired on FACS-Canto-10 cytometers (BD Biosciences, San Jose CA). The results were analyzed using custom Woodlist software (generous gift of Wood BL, University of Washington). The assay is able to detect abnormal populations down to sensitivity of approximately 1 in 1000 events provided at least 100 000 events are acquired in each of the 3 panels used. MRD detected using MFC was defined by the presence of any abnormal bone marrow blast population meeting the above criteria.

Statistics:

VAFs of patient specific mutations quantified at diagnosis and pre-HCT were compared using paired t-test. Kaplan-Meier methods were used to estimate overall survival, and univariate survival comparisons were evaluated using Cox proportional hazards regression. Cumulative incidence functions were used to estimate the incidence of relapse based on MFC and NGS pre-HCT status, treating death in the absence of relapse as a competing risk. Cause-specific Cox regression was used to compare relapse risk based on pre-HCT characteristics. No adjustments were made for multiple comparisons. All analyses were conducted in R v.3.2.3.

Results:

Patient characteristics

Disease and transplant variables for all included patients are described in Table 1. The majority had primary AML (63%) with intermediate risk DRI (68%) and normal karyotype (44%). In total 21 (21%) relapsed and 36 (35%) died, 13 due to leukemia and 23 due to other transplant related causes. The median follow-up amongst survivors from the time of transplant was 13 months (range 6–26 months).

Mutation clearance prior to HCT

Multi-gene NGS was performed at diagnosis and immediately before HCT in 47 patients, 40 (85%) of whom had somatic mutations allowing measurement of the clearance rate of these leukemia alleles. Allele clearance was defined by a reduction in the mutant gene VAF to <5%. These patients had various induction and consolidation therapies (supplementary table 4); however, 44 (93%) received cytarabine based induction and 21 (45%) received at least one cycle of consolidation. Clearance of all mutations was seen in 22/40 (45%) patients. There were 26 patients with multiple mutations at diagnosis, 5 did not clear any mutation, 7 cleared at least one mutation and 14 cleared all mutations. Mutations were rarely cleared if they occurred in the following genes: *JAK2 (0/4, 0%)*, *DNMT3A (3/11, 27%)* and *TET2 (5/10, 50%)* while mutations in the following genes were frequently cleared: *NPM1* (8/9, 88%), *IDH 1/2* (9/11, 82%) and *FLT3* ITD was cleared in 15/20 (75%).

To quantify the absolute change in mutant allele burden we compared the VAF of mutations called at diagnosis with the corresponding mutant VAF measured immediately before HCT (figure 1A). The mean VAF of *RUNX1* (0.47 vs 0.13, p=0.012), *NPM1* (0.14 vs 0.01, p=0.006), *IDH* 1/2 (0.25 vs 0.03, p=0.002), *TET2* (0.41 vs 0.14, p=0.008) and *TP53* (0.32 vs 0.12, p=0.049) were significantly lower pre-HCT, while there was no significant difference in *DNMT3A* (0.24 vs 0.15, p=0.228) and *JAK2* (0.52 vs 0.27, p=0.156) across the two time points.

Comparison of flow cytometry and NGS residual disease assessment

Tandem assessment using both MFC and multi-gene NGS on the same marrow aspirate before HCT was performed on 62 patients allowing for direct comparison between the two assays. Concordant results were seen in 44 (71%) cases. Four had no MRD identified by MFC (F−) but had mutations identified with VAF >5% (M+) (ie. F−/M+) and 14 had detectable MFC MRD but no identified mutations (F+/M−) (table 2).

Patients in the F−M+ group had residual mutations in either *DNMT3A* or *TET2* with VAF >5%. Eight of fourteen patients in the F+/M− had NGS performed at AML diagnosis that identified at least one somatic mutation, which could be tracked at the time-point prior to HCT. On manual review of pre-HCT samples, 6/8 of these patients had residual disease specific alleles; however, these were present at a VAF below the established thresholds (<5%) for mutation calling (table 2).

The residual disease burden of patients who were in CR immediately before transplant was quantified using tandem assessment by NGS and MFC. Seventeen patients were in hematologic CR or CRi at this time-point but had residual mutations identified using NGS and had MRD assessed by MFC (Figure 1B). The average VAF of residual mutations in *DNMT3A* (n=9), *TET2* (n=7) and *JAK2 V617F* (n=4) assessed on bulk bone marrow was markedly higher than the percentage of aberrant blasts measured on the same bone marrow aspirates: 19% vs 0.72%; 29% vs 0.64% and 32% vs 0.53%, respectively (figure 1B). This suggests that residual mutations are likely to be present in non-blast cells in patients who are in hematologic CR/CRi.

Disease variables associated with transplant outcome

Univariate analysis of factors associated with relapse and survival are presented in table 3. Disease related variables and the HCT-CI score were associated with transplant outcomes, while transplant variables such as donor source, HLA matching, conditioning intensity and GVHD prophylaxis were not.

Presence of MFC MRD and residual mutations pre-HCT was associated with relapse and survival. For MFC (n=77), the 18-month relapse incidences for MRD positive (F+) and MRD negative (F−) groups were 37% (95% CI: 21–53%) and 9% (95% CI 2–21%) respectively (p=0.016). For NGS (n=83), the 18-month relapse incidence for those with (M+) and without (M−) residual mutations was 39% (95% CI: 22–56%) and 12% (95% CI: 5–22%) respectively (p= 0.003).

For overall survival, the estimated 18-month OS for F+ and F− was 48% (95% CI: 31–73%) and 73% (95% CI: 57–94%), respectively (p=0.050). For NGS, the estimated 18-month OS for M+ and M− was 54% (95% CI 38–77%) and 69% (95% CI 54–87%), respectively (p=0.059) (figure 2A–D).

Tandem NGS and MFC assessment predicts relapse and survival

Patients with discordant residual disease assessment appeared to have a low relapse incidence (3/18, 16%) compared to those who were positive by both methods (10/20, 50%). The two methods were combined for those who had tandem assessment (n=62). Patients in the F+/M+ group (n=20) had the highest incidence of relapse with an 18-month estimate of 51% (95% CI: 26–71%) compared to those who were F−/M− (n=24) who had an 18-month estimate of 8% (95% CI: 2–24%) and those with discordant results (F−/M+ or F+/M−, n=18) who had a 18-month estimate of 17% (95% CI 4–43%) (overall p=0.008) (Figure 2E).

Using combined assessment, the estimated 18-month survival of patients in the F+/M+ group was 51% (95% CI 30–86%) for (F+/M+), compared to 78% (95% CI 59%–99%) in the F−/M− group and 44% (95% CI 21–92%) in those with discordant RD assessment (F−/M+ or F+/M−) (overall p=0.072) (Figure 2F).

Discussion:

This analysis is the first to show that a targeted multi-gene NGS panel can be used to detect residual mutations in AML patients immediately before allogeneic transplant. For the

first time we compare flow MRD with a multi-gene NGS assay and show that presence of residual mutations as well as atypical blasts are associated with post-transplant relapse and survival. Despite vastly differing sensitivities, the two assays were concordant in 71% of cases; however, the burden of residual disease as measured by residual leukemia alleles was markedly higher than the estimated percentage of aberrant blasts quantified by MFC, suggesting that residual leukemia alleles likely persist in non-blast compartments at the time of remission.

Tracking multiple somatic mutations takes into account both the clonal architecture of AML and the different clearance rates of specific leukemia alleles. Molecular assays that target a single marker include quantitative PCR of core-binding factor fusion transcripts[15] or mutant *NPM1*[16]. Detection of these mutations is associated with outcome; however, assays measuring different markers have different sensitivities in detecting residual leukemia. For instance, measuring residual *FLT3-ITD* alleles is a poor RD marker as this mutation is typically sub-clonal[17]. Conversely, early disease mutations arising in *DNMT3A* may also represent poor disease markers as they often arise in pre-leukemic cells which have unclear malignant potential and have been detected in patients who are in a long-term remission[3,5,16,18]. Multi-gene NGS considers both founder and sub-clonal alleles. NGS techniques are applicable to almost all patients as they can detect and track mutations irrespective of their genomic location. The targeted 28-gene NGS panel applied here was applicable to 49/58 (84%) patients who had at least one somatic mutation identified at diagnosis. A panel with a more extensive gene set and higher analytical sensitivity is likely to improve accuracy in detecting residual sub-clonal and founder mutations and may further improve the concordance rate with flow cytometry.

The clinical significance of residual *DNMT3A and TET2* mutations before HCT is unknown. Patients with *DNMT3A* and *TET2* mutations at diagnosis are highly likely to have these detected at the time of transplant due to the low clearance rate of these alleles with chemotherapy[4,5]. Such patients often had no MRD detected by flow (F−M+ group) and appeared to have a low relapse incidence. This may represent a state of 'clonal remission'. The clinical significance of patients who had residual *DNMT3A* and *TET2* alleles and clearance of high risk kinase mutations *(FLT3, RAS)* requires exploration in a larger cohort.

The high error rate in calling mutations using current NGS methods necessitates setting a high VAF threshold to confidently call mutations. A VAF of 5% was set here based on prior assay validation[14]. Only one other group has used a multi-gene approach to track mutation clearance in AML and a VAF of 2.5% was arbitrarily chosen in that study[4]. MFC can generally detect disease at greater depth (1:1000) and would be expected to have a higher sensitivity than our NGS approach (1:20). Despite this difference in sensitivity we found a high concordance between the assays. The issue of low sensitivity with NGS may be overcome if leukemia specific mutations identified at diagnosis are tracked at serial assessment. However, the threshold at which residual alleles are clinically impactful is unknown. Patients who had MRD detected by MFC but had no mutations called at the pre-defined VAF thresholds (F+M−) often had residual alleles below the necessary threshold for mutation calling. The incidence of relapse among these patients was low suggesting

that a threshold for residual mutations may exist below which they are unlikely to impact transplant outcomes.

The VAF of residual mutations was markedly higher than the quantified percentage of aberrant blasts when compared immediately before HCT. The high VAF of residual alleles suggests that cells in multiple marrow compartments were clonal even in patients who attained morphologic blast clearance (CR/CRi). This group (F−M+) may represent a state of 'clonal remission' where hematopoiesis has reconstituted from progenitors with CHIP-associated mutations but without an aberrant blast population (figure 1b). The clinical significance of this in a pre-transplant setting is unknown. *DNMT3A* mutations can be detected within both lymphoid and myeloid compartments in patients who remain in long term remission following chemotherapy[3]. The only allele whose variant fraction was similar to the quantified percentage of blasts was *NPM1* (supplementary table 5) suggesting that *NPM1* mutations are resident to the blast compartment whereas other alleles are likely to be present in mature myeloid cells and lymphoid cells. This may explain why tracking mutated *NPM* alleles, has been consistently shown to be a valid disease marker in AML[5,19–22]. The clinical significance of 'clonal remission' (F−M+) before HCT requires further exploration.

Important biases pertaining to this analysis include the small cohort size which limits statistical power to perform multivariate analysis and a short follow-up period such that additional relapses may still occur. Analytical issues exist with the application of amplicon-based NGS to monitor residual mutations. The NGS assay applied here was validated to detect somatic mutations at the time of AML diagnosis, rather then to detect low level residual mutations after chemotherapy hence a relatively high variant threshold of 5% was fixed to call mutations. Germline samples were universally unavailable and as a result it is possible that germline polymorphisms have not been efficiently excluded by post analytical bioinformatics methods. This bias is inherent when sequencing liquid tumors where paired germline tissue is not available to filter out private or non-pathogenic polymorphisms. Finally, the NGS panel was restricted to assess only 28 genes and analysis of a more extensive gene list would be expected to increase the likelihood of detecting residual mutations.

Application of multi-gene NGS in the monitoring of residual disease in AML patients undergoing transplant has several benefits. Although the NGS assay was not designed to monitor disease response, monitoring and tracking mutations was prognostic. Our 28-gene assay allowed broad coverage of known mutation hotspots and detected mutations in 84% of patients at diagnosis. The median number of mutations detected was 2 (range 0–5) per patient, which is lower than expected from whole genome sequencing studies, thus the likelihood to detect residual mutations would be greater if more genes were sequenced[23,24]. The clonal composition of hematopoiesis before transplantation appears to be associated with HCT outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References:

1. Welch JS, Ley TJ, Link DC, et al. : The origin and evolution of mutations in acute myeloid leukemia. Cell 150:264–78, 2012 [PubMed: 22817890]

2. Lindsley RC, Mar BG, Mazzola E, et al. : Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. Blood 125:1367–76, 2015 [PubMed: 25550361]

3. Ploen GG, Nederby L, Guldberg P, et al. : Persistence of DNMT3A mutations at long-term remission in adult patients with AML. Br J Haematol 167:478–86, 2014 [PubMed: 25371149]

4. Klco JM, Miller CA, Griffith M, et al. : ASsociation between mutation clearance after induction therapy and outcomes in acute myeloid leukemia. JAMA 314:811–822, 2015 [PubMed: 26305651]

5. Ivey A, Hills RK, Simpson MA, et al. : Assessment of Minimal Residual Disease in Standard-Risk AML. New England Journal of Medicine 0:null

6. Walter RB, Gooley TA, Wood BL, et al. : Impact of pretransplantation minimal residual disease, as detected by multiparametric flow cytometry, on outcome of myeloablative hematopoietic cell transplantation for acute myeloid leukemia. J Clin Oncol 29:1190–7, 2011 [PubMed: 21282535]

7. Araki D, Wood BL, Othus M, et al. : Allogeneic Hematopoietic Cell Transplantation for Acute Myeloid Leukemia: Time to Move Toward a Minimal Residual Disease-Based Definition of Complete Remission? J Clin Oncol 34:329–36, 2016 [PubMed: 26668349]

8. Walter RB, Gyurkocza B, Storer BE, et al. : Comparison of minimal residual disease as outcome predictor for AML patients in first complete remission undergoing myeloablative or nonmyeloablative allogeneic hematopoietic cell transplantation. Leukemia 29:137–44, 2015 [PubMed: 24888275]

9. Dohner H, Estey EH, Amadori S, et al. : Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. Blood 115:453–74, 2010 [PubMed: 19880497]

10. Armand P, Gibson CJ, Cutler C, et al. : A disease risk index for patients undergoing allogeneic stem cell transplantation. Blood 120:905–13, 2012 [PubMed: 22709687]

11. Sorror ML, Maris MB, Storb R, et al. : Hematopoietic cell transplantation (HCT)-specific comorbidity index: a new tool for risk assessment before allogeneic HCT. Blood 106:2912–9, 2005 [PubMed: 15994282]

12. Bacigalupo A, Ballen K, Rizzo D, et al. : Defining the intensity of conditioning regimens: working definitions. Biol Blood Marrow Transplant 15:1628–33, 2009 [PubMed: 19896087]

13. Giralt S, Ballen K, Rizzo D, et al. : Reduced-intensity conditioning regimen workshop: defining the dose spectrum. Report of a workshop convened by the center for international blood and marrow transplant research. Biol Blood Marrow Transplant 15:367–9, 2009 [PubMed: 19203728]

14. Cheng DT, Cheng J, Mitchell TN, et al. : Detection of mutations in myeloid malignancies through paired-sample analysis of microdroplet-PCR deep sequencing data. J Mol Diagn 16:504–18, 2014 [PubMed: 25017477]

15. Yin JA, O'Brien MA, Hills RK, et al. : Minimal residual disease monitoring by quantitative RT-PCR in core binding factor AML allows risk stratification and predicts relapse: results of the United Kingdom MRC AML-15 trial. Blood 120:2826–35, 2012 [PubMed: 22875911]

16. Ivey A, Hills RK, Simpson MA, et al. : Assessment of Minimal Residual Disease in Standard-Risk AML. New England Journal of Medicine 374:422–433, 2016 [PubMed: 26789727]

17. Palmisano M, Grafone T, Ottaviani E, et al. : NPM1 mutations are more stable than FLT3 mutations during the course of disease in patients with acute myeloid leukemia. Haematologica 92:1268–9, 2007 [PubMed: 17768124]

18. Debarri H, Lebon D, Roumier C, et al. : IDH1/2 but not DNMT3A mutations are suitable targets for minimal residual disease monitoring in acute myeloid leukemia patients: a study by the Acute Leukemia French Association. Oncotarget 6:42345–53, 2015 [PubMed: 26486081]

19. Gorello P, Cazzaniga G, Alberti F, et al. : Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (NPM1) gene mutations. Leukemia 20:1103–8, 2006 [PubMed: 16541144]

20. Papadaki C, Dufour A, Seibl M, et al. : Monitoring minimal residual disease in acute myeloid leukaemia with NPM1 mutations by quantitative PCR: clonal evolution is a limiting factor. Br J Haematol 144:517–23, 2009 [PubMed: 19055671]

21. Schnittger S, Kern W, Tschulik C, et al. : Minimal residual disease levels assessed by NPM1 mutation-specific RQ-PCR provide important prognostic information in AML. Blood 114:2220–31, 2009 [PubMed: 19587375]

22. Kronke J, Schlenk RF, Jensen KO, et al. : Monitoring of minimal residual disease in NPM1-mutated acute myeloid leukemia: a study from the German-Austrian acute myeloid leukemia study group. J Clin Oncol 29:2709–16, 2011 [PubMed: 21555683]

23. Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia. New England Journal of Medicine 368:2059–2074, 2013 [PubMed: 23634996]

24. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med 368:2059–74, 2013 [PubMed: 23634996]

Highlights:

- Multi-gene next generation sequencing can be used to track residual disease in acute myeloid leukemia

- Leukemia mutations in *DNMT3A*, *TET2* and *JAK2* are less likely to be cleared than *NPM1*, *IDH* and *FLT3*-ITD.

- Residual disease measured using multi-gene NGS and 10-colour flow cytometry were associated with relapse and survival after HCT
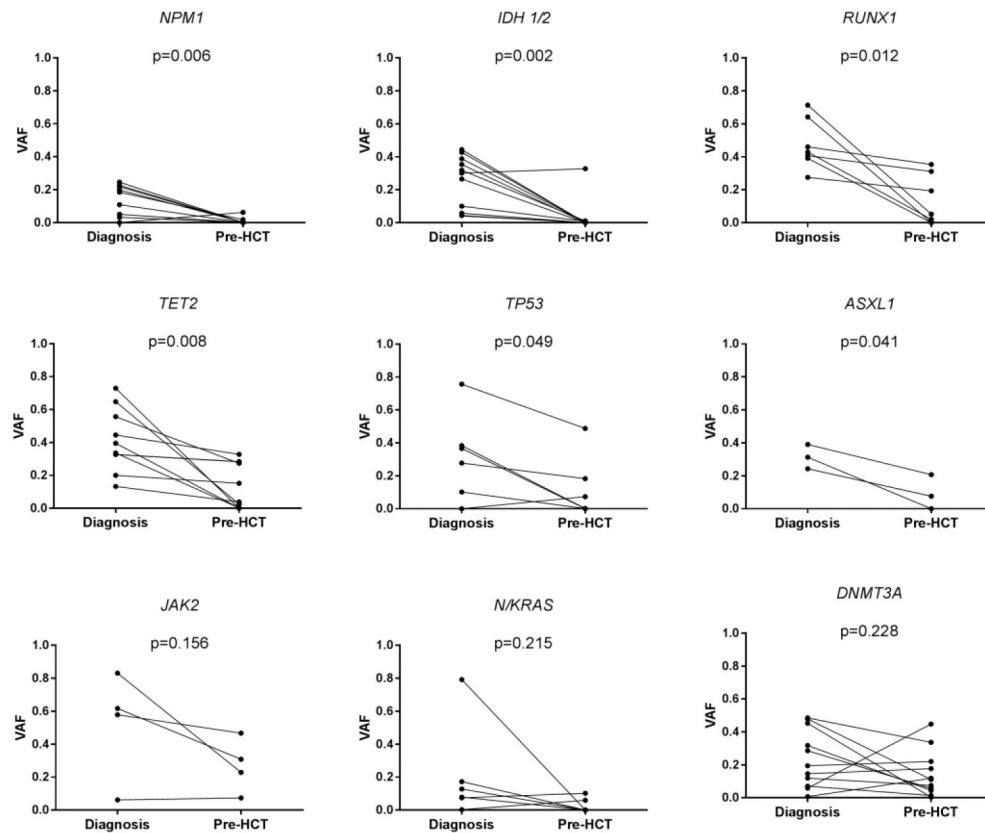
Figure 1A:

Relative change in mutant gene VAF

Mutant gene VAF was compared between diagnosis and immediately before HCT in patients who had mutations identified at AML diagnosis (n=40). A significant reduction in VAF was noted for *NPM1* (n=9), *IDH1* and 2 (n=11), *RUNX1* (n=7), *TET2* (n=10) and *TP53* (n=6) while no change in VAF was seen in mutant *JAK2* (n=4), *N/KRAS* (n=6) and *DNMT3A* (n=11) alleles.
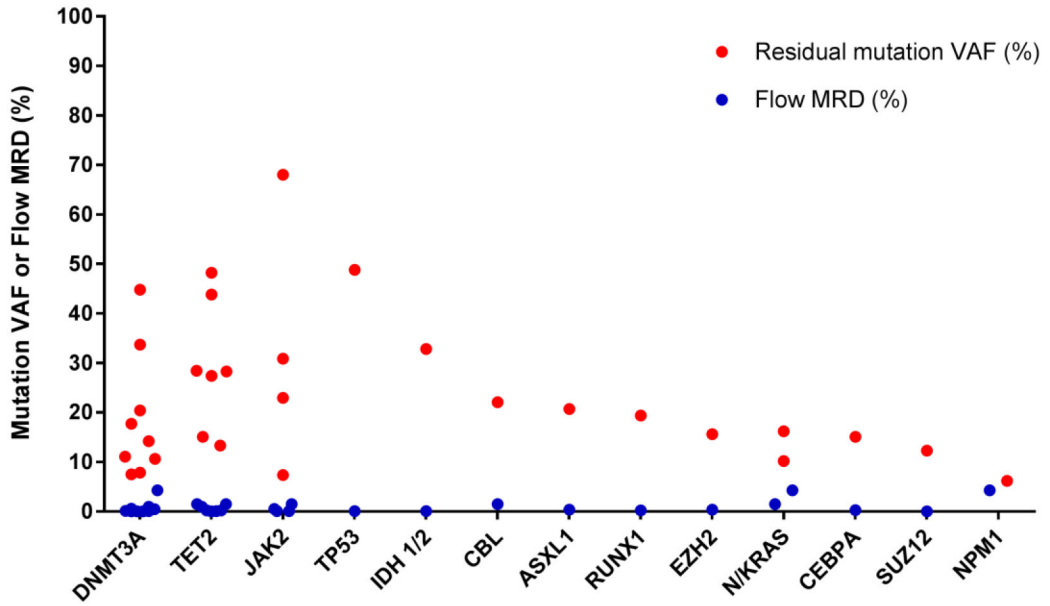
Figure 1B:

Comparison of residual mutation and flow MRD burden pre HCT

Residual disease in the blast compartment quantified by MFC was matched to the VAF of residual mutations quantified by NGS on the same bone marrow sample for patients who were in hematologic CR/CRi at the time of transplant but who had residual mutations called and had MFC residual disease assessment performed (n=17). The VAF (%) of residual mutations (residual mutations defined by VAF ≥ 5%) and the percentage of aberrant blasts are plotted on the same Y-axis grouped by leukemia gene. In the vast majority of cases MFC was quantified to be <1% while residual leukemia alleles were quantified ≥ 5% suggesting that these mutations are present in cells outside of the leukemic blast compartment.
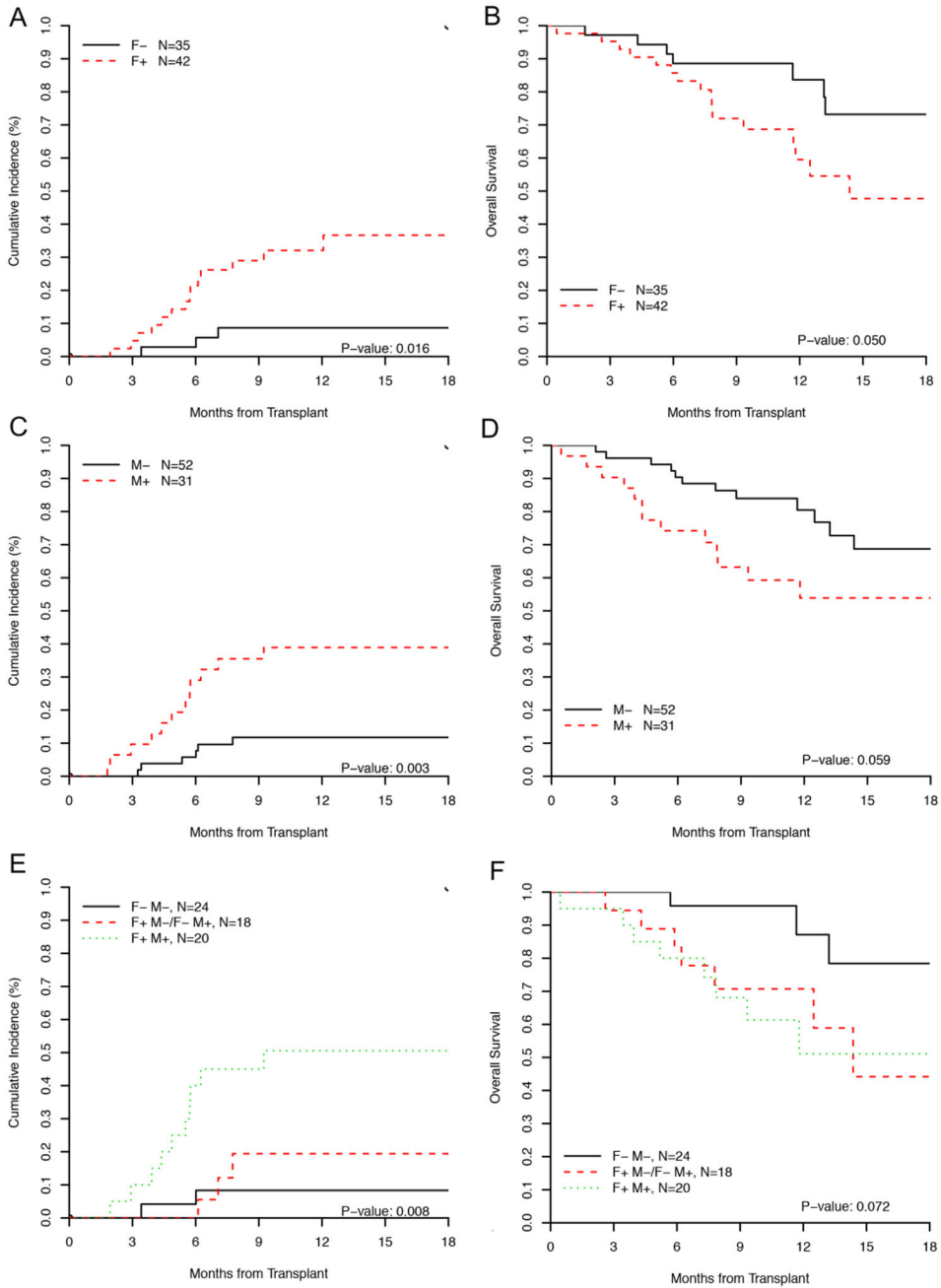
Figure 2:
Transplant outcome stratified by residual disease status.

(A) Cumulative incidence of relapse and (B) overall survival by MFC MRD, (C) Cumulative incidence of relapse and (D) overall survival by NGS, (E) Cumulative incidence of relapse and (F) overall survival by tandem assessment using MFC and NGS. In (E) and (F), patients with discordant assessment are grouped together as their outcome tracked with patients in the F–/M– group.

F denotes MFC, M denotes mutation analysis by NGS and status is categorized as +/−

Table 1:

Characteristics of all patients included in the analysis

Characteristic	N (%)
N	104
Men (%)	51 (49%)
Median age at transplant, years (range)	58 (21–78)
Number of patients with molecular and flow analysis	
Molecular analysis at diagnosis	58 (55.8)
Molecular analysis pre-HCT	83 (79.8)
Paired molecular analysis at diagnosis and pre-HCT	47 (45.2)
Flow MRD analysis pre-HCT	77 (74.0)
Paired flow and molecular analysis pre-HCT	62 (59.6)
AML characteristics	
Primary AML	65 (62.5)
Secondary AML, prior disease	39 (37.5)
Prior MDS	21
Prior MPN	7
Prior CMML	0
Therapy related AML [^]	15
CIBMTR Cytogenetics Grouping	
Favorable	2 (1.9)
Intermediate	88 (84.6)
Adverse	14 (13.5)
Remission status at time of HCT	
CR1	59 (56.7)
CR2	9 (8.7)
CR3	1 (1.0)
CRi	21 (20.2)
Not in CR	14 (13.5)
DRI	
Low	2 (1.9)
Intermediate	71 (68.3)
High	30 (28.8)
Very high	1 (1.0)
HCT-CI	
<3	51 (49)
3	53 (51)
Stem cell source	
Peripheral blood stem cells	73 (70.2)

Characteristic	N (%)
Bone marrow derived stem cells	12 (11.5)
Cord blood	19 (18.3)
CD34+ selection	29 (27.9)
HLA match[#]	
Fully matched	72 (69.2)
Mismatched	32 (30.8)
Conditioning intensity	
Ablative	59 (56.7)
Reduced intensity	45 (43.3)
Transplant	
First	95 (91.3)
Second	9 (8.7)
Outcomes	
Relapse, N (%)	22 (21.2)
Deceased, N (%)	36 (34.6)
Cause of death	
Relapse	13 (12.5)
Infection	10 (9.6)
GVHD	6 (5.8)
Toxicity	2 (1.9)
Organ failure	4 (3.8)
Unknown	1 (0.8)

[^]2 patients developed therapy related AML after initial diagnosis with therapy related MDS

[*]15 patients did not have available *FLT3* mutation testing in the setting of a normal karyotype and thus could not be categorized by the NCCN AML classification.

[#]Number of mismatches at high resolution at HLA class 1 (A, B, C) and class 2 (DR and DQ). All cord blood transplants were mismatched.

Table 2:

Disease profile of patients with discordant results on tandem assessment immediately before HCT

Patient	Assessment at diagnosis		RD assessment pre HCT				Post HCT
	Cytogenetics	Mutations	CR/CRi	MFC MR (%)	Mutations	VAF (%)	Relapse
MFC negative and multi gene NGS positive (F−M+) [∧]							
1	47,XY,+13[21]	*DNMT3A* p.R882C	CR	0	*DNMT3A* p.R882C	11.09	no
2	46,XX[20]	NP	CR	0	*DNMT3A* p.R882H	14.21	yes
3	46,XY,t(X;19), del9(q)	*DNMT3A* p.804L *TET2* p.M1333fs	CR	0	*DNMT3A* p.804L *TET2* p.M1333fs	20.41 13.30	no
		FLT3-ITD[#]			*FLT3*-ITD	<5%[#]	
4	47,XX,+8[9]		CRi	0	*SUZ12* p.H6D	12.31	no
MFC positive and multi gene NGS negative (F+M−) [*]							
1	46, XX [20]	*DNMT3A* p.Q402X	CR	0.35	*DNMT3A* p.Q402X	0.38	no
		NRAS p.G12D,			*NRAS* p.G12D,	0.00	
		IDH1 p.R132H			*IDH1* p.R132H	0.00	
		NPM1 p.L287fs			*NPM1* p.L287fs	0.00	
2	46,XX,inv(16) (p13q22 [20]	*WT1* p.1H469N	CRi	0.01	*WT1* p.H469N	0.00	no
3	Complex[§]	*NRAS* p.Q61K *TP53* p.R333fs	CR	2.00	*NRAS* p.Q61K *TP53* p.R333fs	0.11 0.22	no
		TP53 p.N210fs			*TP53* p.N210fs	0.10	
4	46,XY[20]	*IDH1* p.R132H	CRi	1.20	*IDH1* p.R132H	0.09	no
		TET2 p.P409A			*TET2* p.P409A	3.91	
5	46,XY[20]	*IDH2* p.R172K	CR	1.60	*IDH2* p.R172K	0.15	no
		ASXL1 p.T880fs			*ASXL1* p.T880fs	0.00	
		TET2 p.I873T			*TET2* p.I1873T	0.17	
6	46,XX[20]	*TET2* p.I1873T	CR	2.00	*TET2* p.I1873T	0.19	no
7	46,XY[20]	*NPM1* p.L287fs	CR	1.50	*NPM1* p.L287fs	0.00	no
		FLT3-ITD[#]			*FLT3*-ITD	<5%[#]	
8	46,XY[20]	*IDH* p.R132H	CR	0.28	*IDH* p.R132H	0.46	no

NP: not performed, CR: complete remission, CRi: complete remission with incomplete count recovery, VAF: variant allele frequency, MFC multiparameter flow cytometry

[∧] All 4 patients in the F−M+ group are presented in the table.

[*] Eight patients in the F+M− group had somatic profiling performed at diagnosis that identified mutations which could be tracked. Of the remaining 6 patients in the F+M− group, 5 did not have somatic mutation profiling performed at AML diagnosis and 1 had no mutations called at the time of diagnosis when assessed using the same NGS assay.

[#] *FLT3*-ITD assessment was performed using a qualitative stand-alone PCR assay with a sensitivity of 5%, hence VAF is not reported.

[§] 43–45,X,-X,+3,del(5)(q?15q?33),−7,?del(13)(q12q14),−15,add(16)(q24),−18,−4mar

Table 3:

Univariate analysis of factors associated with overall survival and time-to-relapse after HCT

			Overall Survival			Time-to-Relapse		
		N	Events	HR (95% CI)	p-value	Events	HR (95% CI)	p-value
Residual disease								
Flow MRD	Negative	35	7	reference	0.05	3	reference	0.016
	Positive	42	16	2.44 (1–5.97)		14	4.62(1.32–16.09)	
Multi-gene NGS	Negative	52	13	reference	0.059	6	reference	0.003
	Positive	31	13	2.1 (0.97–4.55)		12	4.35 (1.63–11.6)	
Tandem assessment	F–/M–	24	3	reference	0.072	2	reference	0.008
	F–/M+ or F–/M	18	7	3.96 (1.02–15.37)		3	2.31 (0.38–13.84)	
	F+/M+	20	8	4.53 (1.19–17.18)		10	8.39 (1.83–38.38)	
Blasts >5%	No	90	28	reference	0.011	16	reference	0.007
	Yes	14	8	2.78 (1.26–6.14)		6	3.64 (1.42–9.33)	
Disease variables								
T-AML	No	91	28	reference	0.009	18	reference	0.165
	Yes	13	8	2.87 (1.3–6.35)		4	2.16 (0.73–6.41)	
Secondary AML	No	65	18	reference	0.076	12	reference	0.343
	Yes	39	18	1.81 (0.94–3.48)		10	1.5 (0.65–3.47)	
HCT-CI	<3	51	12	reference	0.006	9	reference	0.184
	3	53	24	2.67 (1.33–5.37)		13	1.78 (0.76–4.17)	
DRI	Low/Intermed	73	21	reference	0.013	10	reference	0.001
	High/very high	31	15	2.34 (1.2–4.57)		12	3.98 (1.71–9.25)	
Transplant variables								
Stem cell source	CB	19	7	reference	0.982	1	reference	0.315
	BM	12	4	0.92 (0.27–3.15)		3	4.92(0.51–47.34)	
	PB	73	25	0.92 (0.4–2.13)		18	4.7 (0.63–35.2)	
HLA matched	Yes	72	23	reference	0.337	18	reference	0.253
	No	32	13	1.4 (0.71–2.76)		4	0.53 (0.18–1.57)	
CD34+ selection	No	75	25	reference	0.86	18	reference	0.228
	Yes	29	11	0.94 (0.46–1.91)		4	0.51 (0.17–1.52)	
Conditioning	Ablative	59	18	reference	0.237	9	reference	0.1
	RIC	45	18	1.48 (0.77–2.86)		13	2.04 (0.87–4.78)	

T-AML: therapy related AML, CB: Cord blood, BM: Bone marrow, PB: peripheral blood; RIC: reduced intensity conditioning, DRI: disease risk index, F: flow MRD, M: Residual mutations using NGS, HLA matched: matched sibling or matched unrelated donors assessed at 10 alleles (A, B, C, DR, DQ) and non-matched includes all mismatched related, unrelated and cord blood transplant.