Pre-transplantation minimal residual disease with cytogenetic and molecular diagnostic features improves risk stratification in acute myeloid leukemia

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ABSTRACT

Our aim was to improve outcome prediction after allogeneic hematopoietic stem cell transplantation in acute myeloid leukemia by combining cytogenetic and molecular data at diagnosis with minimal residual disease assessment by multicolor flow-cytometry at transplantation. Patients with acute myeloid leukemia in first complete remission in whom minimal residual disease was assessed at transplantation were included and categorized according to the European LeukemiaNet classification. The primary outcome was 1-year relapse incidence after transplantation. Of 152 patients eligible, 48 had minimal residual disease at the time of their transplant. Minimal residual disease-positive patients were older, required more therapy to achieve first remission, were more likely to have incomplete recovery of blood counts and had more adverse risk features by cytogenetics. Relapse incidence at 1 year was higher in patients with minimal residual disease (32.6% *versus* 14.4%, *P*=0.002). Leukemia-free survival (43.6% *versus* 64%, *P*=0.007) and overall survival (48.8% *versus* 66.9%, *P*=0.008) rates were also inferior in patients with minimal residual disease. In multivariable analysis, minimal residual disease status at transplantation independently predicted 1-year relapse incidence, identifying a subgroup of intermediate-risk patients, according to the European LeukemiaNet classification, with a particularly poor outcome. Assessment of minimal residual disease at transplantation in combination with cytogenetic and molecular findings provides powerful independent prognostic information in acute myeloid leukemia, lending support to the incorporation of minimal residual disease detection to refine risk stratification and develop a more individualized approach during hematopoietic stem cell transplantation.

Introduction

Disease relapse is the most common cause of treatment failure after allogeneic hematopoietic stem cell transplantation (HCT) for acute myeloid leukemia (AML).[1] In most series, the median time to relapse after HCT is 4-6 months,[2,3] suggesting that identifying patients with a higher risk of relapse early after HCT is important to tailor transplant and post-transplant management strategies with an aim to reduce the relapse incidence (RI).[4] Cytogenetic and molecular abnormalities detected at diagnosis are important prognostic factors for RI, leukemia-free survival (LFS) and overall survival (OS).[5-7] Specific gene abnormalities, such as mutations in *FLT3* and *NPM1* genes, allow subsets of patients with distinct treatment outcomes to be identified, even within homogeneous cytogenetic groups.[8-10] The current standard framework for risk stratification guiding transplant practice in AML has, therefore, been largely based on cytogenetics and a panel of molecular genetic markers, cou-

pled with morphological assessment of bone marrow response to chemotherapy. Recent studies assessing minimal residual disease (MRD) have highlighted the limitations of morphology for reliable determination of remission status and several studies have shown that MRD status independently predicts disease relapse after anti-leukemia treatment.[11-14]

Multiparametric flow cytometry (MFC) has been successfully used to quantify MRD in AML expressing leukemia-associated phenotypes.[13,15] Previous studies have demonstrated that MRD detectable by MFC is a powerful, independent predictor of subsequent relapse and shorter survival for AML patients in complete remission and can be used to risk-stratify both younger and older patients after chemotherapy and following HCT.[16-19] Based on these findings, it is conceivable that outcome prediction after HCT in AML could be improved by a combination of standard prognostic factors, such as cytogenetic and molecular data, with MRD assessment by MFC.

In the present study, we analyzed a large group of consecutive AML patients undergoing HCT in first morphological remission (CR1) for whom cytogenetics, molecular data at diagnosis and MRD assessment by MFC at HCT were available. We aimed to evaluate whether the combination of these determinants would help to optimize risk stratification for relapse in patients with AML undergoing HCT in CR1.

Methods

Study cohort

At our institution, eight-color flow cytometry analysis from bone marrow samples has been part of the standard pre-transplant work-up in AML patients since September 2012. Of 169 consecutive adult AML patients who underwent allogeneic HCT in CR1 from September 2012 through March 2015, 152 (90%) had MFC performed on bone marrow aspirates preceding allogeneic HCT and they were included in the current analyses.

Patients were categorized by the European LeukemiaNet (ELN) classification incorporating both cytogenetic and selected molecular abnormalities at diagnosis, separating AML patients into four distinct genetic risk groups[20-23] (*Online Supplementary Table S1*).

All patients provided written informed consent to transplantation in accordance with the Declaration of Helsinki. The University of Texas MD Anderson Cancer Center institutional review board approved this retrospective analysis.

Flow cytometric immunophenotyping of minimal residual disease

Eight-color flow cytometry analysis was performed using previously described methods.[24,25] In brief, the panel included four tubes as follows: (i) CD7FITC, CD33 PE, CD19 PerCP-Cy5.5, CD34 PE-Cy7, CD13 APC, CD38 BV421, CD45 V500; (ii) HLADR-FITC, CD117 PE, CD4 PerCP-Cy5.5, CD34 PE-Cy7, CD123 APC, CD19-eF780, CD38 BV421, CD45 V500; (iii) HLA-DR-FITC, CD36 PE, CD56 PerCP-Cy5.5, CD34 PE-Cy7, CD64 APC, CD19-eF780, CD14 V450, CD45 V500; and (iv) CD5-FITC, CD2 PE, CD22 PerCP-Cy5.5, CD34 PE-Cy7, CD38 APC, CD19-eF780, CD15 V450, CD45 V500. All antibodies were obtained from Becton Dickinson (San Jose, CA; USA) or eBioscience (San Diego, CA, USA). Samples were acquired on FACSCanto II instruments (BD Biosciences, San Diego, CA, USA) that were standardized daily using CS&T beads. A minimum of 200,000 live events were acquired to achieve a potential sensitivity of at least 10^{-4} (0.01%). MRD was defined as a neoplastic blast population with an abnormal pattern of antigen expression deviating from normal regenerating myeloid progenitors. The abnormal blast population was qualified as a percentage of total events. Any level of an abnormal blast population (≥0.01%) detected by MFC was considered MRD positive.

The first sample aspirated was used for morphological preparations (smears and clot sections), and subsequent draws were sent for flow cytometric analysis and other ancillary testing. This may have affected the quality of some of the samples analyzed by MFC.

Disease characteristics, conditioning regimens and graft-*versus*-host disease prophylaxis

We identified 152 patients in CR1 who had MRD by MFC assessment just prior to HCT. Cytogenetic and molecular data at diagnosis were evaluable for 140 of 152 patients for ELN classification.

The clinical characteristics of the study population, donors, and transplants are summarized in Table 1. Donors were human leukocyte antigen (HLA)-identical siblings in 41 (27%) cases, HLA-matched unrelated in 75 (49.3%), mismatched unrelated in 5 (3.3%), haploidentical in 20 (13.2%) and cord blood in 11 (7.2%) cases. Mismatched unrelated, haploidentical and cord blood patients were analyzed together because of their small numbers.

Sixty-four patients (42.1%) received a reduced intensity conditioning regimen which consisted of: (i) intravenous busulfan either at a dose calculated to target an average daily systemic exposure dose, represented by the area under the concentration *versus* time curve (AUC) of 4,000 μMol-min ±10%, or 100 mg/m^2 with fludarabine 40 mg/m^2 given for 4 days; or (ii) melphalan 100-140 mg/m^2 as a single dose with fludarabine 40 mg/m^2 given for 4 days. Eighty-eight patients (57.9%) received a myeloablative conditioning regimen consisting of intravenous busulfan either at a dose calculated to target an AUC of 5,000-6,000 μMol-min ±10%, or 130 mg/m^2 in combination with fludarabine 40 mg/m^2 given daily for 4 days (66 patients) or the same busulfan treatment in combination with fludarabine, 10 mg/m^2.

Tacrolimus and methotrexate were used as graft-*versus*-host disease prophylaxis in the majority of the patients (73.4%). The recipients of matched unrelated donor grafts and cord blood received rabbit anti-thymocyte globulin (Thymoglobulin, Genzyme, Cambridge, MA, USA) as a part of their conditioning regimen. Graft-*versus*-host disease prophylaxis for recipients of grafts from haploidentical and mismatched unrelated donors consisted of post-transplant cyclophosphamide, tacrolimus, and mycophenolate mofetil.[26]

Statistical analyses

Outcomes analyzed included LFS, cumulative RI, transplant-related mortality and OS. All outcomes were measured from the time of stem cell infusion. LFS was defined as survival without leukemia progression or relapse; patients alive without disease progression or relapse were censored at the time of last contact. OS was based on death from any cause. Surviving patients were censored at the time of last contact. Relapse was defined as leukemia recurrence at any site. LFS and OS were estimated using the Kaplan-Meier method. The probability of relapse was summarized using a cumulative incidence estimate. Non-relapse mortality was considered a competing risk for relapse. All outcomes were treated as time-to-event endpoints. Multivariate analysis was performed using Cox regression. Patients' characteristics that were significant in the univariate models at the 0.10 level and clinically relevant were included in the multivariate model. Backward elim-

ination was implemented until all remaining predictors had a *P*-value less than 0.05. Categorical characteristics were compared using the Fisher exact test, and continuous characteristics were compared with the two-sample *t* test. Statistical analyses were performed with STATA (StataCorp LP, College Station, TX, USA).

Results

The presence of minimal residual disease at transplantation is associated with poor-risk disease features

The cohort with MRD at HSCT had high-risk features including older age, AML with adverse risk features by ELN, requirement of more lines of therapy to achieve CR1 and incomplete count recovery (CRi/p) at HCT compared with 104 MRD-negative patients, as presented in Table 1. MRD-positive, intermediate-risk patients by ELN classification were also less likely to have mutated *NPM1* compared with MRD-negative patients.

Table 1. Patient and disease characteristics.

	MRD-negative, n=104 N.	%	MRD-positive, n=48 N.	%	*P*
Age, median, IQR	54 (40-61)		60 (51-67)		0.005
Age >60 years	31	30	24	50	0.016
Sex					
Male	56	54	29	60	
Female	48	46	19	40	0.4
t-AML	17	16	11	23	0.3
N. of lines of induction					
1	86	83	30	62	
≥2	18	17	18	38	0.006
ELN risk group					0.2
Favorable	12	12	2	5	
Intermediate-I	33	32	17	39	
Intermediate-II	28	27	8	18	
Adverse	22	21	17	39	
Adverse *vs.* others	22	21	17	39	0.06
FLT3 status (intermediate risk patients)					
Wild-type	27	49	15	60	
Mutated	28	51	10	40	0.4
NPM1 status (CN patients)					
Wild-type	30	57	21	88	
Mutated	23	43	3	12	0.008
Count recovery in CR1					
CRi/p	10	10	28	58	
CR w/count recovery	94	90	20	42	<0.001
Time to HCT from diagnosis					
Median, days (IQR)	159	131-233	190	152-323	0.01
Conditioning intensity					
MAC	63	61	24	50	
RIC	40	39	24	50	0.2
Source of stem cells					
Peripheral blood	56	54	27	56	
Bone marrow	41	39	17	35	
Cord blood	7	7	4	9	0.9
Donor					0.8
MRD	28	27	12	25	
MUD	52	51	23	48	
MMUD	23	22	13	27	
Follow-up after HCT, survivors					0.5
Median, days	454	348-696	531	346-757	

MRD: minimal residual disease; IQR: interquartile range; t-AML: therapy-related AML; ELN: European LeukemiaNet; CN: normal cytogenetics; CR1: first complete remission; CRi/p: complete remission without count recovery; HCT: hematopoietic stem cell transplantation; MAC: myeloablative conditioning; RIC: reduced intensity conditioning; MRD: matched related donor; MUD: matched unrelated donor; MMUD: mismatched unrelated donor.

Minimal residual disease-positive patients are more likely to relapse within 1 year after transplantation

Overall, the 1-year RI was higher among MRD-positive patients than among MRD-negative ones [32.6% *versus* 14.4%, respectively: hazard ratio (HR) =3.1, 95% confidence interval (CI): 1.5-6.5; *P*=0.002] (Figure 1A). Among patients who were MRD-positive at HCT, no significant effect of increasing levels of MRD was observed on RI, LFS or OS. This observation held true when MRD was evaluated as a continuous variable (on a log scale) and as a categorical variable using the quartiles of MRD in our

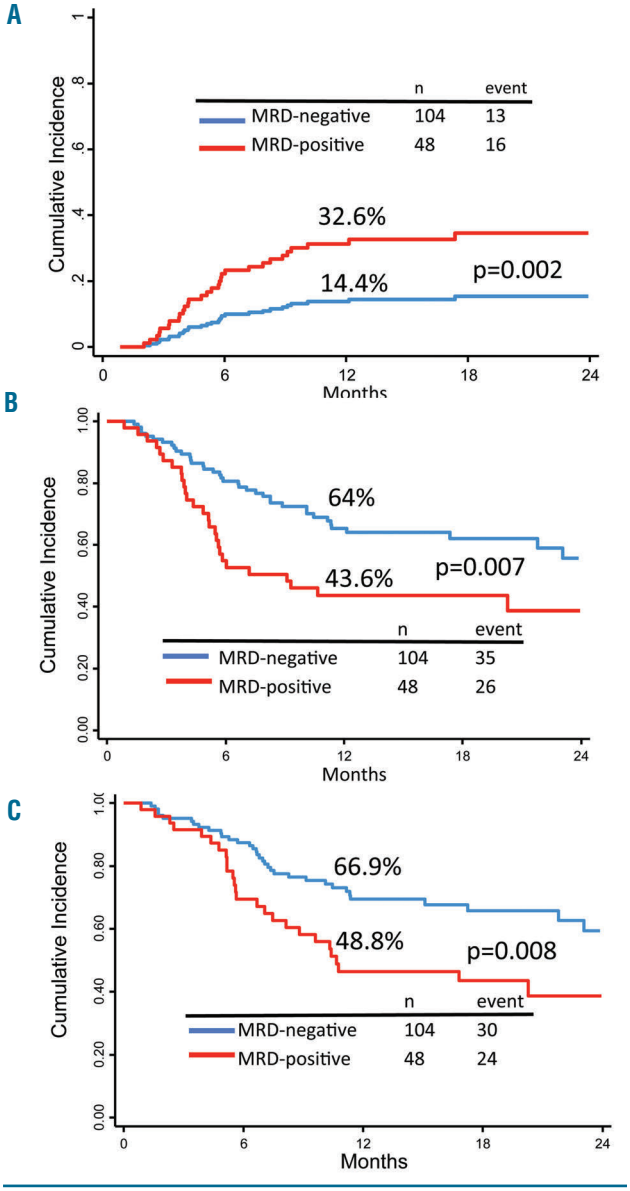

Figure 1. The presence of MRD using MFC at HCT increased (A) **1-year RI while it decreased** (B) **1-year LFS** (C) **OS compared with those in MRD-negative patients at HCT.**

cohort as ≤0.3%, >0.3% to 1.3%, >1.3% to 2% (*Online Supplementary Figure S1*).

Similarly, LFS and OS estimates at 1-year were inferior for patients who were MRD-positive at HCT compared with the MRD-negative group (Figure 1B,C). The cumulative incidence of non-relapse mortality at 1 year was 22% and there was no difference between MRD-negative and MRD-positive patients (*P*=0.97).

Univariate analysis revealed that older age and adverse risk according to the ELN classification were other poor prognostic factors for 1-year RI, as presented in Table 2. There was no difference between intermediate-I and –II risk patients for 1-year RI (HR=0.5, 95% CI: 0.2-1.7; *P*=0.3) and none of the patients with a favorable risk according to the ELN classification relapsed at 1 year after HCT.

Analyses for 1-year LFS and OS revealed that ELN-defined adverse risk, MRD at HCT, older age, CRi/p at HCT and use of reduced intensity conditioning were poor prognostic factors (Table 2). LFS and OS at 1 year were similar for intermediate-I and -II risk patients.

The variables significant in univariate analysis and/or clinically relevant were forced into the multivariate regression model for 1-year RI. We added an interaction term for the MRD status at HCT and ELN risk categorization at diagnosis into the model. Multivariate regressions confirmed the independent prognostic value of MRD at HCT, ELN-defined adverse risk and use of reduced intensity conditioning for 1-year RI, as presented in Table 3. The interaction term was also significant indicating that the effect of MRD on 1-year RI was different for different ELN-defined risk groups (*P*=0.013).

Multivariate regression analysis for 1-year LFS and OS also revealed the independent prognostic impact of MRD at HCT, adverse risk according to the ELN classification and CRi/p at HCT (Table 3).

The presence of minimal residual disease at transplantation identifies a subgroup of intermediate-risk patients with poor prognosis for early relapse after transplantation

MRD-negative and MRD-positive adverse risk patients had a high 1-year RI independently of their MRD status at HCT (31.6% *versus* 31.8% respectively, *P*=0.98) (Figure 2A). However, patients in the intermediate risk group had different prognoses depending on their MRD status at HCT. MRD-positive intermediate-I/II risk patients had a 1-year RI of 42.7% while MRD-negative intermediate-I/II risk patients had a 1-year RI of only 6.9% (*P*<0.001) (Figure 2B).

Table 2. Univariate regression analyses for the impact of prognostic factors on relapse incidence, leukemia-free survival and overall survival.

	1-year RI			1-year TRM			1-year LFS			1-year OS		
	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
Age (>60 *vs.* ≤60 years)	2.1	1.03-4.4	0.04	1.5	0.8-3.1	0.2	2.0	1.2-3.4	0.005	2.1	1.2-3.6	0.006
t-AML (yes *vs.* no)	2.1	0.95-4.6	0.07	1.5	0.7-3.3	0.3	1.9	1.1-3.4	0.02	1.6	0.9-2.9	0.1
Line of induction (>1 *vs.* 1)	2.1	0.98-4.4	0.057	0.7	0.3-1.7	0.4	1.3	0.7-2.2	0.4	1.3	0.7-2.4	0.3
MRD-positive vs. MRD-negative	3.1	1.5-6.5	0.002	1.01	0.5-2.2	0.97	2.0	1.2-3.5	0.007	2.1	1.2-3.5	0.008
Intermediate-II *vs.* intermediate-I (ELN)	0.5	0.2-1.7	0.3	0.9	0.4-2.5	0.9	1.4	0.6-2.9	0.4	1.3	0.6-2.8	0.5
Favorable *vs.* intermediate-I (ELN)	NE			1.1	0.3-4.3	0.9	0.7	0.2-2.3	0.5	0.7	0.2-2.6	0.6
Adverse *vs.* intermediate-I/II (ELN)	2.0	0.9-4.2	0.08	1.4	0.6-3.1	0.4	1.8	1.04-3.1	0.035	1.7	0.9-3.0	0.08
Adverse *vs.* others (ELN)	2.3	1.1-5.0	0.03	1.4	0.6-3.0	0.4	1.9	1.1-3.3	0.002	1.8	1.005-3.2	0.048
CRi/p *vs.* CR w/count recovery	1.7	0.8-3.6	0.2	2.2	1.1-4.4	0.03	2.2	1.3-3.7	0.003	2.6	1.5-4.4	0.001
Conditioning intensity												
Myeloablative	1.0			1.0			1.0			1.0		
Reduced intensity	1.8	0.8-3.7	0.1	1.7	0.9-3.4	0.1	1.9	1.1-3.1	0.01	1.7	1.01-2.9	0.045
Donor												
Matched related	1.0			1.0			1.0			1.0		
Matched unrelated	0.8	0.3-1.8	0.6	1.5	0.5-4.2	0.4	1.0	0.5-2.0	0.9	1.2	0.6-2.4	0.6
Mismatched	0.7	0.2-1.9	0.5	3.1	1.1-8.8	0.03	1.6	0.8-3.1	0.2	1.9	0.9-4.1	0.08

RI: relapse incidence; TRM: transplant-related mortality; LFS: leukemia-free survival; OS: overall survival; HR: hazard ratio; CI: confidence interval; MRD: minimal residual disease; t-AML: therapy-related AML; ELN: European LeukemiaNet; CRi/p: complete remission without count recovery.

Table 3. Multivariate regression model for 1-year relapse incidence, leukemia-free survival and overall survival*.

	1-year RI			1-year LFS			1-year OS		
	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
CRi/p *vs.* CR w/count recovery				3.6	1.6-8.1	0.002	4.6	2.0-10.3	<0.001
MRD-positive *vs.* MRD-negative	6.4	1.9-21.4	0.003	2.2	1.05-4.5	0.037	2.5	1.1-5.4	0.02
Adverse *vs.* others (ELN)	6.7	2.1-21.7	0.001	1.8	1.01-3.1	0.047			
RIC *vs.* MAC	2.4	1.1-5.6	0.03						

RI: relapse incidence; LFS: leukemia-free survival; OS: overall survival; HR, hazards ratio; CI: confidence interval; MRD: minimal residual disease; t-AML: therapy-related AML; ELN: European LeukemiaNet; CRi/p: complete remission without count recovery; MAC: myeloablative conditioning; RIC: reduced intensity conditioning. *The variables included were age older than 60 years, t-AML, line of induction chemotherapy, MRD, risk groups according to ELN and conditioning intensity for RI. For leukemia-free-survival; age older than 60 years, t-AML, CRi/p, MRD, risk groups according to ELN and conditioning intensity were included. For overall survival, age older than 60 years, CRi/p, MRD and cytogenetics according to ELN were included.

These results enabled us to identify two risk groups for 1-year RI: (i) a high-risk group with a 1-year RI of 36% including patients with adverse risk according to ELN criteria and those with intermediate-I/II risk who were MRD-positive; (ii) a lower risk group with a 1-year RI of 6.9% including intermediate-I/II risk, MRD-negative patients and favorable risk patients (Figure 3A). The favorable risk group that did not have any relapse at 1 year on follow-up was not included in this risk group classification.

In the adverse risk group, the LFS rate at 1 year was 48.7% *versus* 31.4% (P=0.17) and OS was 58.4% *versus* 37.7% (P=0.16) in MRD-negative and MRD-positive patients, respectively; these differences in outcome expectation did not reach statistical significance. However, MRD-positive, intermediate-I/II risk patients had a significantly inferior 1-year LFS of 46.8% and 1-year OS of 47% compared with the 68.9% and 73.2%, respectively, observed in MRD-negative patients (P=0.02 and P=0.03). This difference, which was seen for LFS and OS, but not for RI can be explained by the fact that LFS and OS are composite outcomes taking into account not only RI but also transplant-related mortality. Therefore, other patient-, disease- and transplant-related characteristics in addition to post-transplant relapse therapy might have an impact on LFS and OS.

We then analyzed the impact of risk groups defined by the ELN classification and MRD status at HCT on LFS and OS and found significantly different results for outcomes in high and low risk groups, similar to RI (Figure 3B,C).

Intermediate-risk minimal residual disease-negative patients who harbor the *FLT3*-ITD mutation enjoy a lower risk of relapse after transplantation

Intermediate risk patients with mutated *FLT3*-internal tandem duplication (*FLT3*-ITDmut) who were MRD-negative at HCT had a lower 1-year RI than that of patients who were MRD-positive at HCT (7.4% *versus* 45.7%, P=0.014). These results were comparable with outcomes

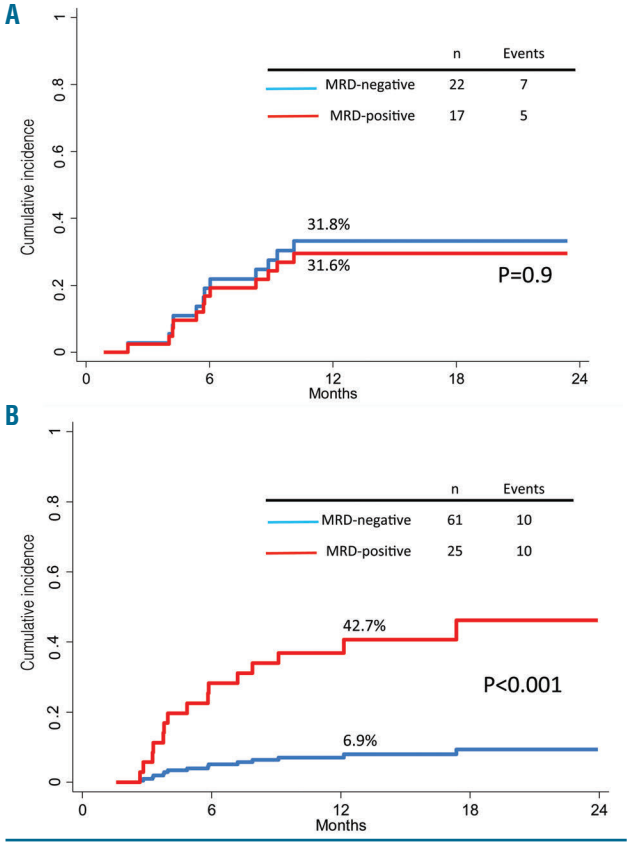

Figure 2. Prognostication based on MFC assessment of MRD. (A) The presence of MRD assessed using MFC at HCT in patients in the adverse risk group did not reveal distinct subgroups for 1-year RI. (B) Intermediate risk patients, however, had a higher RI at 1-year, comparable to that of adverse risk patients, if they were MRD-positive at HCT.

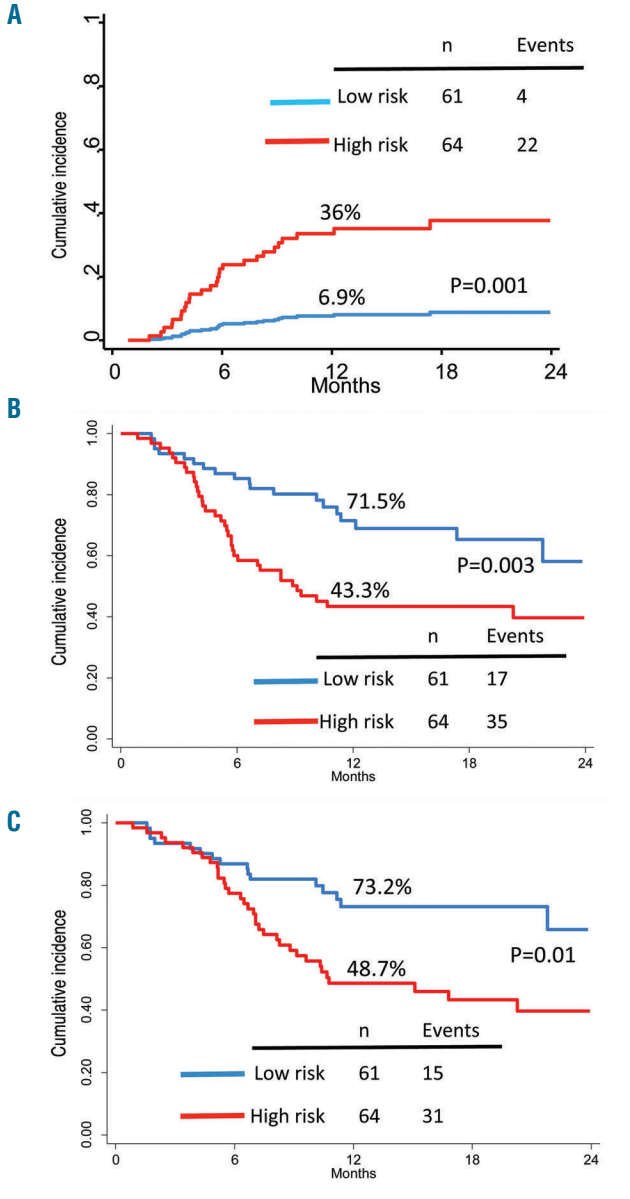

Figure 3. Prognostication based on ELN classification diagnostic cytogenetic/molecular data and MFC assessment of MRD. (A) Two risk groups were identified for 1-year RI using the ELN classification based on diagnostic cytogenetic/molecular data and MRD assessment by MFC at HCT: a high risk group, including intermediate I/II MRD-positive and adverse risk patients, with a RI of 36%, and a low-risk group, including intermediate I/II MRD-negative and favorable risk patients, with a RI of 5.5%%. This risk classification by ELN and MRD at HSCT also predicted (B) LFS and (C) OS outcomes after HCT.

observed in patients with wild-type *FLT3*-ITD (*FLT3*-ITDwt) divided according to their MRD status at HCT (4% *versus* 40.3% for MRD-negative and MRD-positive, respectively; *P*=0.017) (Figure 4). The impact of MRD by MFC could not be analyzed in patients with *NPM1* mutations because of the significant association between *NPM1* mutation and MRD-negative status at HCT.

Risk stratification by disease and transplant characteristics predicts post-transplant relapse incidence

We explored the possibility of developing a predictive model of 1-year relapse outcome in AML patients using MRD status at HCT. Considering the significance of the interaction term at multivariate regression indicating the different prognostic impact of MRD at HCT on 1-year RI among different ELN-defined risk groups, we re-ran multivariate regressions with variables generated by the combination of cytogenetics and MRD status at HCT: (i) high risk including adverse risk and intermediate risk MRD-positive patients and (ii) low risk including intermediate risk MRD-negative patients. Each factor was assigned a score proportional to the regression coefficient obtained from the multivariable regression model (*Online Supplementary Table S1*). Accordingly, a score of 1 was assigned to the risk factor of using reduced intensity conditioning. Then, a score of 2 was assigned to those with high-risk disease.

A post-transplant relapse risk index was calculated as the sum of these weighted scores, and this index was then categorized into three risk groups: low (score = 0 or 1), intermediate (score = 2), high (score = 3). The 1-year RI was 6.9% in patients with a low relapse risk index, 26.9% in patients with an intermediate index, and 47.2% in patients with a high relapse risk index (*P*< 0.001; *Online Supplementary Figure S2*).

Discussion

Our study is unique for investigating the impact of MFC-determined MRD status at HCT on 1-year RI not only by adjusting for the cytogenetic risk at diagnosis but also for distinct molecular abnormalities including *FLT3*-ITD mutation per ELN classification. The analyses including 152 patients who underwent HCT in CR1 over the last 3 years at our institution confirm that MRD detected by MFC at HCT identifies a group with a poor prognosis with regards to relapse at 1 year after HCT. Our results were comparable to those recently reported showing that the presence of MRD determined by MFC increases the risk of relapse not only after myeloablative conditioning but also after reduced intensity conditioning regimens and it signifies a poor prognosis in addition to that conferred by other patient- and disease-related characteristics including cytogenetics.[27] Moreover, our results suggest that the impact of MRD at HCT on RI differs in distinct prognostic groups defined by using diagnostic cytogenetics and molecular findings.

Assessment of MRD at HCT appeared to have the potential to differentiate a large group of patients with intermediate risk features according to the ELN classification, including those with *FLT3*-ITD mutations. MRD-positive intermediate risk patients represented a worse prognostic group with a 1-year RI of 42.7% compared with MRD-negative counterparts who had a 1-year RI of 7.4%. This observation remained the same when *FLT3*-ITD mutations were taken into account. Patients with *FLT3*-ITDmut, who are known to have a high risk of relapse after HCT,[28,29] enjoyed a lower RI of 7.4% at 1 year if MRD-negative compared with 45.7% if MRD-positive. On the other hand, intermediate risk patients with *FLT3*-ITDwt, a group that is thought to have better results than *FLT3*-ITDmut patients, had a 1-year RI of 40.3% if they were MRD-positive at HCT, comparable to outcomes observed in *FLT3*-ITDmut, MRD-positive patients. These findings suggest that MRD status at HCT alters the initial prognosis dictated by genetic abnormalities at diagnosis within intermediate risk patients. On the other hand, we could not investigate whether *NPM1*-mutated intermediate risk patients had worse outcomes if they were MRD-positive at HCT by MCF because of the significant association of MRD-negative status and *NPM1* mutation. We believe that addition of further genetic markers (e.g., *DNMT3*, *TET2*, *ASXL1*, *RUNX* mutations) and novel molecular abnormalities emerging from next-generation sequencing may further refine the accuracy of patient risk stratification by MRD using MFC after transplantation.

Differently from what we observed for the intermediate risk group, MRD assessment at HCT did not identify different prognostic groups for 1-year RI in patients with adverse risk according to the ELN classification. These results are similar to those of previously published studies in the non-transplant setting showing that MRD determined by MFC had better prediction to identify prognostic groups in intermediate risk patients.[30,31] We were not able to investigate the prognostic impact of MRD determined by MFC in the favorable risk group since there were few favorable risk patients who were MRD-positive in our series. Moreover, none of the favorable risk patients had experienced relapse by 1 year after HCT. MRD detection using more sensitive quantitative polymerase chain reaction assays[32] merits further investigation in this group.

As a result, given the limitations inherent in a retrospectively designed study, the implementation of MRD assessment by MFC at HCT allowed us to simplify risk groups

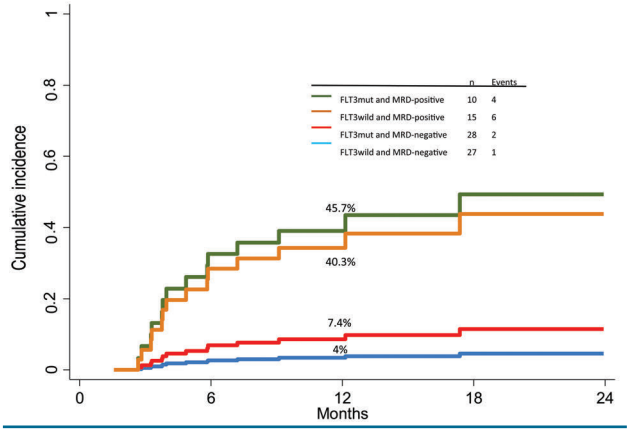

Figure 4. Effect of the *FLT3*-ITD mutation on relapse incidence. *FLT3*-ITD*mut* MRD-negative patients had a comparable 1-year RI (7.4%) to that of *FLT3*-ITD*wt* MRD-negative patients (4%) (*P*=0.8). Similarly, *FLT3*-ITD*mut* MRD-positive and *FLT3*-ITD*wt* MRD-positive patients had high incidences of relapse at 1 year (45.7% and 40.3%, respectively), which were not statistically different (*P*=0.4).

for RI at 1 year using the ELN classification, which incorporates both cytogenetic and selected molecular abnormalities: a low risk group with a 1-year RI of 6.9%, including intermediate-I/II risk MRD-negative patients and a high risk group with a 1-year RI of 36% including intermediate-I/II risk MRD-positive and adverse risk patients.

In our study, the use of reduced intensity conditioning was also a poor prognostic factor for 1-year RI suggesting that patients at high risk of early relapse should be considered for myeloablative conditioning if they are medically fit to tolerate the regimen. A recent Blood and Marrow Transplant Clinical Trial Network phase III randomized clinical trial showed significantly improved RI in AML patients if transplanted with myeloablative conditioning regimens rather than reduced intensity conditioning.[33] Our group also reported that HCT with myeloablative conditioning using pharmacokinetics to target an average daily systemic exposure dose in a timed sequential approach allows even older patients to tolerate more intensive regimens without increased regimen-related toxicity.[34] These results support the concept of treating high risk patients with myeloablative conditioning rather than reduced intensity conditioning.

In addition to conditioning intensity modification, the MRD assessment at HCT combined with diagnostic cytogenetic and molecular characteristics may help to identify the target population in which to investigate innovative approaches for pre-emptive strategies to decrease the risk of relapse and improve transplant outcomes. A recent study by Platzbecker *et al.* showed that hematologic relapse after HCT could be prevented with the pre-emptive use of azacitidine and donor lymphocyte infusion in high risk patients defined by losing chimerism in the post-transplant setting.[35] Similarly, post-transplant maintenance therapy, with various agents including DNA methytransferase inhibitors, deacetylase inhibitors and tyrosine kinase inhibitors, has been investigated to determine its efficacy at decreasing relapse and improving post-transplant outcomes.[36-40] Despite the encouraging results reported, pre-emptive strategies including pharmacological, immunological and cellular therapies pose the dilemma of administering potentially toxic therapy without evidence of relapse. Proper risk assessment with the use of MRD status determined by MFC at HCT could overcome this potential problem.

One important question implicit in the detection of MRD prior to HCT is whether those patients should receive additional pre-transplant treatment with the goal of achieving MRD-negative status or should such patients proceed to HCT without delay. To date, studies evaluating the role of post-remission chemotherapy before HCT with myeloablative or reduced intensity conditioning have not shown any improvement in post-transplantation outcomes.[41-44] Information on MRD at HCT was not available in any of those retrospective studies and it is unknown whether additional post-remission therapy before HCT could benefit a subset of patients who are MRD-positive.

In conclusion, our study indicates that, in AML, the combination of diagnostic cytogenetic/molecular findings and MRD status determined by MFC at HCT enable a better definition of distinct prognostic categories for transplant outcomes. This approach may potentially lead to an improvement in tailoring the intensity of transplantation and use of post-transplant interventions to prevent relapse, with the aim of preventing both under-treatment as well as overtreatment of AML patients.

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