Annals of Hematology (2018) 97:1757–1765
https://doi.org/10.1007/s00277-018-3373-y

ORIGINAL ARTICLE

Digital droplet PCR-based absolute quantification of pre-transplant *NPM1* mutation burden predicts relapse in acute myeloid leukemia patients

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Received: 22 April 2018 / Accepted: 12 May 2018 / Published online: 22 May 2018

Abstract

Allogeneic hematopoietic stem cell transplantation is an established consolidation therapy for patients with acute myeloid leukemia. However, relapse after transplantation remains a major clinical problem resulting in poor prognosis. Thus, detection of measurable ("minimal") residual disease to identify patients at high risk of relapse is essential. A feasible method to determine measurable residual disease may be digital droplet PCR (ddPCR) that allows absolute quantification with high sensitivity and specificity without the necessity of standard curves. Using ddPCR, we analyzed pre-transplant peripheral blood and bone marrow of 51 *NPM1*-mutated acute myeloid leukemia patients transplanted in complete remission or complete remission with incomplete recovery. Mutated *NPM1* measurable residual disease-positive patients had higher cumulative incidence of relapse ($P < 0.001$) and shorter overall survival ($P = 0.014$). Restricting the analyses to patients receiving non-myeloablative conditioning, mutated *NPM1* measurable residual disease positivity is associated with higher cumulative incidence of relapse ($P < 0.001$) and shorter overall survival ($P = 0.006$). Positive mutated *NPM1* measurable residual disease status determined by ddPCR before allogeneic stem cell transplantation is associated with worse prognosis independent of other known prognostic markers—also for those receiving non-myeloablative conditioning. In the future, mutated *NPM1* measurable residual disease status determined by ddPCR might guide treatment and improve patients' outcomes.

Keywords Acute myeloid leukemia · Measurable residual disease · Digital droplet PCR · Allogeneic hematopoietic stem cell transplantation

Introduction

In acute myeloid leukemia (AML), up to 60% of younger (< 60 years) and 85–95% of older patients (≥ 60 years) fail to attain long-term survival [1–4]. Suffering relapse after achieving a complete remission (CR) remains a major clinical challenge. Thus, identifying patients at high risk of relapse by detecting measurable ("minimal") residual disease (MRD) is of growing interest [2, 5–7]. The importance is also highlighted in the 2017 recommendations of the European LeukemiaNet (ELN) for diagnosis and management of AML defining a new response category named "complete remission without minimal residual disease" [8]. Consequently, establishing novel, reliable, and reproducible methods for MRD detection is an emerging research field. Multiparameter flow cytometry (MFC), a method based on immunophenotypic

Marius Bill and Juliane Grimm contributed equally to this work.

Presented in part and published in abstract form at the 21st Congress of the European Hematology Association (EHA) in Copenhagen, Denmark, the Annual Meeting of the Deutsche Gesellschaft für Hämatologie und Medizinische Onkologie (DGHO) in Leipzig, Germany, and the 57th Annual Meeting and Exposition of the American Society of Hematology (ASH) in Orlando, FL, USA.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00277-018-3373-y) contains supplementary material, which is available to authorized users.

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differences between AML and healthy hematopoietic cells, offers only limited sensitivity and depends on specialized, centralized laboratories. Besides MFC, quantitative reverse transcription polymerase chain reaction (RT-qPCR) has been established to measure MRD in recent years [5, 9–14]. MRD assessment by RT-qPCR offers a higher sensitivity and seems to be more robust in daily clinical use than MFC, but its application is restricted to AML subpopulations with distinct molecular alterations, such as *NPM1* mutations [5, 9–11, 13, 15–17]. Another limitation of RT-qPCR is the necessity of standard curves for absolute quantification, which complicates the direct comparison of results [15]. Digital droplet PCR (ddPCR) is a novel technique that may overcome this obstacle allowing a highly sensitive and specific absolute quantification without the need for standard curves. However, data on absolute quantification of *NPM1* mutations as MRD marker using ddPCR are still limited and further studies are needed to evaluate the feasibility of ddPCR application for MRD detection.

The MRD status prior to allogeneic hematopoietic stem cell transplantation assessed by multiparameter flow cytometry (MFC) was previously showed to be an important prognosticator [18, 19]. Araki et al. showed that the outcome of pre-transplant MRD-positive (MRD^{pos}) patients in hematological complete remission (CR) is comparable to that of patients transplanted with active disease [18]. Patients in these two studies were mainly younger and consequently received myeloablative conditioning regimes. However, allogeneic hematopoietic stem cell transplantation in older AML patients is of growing importance since it may improve survival in selected patients [20]. Data analyzing the impact of MRD, especially assessed apart from MCF, in patients receiving non-myeloablative conditioning regimens, which made allogeneic hematopoietic stem cell transplantation available to older and comorbid patients, are lacking.

Here, we performed—to our knowledge for the first time—absolute quantification of *NPM1* mutations as MRD marker using ddPCR in AML patients consolidated with allogeneic hematopoietic stem cell transplantation including 44 patients that received non-myeloablative conditioning. Our results constitute the application of ddPCR for mutated *NPM1* MRD detection to reliably identify patients at high risk of relapse offering the potential to guide future treatment decisions.

Patients and methods

Patients and treatment

We identified 51 AML patients with a *NPM1* mutation and pre-treatment bone marrow available, who received allogeneic hematopoietic stem cell transplantation at the University Hospital Leipzig between January 2001 and January 2016. In these patients, directly before hematopoietic stem cell transplantation, either bone marrow ($n = 11$, 21.6%; median 10 days, range 5–24 days) or peripheral blood ($n = 40$, 78.4%; median 6.5 days, range 0–20 days) was available. Patients received standard cytarabine-based chemotherapies and were transplanted in CR ($n = 41$, 80.4%) or in CR with incomplete recovery (CRi; $n = 10$, 19.6%). Written informed consent for participation in studies was obtained in accordance with the Declaration of Helsinki.

Forty-four patients (86.3%) received non-myeloablative, one patient (1.9%) reduced intensity, and six patients (11.8%) received myeloablative conditioning [21–24].

For further information on treatment protocol and definition of clinical endpoints, see supplementary material.

Cytogenetics and molecular analyses of *NPM1*, *FLT3*, and *CEBPA*

Pre-treatment bone marrow cytogenetics were determined using standard techniques for banding and fluorescence in situ hybridization.

At time of diagnosis, all patients were screened for mutations in the *NPM1* gene as previously described [24]. Exon 12 of positively screened patients was sequenced using Sanger method as previously described [21].

Presence of an internal tandem duplication (ITD) in the *FLT3* gene and mutations in CCAAT/enhancer-binding protein alpha (*CEBPA*) gene were also determined as previously described [21]. Patients were grouped into four genetic groups according to the European LeukemiaNet standardized reporting system of 2010 [2].

Absolute quantification by ddPCR

Complementary DNA (cDNA) was synthesized from RNA, which was isolated from pre-transplant bone marrow or peripheral blood as previously described [21]. Two separate PCR reactions were performed for *NPM1* mutation and for *ABL1* quantification (for details, see supplementary material). To achieve a higher specificity for the *NPM1* mutation detection, we applied a competitive probe approach, using wild-type and mutation-specific probes in each well. Droplets were generated using the QX200 AutoDG Droplet Digital (BioRad, Munich, Germany). PCR was performed as described in the supplementary material. Droplets were read with a QX200™ Droplet Reader (BioRad). Copy numbers of *NPM1* mutations were normalized to *ABL1* copy numbers. Samples were measured in triplicates. In concordance with previous studies, we only included samples with at least 1000 *ABL1* copies per well [12].

All samples with an average mutation burden ≤ 0.01% or < 3 positive droplets in three wells were defined negative according to the manufacturer's recommendations.

Statistical analyses

Statistical analyses were performed using the R statistical software platform (version 3.3.2). For further details, see supplementary material.

Results

Patient characteristics

We identified 51 AML patients with a *NPM1* mutation at diagnosis. The distribution of the mutation types—with 50 (98.0%) type A, one (2.0%) type D—did not differ from a previous study ($P = 0.19$) [17]. The median age of the patients was 61.6 years (range 32.6–73.9 years) at diagnosis. Further patients' characteristics are shown in Table 1.

Patients were in first ($n = 31$; 60.8%) or second ($n = 10$; 19.6%) CR or CRi ($n = 10$; 19.6%). Conditioning regimens were myeloablative ($n = 6$; 11.8%), reduced intensity ($n = 1$; 1.9%), or non-myeloablative ($n = 44$; 86.3%). On the day of transplantation, 11 patients (21.6%) received granulocyte colony-stimulating factor (G-CSF)-stimulated stem cells from HLA-matched related donors. Forty patients (78.4%) received G-CSF-stimulated stem cells from either HLA-matched ($n = 27$; 52.9%) or HLA-mismatched ($n = 13$; 25.5%) unrelated donors.

MRD status at transplantation

Using ddPCR for absolute quantification of *NPM1* mutation and *ABL1* copies, we found 17 of the 51 patients (33.3%) to be mutated *NPM1* MRD^{pos} prior to hematopoietic stem cell transplantation. The mutation burden showed a broad variation with a median of 0.29% mutated *NPM1* copies per *ABL1* copies (range 0.02–104.0%). Only one patient of the *NPM1* mutation MRD-negative (MRD^{neg}) cohort had mutated *NPM1* copies, but two log ranges below the 0.01% cutoff, and thus was defined as *NPM1* mutation MRD^{neg}.

Associations of clinical and transplant-related characteristics of the AML patients and mutated *NPM1* MRD status at transplantation are shown in Table 1. No significant differences at time of diagnosis were detectable, except that *NPM1* mutation MRD^{pos} patients were more often female (see Table 1).

At the time of allogeneic stem cell transplantation, *NPM1* mutation MRD^{pos} patients were less often in CR1 and more frequently in CR2 (19.4 vs 60.0%, $P = 0.04$). The frequency of patients with CRi did not differ between the MRD^{pos} and MRD^{neg} groups (24.9 vs 14.7%, $P = 0.27$, Table 1).

Outcome analysis

For the whole cohort, we observed a cumulative incidence of relapse of 25.6% with a median time to relapse of 101 days after transplantation (Fig. 1), and an overall survival of 61.6% 2 years after transplantation. In our cohort after transplantation, 15 patients (29.4%) relapsed, of whom 8 subsequently died, and additional 12 patients died because of non-relapse mortality. The median follow-up for patients alive was 2.2 years after transplantation.

We observed a significant difference in cumulative incidence of relapse ($P < 0.001$) and overall survival ($P = 0.014$) after allogeneic hematopoietic stem cell transplantation between pre-transplant mutated *NPM1* MRD^{pos} and MRD^{neg} patients (Fig. 2). The observed 2-year cumulative incidence of relapse was 64.7 vs 6.0% translating into an overall survival of 38.8 vs 71.7% in the pre-transplant mutated *NPM1* MRD^{pos} and MRD^{neg} patients, respectively. In multivariate analyses, mutated *NPM1* MRD^{pos} was the only prognostic factor associated with higher cumulative incidence of relapse (hazard ratio 21.1, confidence interval 4.9–91.6, $P < 0.001$) and also the only prognostic factor associated with shorter overall survival (hazard ratio 2.9, confidence interval 1.2–7.1, $P = 0.020$, Table 2).

In our cohort, 44 patients (median age 63.9 years, range 32.6–73.9 years) received non-myeloablative conditioning prior to allogeneic hematopoietic stem cell transplantation. Fourteen of the 44 patients receiving non-myeloablative conditioning were mutated *NPM1* MRD^{pos} (31.8%) of whom 11 relapsed after transplantation, resulting in a higher cumulative incidence of relapse (64.3 vs 6.8% 2 years after transplantation, $P < 0.001$) and shorter overall survival (39.0 vs 71.6% 2 years after transplantation, $P = 0.006$, Fig. 3) in the pre-transplant mutated *NPM1* MRD^{pos} and MRD^{neg} patients, respectively.

False positive and false negative identified patients

In the group of mutated *NPM1* MRD^{pos} patients, four of 17 patients (23.5%) did not relapse. However, three of these patients died due to treatment-related complications. Two of these patients died within 100 days after transplantation. Median time to relapse for all patients in our cohort was 101 days after transplantation (Fig. 1), suggesting that these patients might have died too early to experience relapse.

In the mutated *NPM1* MRD^{pos} group, two patients experienced relapse relatively late after transplantation (789 and 820 days). We tested if a later time point of relapse after transplantation was associated with a lower pre-transplant mutation burden and consequently lower level of residual

Table 1 Associations of pre-transplant mutated *NPM1* MRD status with clinical and transplant characteristics of 51 *NPM1*-mutated AML patients that received hematopoietic stem cell transplantation in CR or CRi

Characteristics	All patients, $n = 51$	Mutated *NPM1* MRD positive, $n = 17$	Mutated *NPM1* MRD negative, $n = 34$	*P* value
Mutated *NPM1/ABL1* copies, %				
Median	0.29	0.29		
Range	0.000006–104.0	0.02–104.0		
Age, years				
Median	61.6	59.0	63.9	0.22
Range	32.6–73.9	43.5–68.8	32.6–73.9	
Sex, *n* (%)				
Female	25 (49.0)	12 (70.6)	13 (38.2)	0.04
WBC at diagnosis, $\times 10^9$/l				
Median	37.8	35.1	40.7	0.98
Range	1.0–324.0	1.0–137.4	2.4–324.0	
Platelet count at diagnosis, $\times 10^9$/l				
Median	72.0	72.5	72	0.90
Range	3.0–238.0	3.0–207	15–238.0	
Hemoglobin at diagnosis, g/dl				
Median	9.2	8.4	9.5	0.59
Range	4.5–14.4	4.5–13.3	5.4–14.4	
Peripheral blast at diagnosis, %				
Median	38.0	27.5	40	0.98
Range	2.0–97.0	2.0–93.0	2.0–97.0	
Bone marrow blasts at diagnosis, %				
Median	66.0	65.0	66.0	0.39
Range	20.0–95.0	20.0–90.0	22.0–95.0	
2010 ELN genetic group, *n* (%)				
Favorable	28 (60.9)	11 (68.8)	17 (56.7)	0.14
Intermediate-I	11 (23.9)	4 (25.0)	7 (23.3)	
Intermediate-II	6 (13.0)	0 (0.0)	6 (20.0)	
Adverse	1 (2.2)	1 (6.3)	0 (0.0)	
FLT3-ITD at diagnosis, *n* (%)				
Absent	31 (60.8)	11 (64.7)	20 (58.8)	0.77
Present	20 (39.2)	6 (35.3)	14 (41.2)	
CEBPA at diagnosis, *n* (%)				
Wild-type	40 (88.9)	13 (81.2)	27 (93.1)	0.33
Mutated	5 (11.1)	3 (18.8)	2 (6.9)	
Remission status at transplantation, *n* (%)				
CR1	31 (60.8)	6 (35.3)	25 (73.5)	0.03
CR2	10 (19.6)	6 (35.3)	4 (11.8)	
CRi	10 (19.6)	5 (29.4)	5 (14.7)	
WBC pre-transplantation*, $\times 10^9$/l				
Median	3.7	2.3	4.3	0.13
Range	0–9.1	0.1–6.5	0–9.1	
Conditioning, *n* (%)				
Myeloablative	6 (11.8)	3 (17.6)	3 (8.8)	0.67
Reduced intensity	1 (1.9)	0	1 (2.9)	
Non-myeloablative	44 (86.3)	14 (82.4)	30 (88.2)	
Donor, *n* (%)				
HLA-matched related	11 (21.6)	4 (23.5)	7 (20.6)	0.96
HLA-matched unrelated	27 (52.9)	9 (52.9)	18 (52.9)	
HLA-mismatched unrelated	13 (25.5)	4 (23.5)	9 (26.5)	

MRD, measurable residual disease; *CR*, complete remission; *CRi*, complete remission without peripheral recovery; *ELN*, European LeukemiaNet classification 2010; *HLA*, human leukocyte antigen; *NPM1*, nucleophosmin 1; *ABL1*, Abelson murine leukemia viral oncogene homolog 1; *FLT3*, fms like tyrosine kinase 3; *ITD*, internal tandem duplication; *CEBPA*, CCAAT/enhancer-binding protein alpha; *WBC*, white blood count

*WBC from the day of blood or bone marrow collecting for analyses

disease, but no correlation of time from transplantation to relapse and mutated *NPM1/ABL1* copy numbers was observed ($P = 0.54$). However, the absolute number of relapsed patients in the MRD^{pos} group was small ($n = 13$) preventing further analyses.

Two patients out of 51 patients (6%) were MRD^{neg} prior to hematopoietic stem cell transplantation but nonetheless experienced relapse 68 and 244 days after transplantation, respectively. This may indicate the sensitivity limits of the assay. Another possible explanation is that relapse rose from a

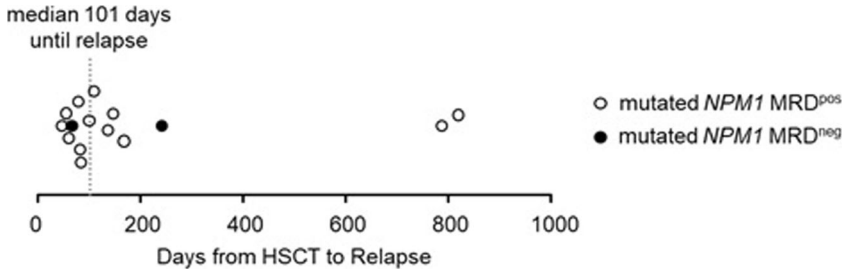

Fig. 1 Days until relapse after hematopoietic stem cell transplantation according to mutated *NPM1* measurable residual disease (MRD) status

NPM1 wild-type subclone. Although a high stability for *NPM1* mutations during clonal evolution is described, up to 9% of paired diagnosis/relapse samples did not show a detectable *NPM1* mutation in relapse samples in previous studies [12, 25]. However, there were no matched relapse samples available from these two patients to test for *NPM1* mutations at relapse.

The patient, who was MRD^{neg} but had positive mutated *NPM1* transcripts below the cutoff, was alive and in CR 5 years after allogeneic hematopoietic stem cell transplantation.

Discussion

Previous studies in AML patients receiving chemotherapy-based consolidation already indicated that mutated *NPM1* burden is an eligible MRD marker due to the high mutation frequency and a relatively high stability during clonal evolution (91–100% in paired diagnosis/relapse samples) [9, 12, 25]. Here, we investigated the prognostic impact of the pre-transplant mutated *NPM1* MRD status on outcome in AML patients that received allogeneic hematopoietic stem cell transplantation. We show that pre-transplant mutated *NPM1* MRD^{pos} associates with higher cumulative incidence of relapse and shorter overall survival independently of other clinical characteristics. These findings are in line with other studies which also found that the prognostic influence of mutated *NPM1* MRD outweighs the impact of clinical characteristics at diagnosis, including the diagnostic presence of *FLT3*-ITD, which typically impairs the prognosis in co-occurrence with *NPM1* mutations [5, 13, 17, 26–29].

In our study, we observed that MRD^{neg} patients were more likely to be in CR1, while 60% of patients transplanted in CR2 were mutated *NPM1* MRD^{pos}. This could indicate that patients who have experienced hematological relapse before might be more difficult to get into a deep molecular response in a later CR. Due to the small number of patients in CR2, we were not able to investigate whether the different distribution of CR1 and CR2 patients in the MRD^{pos} and MRD^{neg} group led to relapse or survival differences. However, previous studies showed that the prognostic impact of MRD status is comparable between patients in CR1 or CR2 [5, 26, 30]. In the studies by Ivey et al. [5] and Krönke et al. [17], it was shown that after two cycles of induction chemotherapy, mutated *NPM1* MRD^{pos} is an independent prognostic factor for higher risk of relapse and shorter overall survival. However, both studies were conducted in AML cohorts which were mainly consolidated using chemotherapy-based regimens [5, 17]. A retrospective study by the Acute Leukemia French Association Group showed that mutated *NPM1* MRD can also be applied to determine AML patients who particular benefit

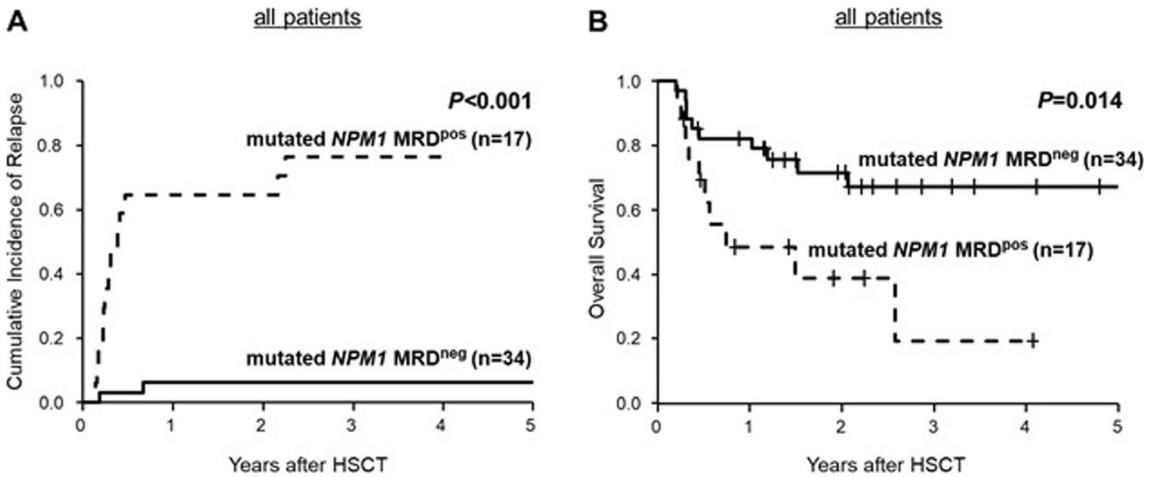

Fig. 2 Cumulative incidence of relapse (**a**) and overall survival (**b**) after allogeneic stem cell transplantation in CR or CRi according to pre-transplant mutated *NPM1* measurable residual disease (MRD) in *NPM1*-mutated AML patients

Table 2 Multivariate analyses of 51 *NPM1*-mutated AML patients that received allogeneic stem cell transplantation in CR or CRi

Variable	Cumulative incidence of relapse		Overall survival	
	HR[a] (95% CI)	*P*	HR[a] (95% CI)	*P*
Mutated *NPM1* MRD (positive vs. negative) prior to transplantation	21.1 (4.9–91.6)	< 0.001	2.9 (1.2–7.1)	0.020

NPM1, nucleophosmin 1; *MRD*, measurable residual disease; *CI*, confidence interval; *HR*, hazard ratio

[a] HR, hazard ratio, < 1 (> 1) indicate lower (higher) risk for an event for the first category listed for the dichotomous variables. Variables considered in the models were those significant at $\alpha = 0.20$ in univariable analyses. For OS endpoint, variables considered were hemoglobin count at diagnosis, peripheral blood blasts at diagnosis, bone marrow blasts at diagnosis, and mutated *NPM1* MRD (positive vs negative) while for CIR endpoint, variables considered were disease origin (secondary vs de novo) and mutated *NPM1* MRD (positive vs negative)

from transplantation compared to chemotherapy-based consolidation [28]. They detected improved disease-free and overall survival after allogeneic hematopoietic stem cell transplantation only for patients with a < 4-log reduction of the mutated *NPM1* MRD in peripheral blood after induction chemotherapy, while no such benefit was shown for patients with an > 4-log reduction [28].

In our study, we show that pre-transplant mutated *NPM1* MRD^{pos} status independently predicts poor outcome which is consistent with previous studies assessing the pre-transplant *NPM1* mutation MRD in AML cohorts receiving myeloablative or reduced intensity conditioning [26, 27]. However, a distinguishing feature of our study is the large proportion (86.3%) of older patients (median age 61.6 years) who mainly received non-myeloablative conditioning. When we restricted our analysis to patients receiving non-myeloablative conditioning, *NPM1* mutation MRD^{pos} identified patients with a high cumulative incidence of relapse and subsequent shorter overall survival (Fig. 3).

In our study, we applied the novel ddPCR methodology allowing robust and sensitive absolute quantification of mutated *NPM1* copy numbers. With this highly specific technique, 97% of MRD^{neg} patients did not have any traceable mutated *NPM1* copies (one patient was designated MRD^{neg} applying a 0.01% mutated *NPM1*/*ABL1* cutoff). This might also be an advantage compared to the other studies assessing the mutated *NPM1* MRD status prior to transplantation since they used standard RT-qPCR and applied higher cutoffs to determine MRD^{pos} and MRD^{neg} (0.1 and 1%) [26, 27]. Due to restricted quantity of patient material, we could only perform comparative RT-qPCR quantification prior to transplantation for a small number of patients (see supplementary material).

A recent study already indicated that ddPCR is an eligible method to determine mutated *NPM1* MRD applying a multiplex PCR with mutation-specific primers [31]. Here, we used competitive probes specific for the wild-type or mutated sequence, showing that this approach is feasible for mutated *NPM1* MRD detection with ddPCR.

The current studies on mutated *NPM1* MRD prior to transplantation are heterogeneous concerning the quantification method, the cutoff but also with regard to used material (our study, bone marrow and peripheral blood

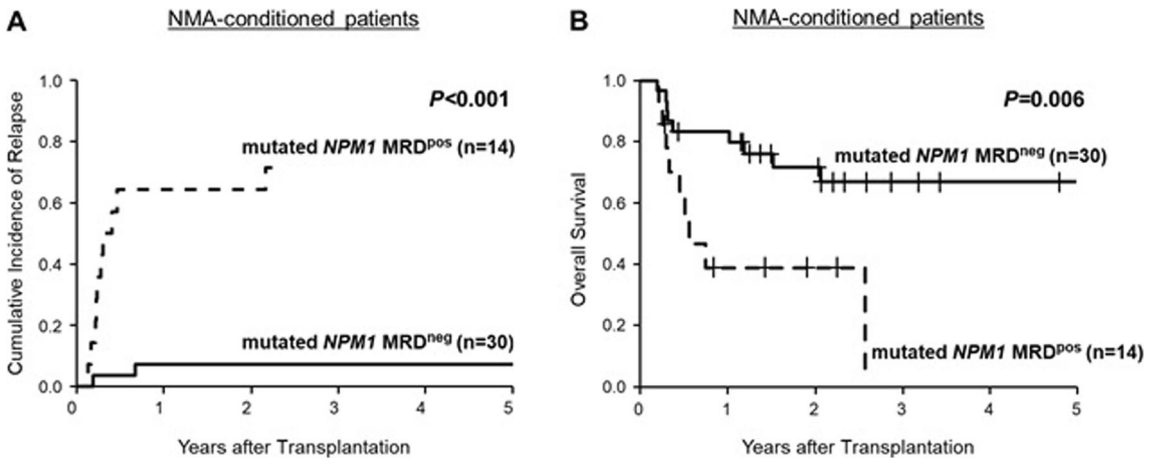

Fig. 3 Cumulative incidence of relapse (**a**) and overall survival (**b**) after allogeneic stem cell transplantation in CR or CRi according to pre-transplant mutated *NPM1* measurable residual disease (MRD) in *NPM1*-mutated AML patients that received non-myeloablative (NMA) conditioning

vs bone marrow [26] vs bone marrow and peripheral blood [27]) and time of sampling (our study, 24 vs 7 days [26] vs 2 months [27] prior to transplantation). Thus, prospective trials are needed to standardize the optimal material, time points, and cutoffs for a meaningful and comparable application of pre-transplant *NPM1* mutation MRD detection in clinical routine.

Prospective clinical trials should also be conducted to identify potential treatment options to improve the prognosis of AML patients who are mutated *NPM1* MRD^{pos} prior to hematopoietic stem cell transplantation. To date, it is uncertain whether mutated *NPM1* MRD^{pos} patients might benefit from additional therapy prior to transplantation or intensification of the conditioning regimen [32–34]. After transplantation, interventions for MRD^{pos} transplanted patients are also conceivable, e.g., accelerated tapering of immunosuppression, administration of donor lymphocyte infusions or demethylating agents [35–37], and should be tested in future clinical trials. Patients transplanted with *NPM1* mutation MRD^{pos} should be continuously monitored after allogeneic hematopoietic stem cell transplantation, e.g., by mutated *NPM1* MRD and/or chimerism analysis, to detect imminent hematological relapse at the earliest stage possible. In our study, relapse occurred within a short period of time after transplantation (median of 101 days).

Here, we could show that mutated *NPM1* MRD^{pos} independently predicts higher cumulative incidence of relapse and shorter overall survival. Our results emphasize that older AML patients transplanted following non-myeloablative conditioning have particular dismal prognosis when mutated *NPM1* MRD is detectable prior to hematopoietic stem cell transplantation. The presented study underlines that ddPCR is an eligible method to routinely determine mutated *NPM1* MRD status in AML patients. Future prospective trials might help to address the issue of heterogeneous sampling time points and methodology to facilitate the comprehensive application of mutated *NPM1* MRD detection in AML routine diagnostics. Additionally, evidence-based treatment regimens before and after hematopoietic stem cell transplantation are needed to improve the poor outcome of patients transplanted with traceable mutated *NPM1* MRD.

Acknowledgements The authors thank Ines Kovacs, Kathrin Wildenberger, Scarlett Schwabe, Christine Günther, Daniela Bretschneider, Evelin Hennig, and Christel Müller for their assistance.

Authors' contributions M.B., J.G., and S.S. designed, performed experiments, analyzed, and interpreted data. L.K., K.G., J.S., S.B., and J.H. performed experiments. M.J. analyzed data. T.L., M.C., G.B., V.V., W.P., and GN.F. provided administrative and technical support. M.B., J.G., and S.S. wrote and all authors reviewed and approved the manuscript. D.N. and S.S. supervised the study.

Funding information This work was supported by the Deutsche José-Carreras-Stiftung (#04R/2016 and #PS15/05 J.G.), Verein zusammen gegen den Krebs e.V., Ein Herz für Kinder e.V., and the Medical Faculty of the University of Leipzig (#990101-089).

Compliance with ethical standards

Written informed consent for participation in studies was obtained in accordance with the Declaration of Helsinki.

Conflict of interest The authors declare that they have no conflict of interest.

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