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Quantification of minimal disseminated disease by quantitative polymerase chain reaction and digital polymerase chain reaction for *NPM-ALK* as a prognostic factor in children with anaplastic large cell lymphoma

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ABSTRACT

Detection of minimal disseminated disease is a validated prognostic factor in ALK-positive anaplastic large cell lymphoma. We previously reported that quantification of minimal disease by quantitative real-time polymerase chain reaction (RQ-PCR) in bone marrow applying a cut-off of 10 copies *NPM-ALK*/10^4 copies of *ABL1* identifies very high-risk patients. In the present study, we aimed to confirm the prognostic value of quantitative minimal disseminated disease evaluation and to validate digital polymerase chain reaction (dPCR) as an alternative method. Among 91 patients whose bone marrow was analyzed by RQ-PCR, more than 10 normalized copy-numbers correlated with stage III/IV disease, mediastinal and visceral organ involvement and low anti-ALK antibody titers. The cumulative incidence of relapses of 18 patients with more than 10 normalized copy-numbers of *NPM-ALK* was 61±12% compared to 21±5% for the remaining 73 patients (P=0.0002). Results in blood correlated with those in bone marrow (r=0.74) in 70 patients for whom both materials could be tested. Transcripts were quantified by RQ-PCR and dPCR in 75 bone marrow and 57 blood samples. Copy number estimates using dPCR and RQ-PCR correlated in 132 samples (r=0.85). Applying a cut-off of 30 copies *NPM-ALK*/10^4 copies *ABL1* for quantification by dPCR, almost identical groups of patients were separated as those separated by RQ-PCR. In summary, the prognostic impact of quantification of minimal disseminated disease in bone marrow could be confirmed for patients with anaplastic large cell lymphoma. Blood can substitute for bone marrow. Quantification of minimal disease by dPCR provides a promising tool to facilitate harmonization of minimal disease measurement between laboratories and for clinical studies.

Introduction

ALK-positive anaplastic large cell lymphomas (ALCL) in children and adolescents are characterized by translocations involving the *ALK* gene on chromosome 2p23.[1] About 90% of ALK-positive ALCL carry the translocation t(2;5)(p23;q35) resulting in the fusion gene *NPM-ALK*.[2-4] Between 25% and 35% of patients relapse with current treatment protocols.[5-9]

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Detection of blasts in bone marrow (BM) and blood by cytomorphological analysis is a rare event in ALCL.[6,7] The chimeric fusion gene transcript *NPM-ALK* has been used to investigate the prognostic value of submicroscopic minimal disseminated disease (MDD) in BM and blood at diagnosis in independent cohorts of patients.[10-13] Polymerase chain reaction (PCR) analysis allows the reliable detection of one circulating tumor cell among 100,000 normal cells.[10] The detection of MDD by qualitative PCR in BM or blood (55% of patients) conferred a relapse risk of 50% in several studies.[10-12,14] Measurement of minimal residual disease (MRD) using the qualitative PCR assay enabled identification of a very high-risk group of patients.[14]

We previously reported the possibility of identifing patients bearing a very high risk of relapse already at diagnosis by quantification of fusion gene transcripts using an *NPM-ALK*-specific quantitative real-time (RQ)-PCR assay. Applying a cut off of 10 copies *NPM-ALK* per 10^4copies of the reference transcript *ABL1* (normalized copy numbers, NCN), 16 patients (22%) with more than 10 NCN *NPM-ALK* in the BM at diagnosis had a 5-year probability of event-free survival of 23±11% compared to 78±6% for the 58 patients with NCN below the cut-off. MDD levels measured in blood provided comparable results.[12]

The Japanese NHL study group recently reported the outcomes of 60 ALCL-patients according to MDD in blood or BM using the same cut-off of 10 NCN *NPM-ALK*.[13] The patients received comparable therapies. Compared to the Berlin-Frankfurt-Münster (BFM) group, however, more patients showed MDD levels above 10 NCN. The progression-free survival of 37 patients with >10 NCN *NPM-ALK* in blood or BM was 58±12% compared to 85±6% for the 22 patients with ≤10 NCN.[13]

The differences regarding the prognostic value of MDD assessment by RQ-PCR in these two studies illustrate the need for standardization before the implementation of quantification of *NPM-ALK* transcripts for initial risk assessment or MRD evaluation in clinical studies. Currently, quantitative values from different laboratories cannot be directly compared to each other, whereas MRD quantification within one laboratory has been reported to enable the course of the disease to be monitored.[15-17]

To achieve comparability of MRD quantification for *NPM-ALK* obtained by RQ-PCR in different reference laboratories, extensive protocol harmonization is necessary, as was done for the quantification of *BCR-ABL1* fusion gene transcripts in acute lymphoblastic leukemia and chronic myelogenous leukemia.[18,19] Since quantification is performed at the lowest end of the necessary standard curve in *NPM-ALK*-specific RQ-PCR, a quantitative PCR approach with improved reproducibility without the need for a standard curve would be advantageous. Digital PCR (dPCR) may represent a quantitative PCR method that could be used as a replacement for RQ-PCR for *NPM-ALK* copy number estimation in ALK-positive ALCL. dPCR is a quantitative PCR method based on the distribution of the target RNA or DNA molecules in many partitions.[20] The amount of partitions with a positive PCR results allows the concentration of a given target to be determined without the need for standard curve calibration.[21]

The aim of this work was to validate the prognostic meaning of quantitative MDD measurements of *NPM-ALK* fusion gene transcripts by RQ-PCR in an independent cohort of uniformly treated ALK-positive ALCL patients of the BFM group. In addition, in an effort to facilitate quality-controlled quantification between different laboratories, a dPCR assay for quantification of *NPM-ALK* transcripts was developed and validated.

Methods

Patients

Patients with ALCL from Austria, Germany and Switzerland enrolled in the ALCL99 trial or the NHL-BFM registry 2012 between January 2006 and December 2016 were eligible after confirmation of NPM-ALK positivity of the ALCL. Both studies were approved by the institutional ethics committee of the primary investigators. Informed consent from the patients/caregivers to the studies included consent for future research on MDD.

Controls and cell lines

Blood from 20 healthy donors and eight ALK-negative ALCL patients included in ALCL99 or the NHL-BFM registry served as negative controls after written informed consent.

The cell lines HL-60 (acute myeloid leukemia), SU-DHL-1 and Karpas-299 (NPM-ALK-positive ALCL) were obtained from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ, Braunschweig, Germany).

Complementary DNA synthesis and quantitative real-time polymerase chain reaction

Complementary DNA (cDNA) synthesis and RQ-PCR were performed as described previously.[12] In total four replicates were analyzed (two with undiluted cDNA and two with 1+1 diluted cDNA, as an additional control for RQ-PCR inhibition). Samples which were positive for *NPM-ALK* in one to three of four replicates only or had NCN below one copy were considered as low positive not quantifiable. Negativity for *NPM-ALK* in all four replicates was considered as negative.

Digital polymerase chain reaction assay

Primer and probe sequences for the *NPM-ALK*- and *ABL1*-specific dPCR assay were identical to those used for the RQ-PCR assay.[12] Probes used for dPCR were ordered with 5'FAM™ as the reporter dye and the double quencher dyes ZEN™ and 3'Iowa-Black®FQ (IDT, Leuven, Belgium).

Ten microliters of dPCR™ supermix for probes (no dUTP; BIO-RAD, Munich, Germany), 0.6 μL forward primers, 0.6 μL reverse primers (10 μM, final concentration 300 pM) and 1 μL probe (final concentration 250 pM) were used in a reaction volume of 20 μL. Droplets were generated with the QX-200 droplet generator (BIO-RAD, Munich, Germany). The PCR was performed at 95°C for 10 min for enzyme activation, 44 cycles at 94°C for 30 s, followed by 1 min at 54.1°C for annealing and extension, and enzyme inactivation at 98°C for 10 min. Droplets were measured with the QX200 droplet reader and were analyzed with Quanta Soft pro analysis software (BIO-RAD, Munich, Germany). Four replicates per sample were measured. Only replicates with ≥10,000 accepted droplets were included in the analysis. The threshold for discrimination between positive and negative droplets was set manually with an adequate distance from the background. cDNA from the ALK-positive cell line SU-DHL-1 (positive control), HL-60 (negative control) and no template controls were included in each measurement. Copy numbers were normalized to 10,000 copies of the reference gene *ABL1* (NCN). Samples with <1,000 copies of *ABL1* were excluded. Samples with detectable fusion gene transcripts in one to three, but not in all four replicates were defined as low positive, not quantifiable. Samples were defined as negative if no positive droplets were observed.

Statistical analysis

Event-free survival and overall survival were analyzed using the Kaplan-Meier method with differences compared by the log-rank test. Cumulative incidence functions for relapse were constructed using the method of Kalbfleisch and Prentice. Functions were compared with the Gray test. Quantification by RQ-PCR and dPCR was compared using Spearman correlation. All analyses were performed using SAS (SAS-PC, version 9.4, SAS Institute Inc., Cary, NC, USA).

Results

Quantification of *NPM-ALK* fusion gene transcripts by quantitative real-time polymerase chain reaction (validation cohort)

Patients' characteristics

MDD was quantified by RQ-PCR in initial BM samples from 91 *NPM-ALK*-positive ALCL patients. Parallel blood samples for quantification were available from 70 of those patients. The clinical and biological characteristics of the 91 patients are shown in Table 1. Twenty-six of the 91 patients relapsed, one patient died from initial tumor complications. The cumulative incidence of relapse at 3 years of the 91 patients was 29±5%, the event-free survival at 3 years was 70±5% and the overall survival 92±3%. More than 10 NCN *NPM-ALK* were measured in the BM of 18 patients and ≤10 NCN *NPM-ALK* were detected in the remaining 73 patients.

The detection of >10 NCN *NPM-ALK* in BM correlated with stage III/IV disease, mediastinal and visceral organ involvement, as well as low anti-ALK antibody titers (Table 1). No association of *NPM-ALK* copy numbers above 10 NCN and histological subtype was observed (Table 1).

Prognostic impact of quantitative minimal disseminated disease in bone marrow

The cumulative incidence of relapse of 18 patients with more than 10 NCN *NPM-ALK* in BM was 61±12% compared to 21±5% for the remaining 73 patients (P=0.0002), The event-free survival rates at 3 years were 33±11% and 79±5%, respectively (P<0.0001), the overall survival rates were 83±9% and 94±3%, respectively (P=0.099) (*Online Supplementary Figure S1*). Application of the cut-off of 10 NCN *NPM-ALK* allowed the separation of a group of patients with a very high risk of relapse in the validation cohort.

Prognostic impact of quantitative minimal disseminated disease in blood

In 70 of the 91 patients for whom MDD was measured in the BM, *NPM-ALK* transcripts could be measured in blood, as well. The results for blood and BM in the same patients correlated (r=0.74). Notably, more patients were MDD-positive and showed higher copy numbers in blood compared to BM (Figure 1).

At 3 years the cumulative incidence of relapse of the 70 patients for whom MDD measurements were available in both BM and blood was 26±5%, the event-free survival was 74±5% and the overall survival 94±3%. To analyze a possible influence of the biological medium used for the quantitative MDD measurement on the detection of very high-risk patients, outcome was compared according to quantitative MDD in blood and BM using the same cut-off among these 70 patients. The cumulative incidence of relapse of the 17 patients with >10 NCN *NPM-ALK* measured in blood was 59±13% compared to 15±5% in 53 patients with ≤10 NCN *NPM-ALK* (P=0.0004). In comparison, the cumulative incidence of relapse of the 13 patients with >10 NCN *NPM-ALK* measured in BM was 62±14% compared to 18±5% in the 57 patients with ≤10 NCN (P=0.0007) (Figure 2).

Establishment and validation of a digital droplet polymerase chain reaction assay

To overcome some limitations of RQ-PCR we tested an *NPM-ALK*-specific dPCR assay for fusion gene and reference gene quantification.

A gradient PCR was performed to optimize the performance of the dPCR assays for *NPM-ALK* and *ABL1*.[12] The amplification and elongation temperature was set to

Table 1. Association of the quantity of *NPM-ALK* transcripts in bone marrow with patients' characteristics, clinical and biological risk factors in the validation cohort.

		MDD		
	All patients	≤10 NCN *NPM-ALK*	>10 NCN *NPM-ALK*	*P*
	91	73	18	
Gender, n (%)				
Male	59	47 (64%)	12 (67%)	1.0
Female	32	26 (36%)	6 (33%)	
Stage*, n (%)				
I	5	5 (7%)	0 (0%)	0.002
II	20	20 (27%)	0 (0%)	
III	56	42 (58%)	14 (78%)	
IV	6	2 (3%)	4 (22%)	
n.a.	4	4 (5%)		
Age, n (%)				1.0
Age <10 years	24	19 (26%)	5 (28%)	
Age ≥10 years	67	54 (74%)	13 (72%)	
CNS, n (%)				
Negative	83	67 (100%)	16 (100%)	
Positive	-	-	-	
Bone marrow, n (%)				0.004
Negative	86	72 (99%)	14 (78%)	
Positive	5	1 (1%)	4 (22%)	
Bone, n (%)				0.14
No	77	64 (88%)	13 (72%)	
Yes	14	9 (12%)	5 (28%)	
Skin, n (%)				1.0
No	73	58(80%)	15 (83%)	
Yes	18	15 (20%)	3 (17%)	
Mediastinum, n (%)				0.001
No	52	48 (66%)	4 (22%)	
Yes	39	25 (34%)	14 (78%)	
Viceral organs**, n (%)				0.01
No	68	59 (81%)	9 (50%)	
Yes	23	14 (19%)	9 (50%)	
Histology				0.59
Non-common	35	27 (39%)	8 (47%)	
Common	52	43 (61%)	9 (53%)	
Anti-ALK titer				0.03
≤750	24	15 (28%)	9 (60%)	
>750	44	38 (72%)	8 (40%)	

MDD: minimal disseminated disease; NCN: normalized copy number; n.a.: not available; CNS: central nervous system. * St. Jude staging system; ** liver, spleen, lung.

54.1°C after optimization. A serial limited 10-fold dilution of the ALK-positive cell line Karpas 299 in peripheral blood mononuclear cells from 10^{-1} to 10^{-5} was performed to evaluate the sensitivity and specificity of the dPCR assay (Table 2). Normalized copies of *NPM-ALK* were detected in cDNA prepared from dilutions of 10 ALK-positive cells in 1,000,000 normal cells in one out of four replicates. The peripheral blood mononuclear cells used for dilution had no detectable copies of *NPM-ALK*.

To estimate the rate of false positivity, blood from 20 healthy controls and BM or blood from eight ALK-negative ALCL patients was analyzed. No positive droplets were detected in BM or blood from ALK-negative ALCL patients or 20 healthy controls. All samples were negative by RQ-PCR. Since higher copy numbers of *NPM-ALK* were measured by dPCR compared to RQ-PCR, the cut-off for outcome analysis was set at 30 NCN for the dPCR.

Comparison of *NPM-ALK* quantification by digital and quantitative real-time polymerase chain reaction analyses in bone marrow and blood samples

Measurement of circulating tumor cells using RQ-PCR and dPCR was possible in 132 initial BM (n=75) or blood (n=57) samples from ALCL patients. Forty-five samples were negative by both RQ-PCR as well as dPCR (31/75 BM, 14/57 blood samples). Of the 75 BM samples, 19 and 15 were low positive, not quantifiable by dPCR and RQ-PCR, respectively. Of the 57 blood samples, 21 and 14 were low positive by dPCR and RQ-PCR, respectively. The results of dPCR and RQ-PCR correlated with a correlation coefficient of r=0.85 (Figure 3). Above the threshold of 10 NCN *NPM-ALK* measured by RQ-PCR the copy numbers obtained by both methods were highly correlated with a correlation coefficient of r=0.96, but generally higher with dPCR.

Comparison of patient stratification according to the results of quantitative real-time and droplet polymerase chain reaction analyses in bone marrow and blood

The cumulative incidence of relapse, event-free survival and overall survival of patients with dPCR quantification of MDD in BM (n=75) and blood (n=57) by dPCR using a threshold of 30 NCN are shown in Figure 4. The comparable data according to RQ-PCR, using the cut-off of 10 NCN, were almost identical. The patients' distribution according to MDD measured in BM was concordant with both quantification methods (Table 3). Among the 57 patients for whom MDD was quantified in blood, only one patient had a discordant result with ≤30 (dPCR) but >10 NCN (RQ-PCR) (Table 3b).

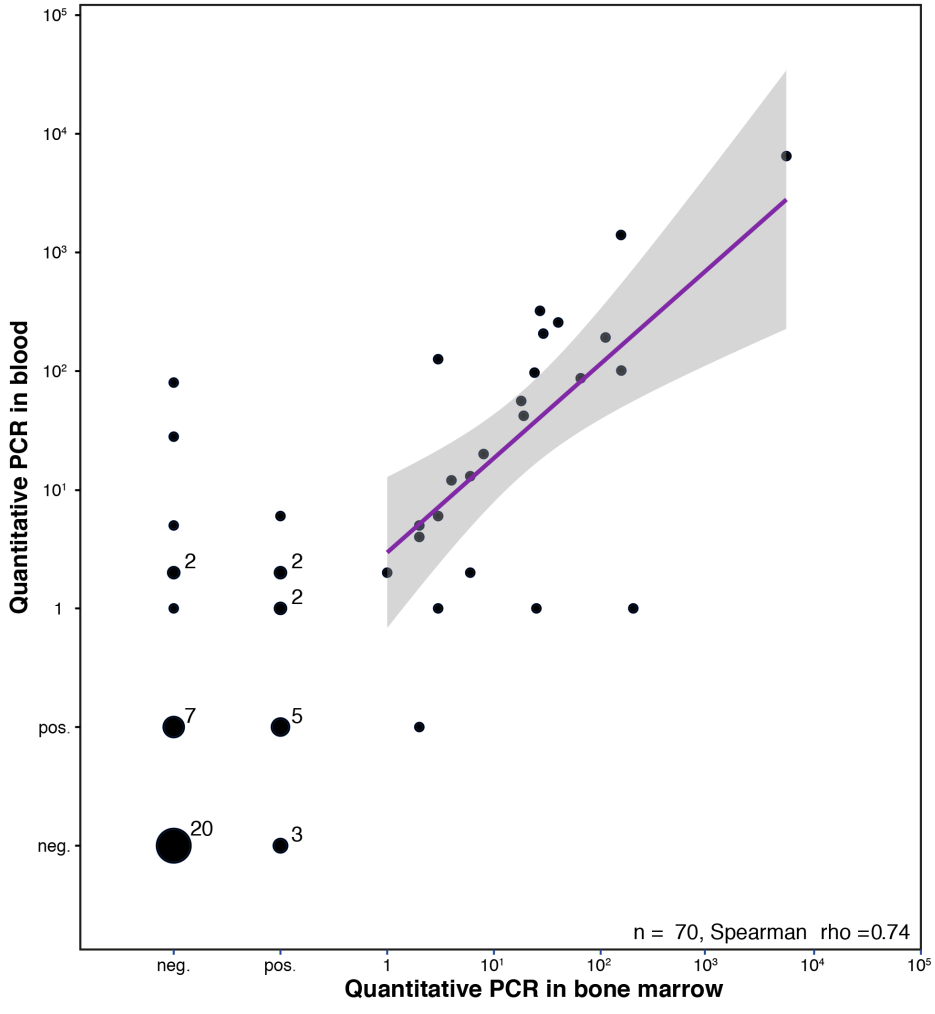

Figure 1. Normalized copy numbers of *NPM-ALK* in blood and bone marrow measured by quantitative real-time polymerase chain reaction. Copy numbers of *NPM/ALK*/10^4 copy numbers of *ABL1* were measured in 70 patients in initial blood and bone marrow samples. PCR: polymerase chain reaction.

Discussion

In our previous study, quantitative measurement of *NPM-ALK* fusion gene transcripts in blood or BM using a cut-off of 10 NCN in the RQ-PCR analysis allowed us to identify the group of patients with the highest risk of relapse. We were able to confirm these results in the validation cohort of uniformly treated *NPM-ALK*-positive ALCL patients. As in our previous analysis, only 20% of patients had more than 10 NCN *NPM-ALK* detectable in BM. Two-thirds of those patients relapsed in both series of altogether 175 ALCL patients.

When comparing the event-free survival of the very high-risk group determined by quantification of MDD in blood between the two cohorts, the EFS of high-risk patients was somewhat higher in the validation cohort than in the earlier cohort. This difference might be attributable to a selection bias with a higher event-free survival in the current cohort analyzed in blood compared to the previously reported cohort (previous cohort 61±6%, current cohort 74±5%). In the current validation cohort, MDD results measured in blood and BM of the same patients were comparable and had the same prognostic impact. For future studies, investigation of peripheral blood could therefore be sufficient for quantitative MDD evaluation. This is especially helpful bearing in mind the application of MRD to follow the course of disease in very high-risk patients or after relapse.

Compared to the earlier cohort including patients diagnosed until 2005, the survival of the very high-risk patients, as defined by more than 10 NCN in BM, improved in the validation cohort (83% compared to 46%). New therapeutic options became available for patients with relapsed ALCL, ranging from vinblastine monotherapy, brentuximab vedotin, ALK kinase inhibitors to PD-L1 checkpoint inhibitors.[15,17,22-28] In addition, allogeneic blood stem cell transplantation was increasingly used for consolidation in relapse.[29-32]

Our results show that separation of patients with a high risk of relapse can be achieved by quantification of MDD in patients with ALCL with the prerequisites that quantitative PCR evaluation is performed in the same laboratory

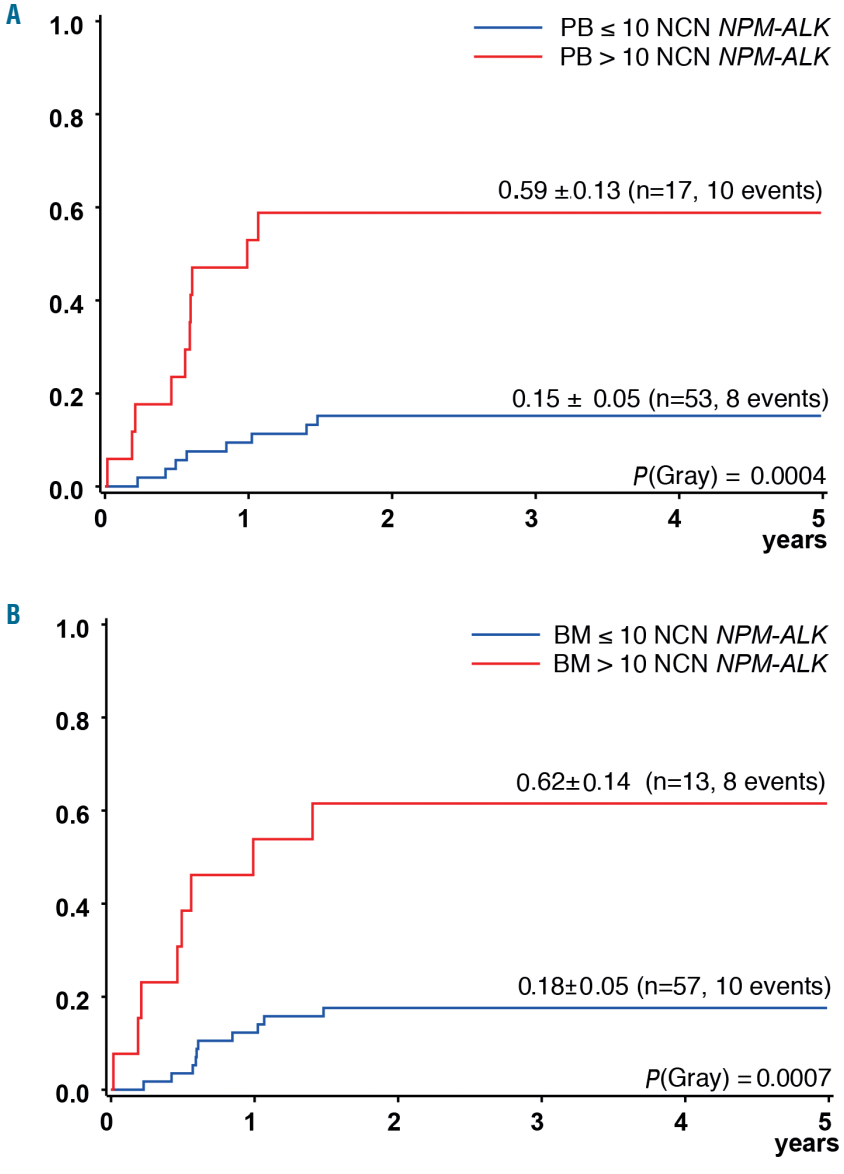

Figure 2. Outcome according to *NPM-ALK* copy numbers measured by quantitative real-time polymerase chain reaction in blood and bone marrow. Cumulative incidence of relapse (according to a cut-off of 10 normalized copy numbers of *NPM/ALK*/10^4 copy numbers of *ABL1*) measured in initial (A) blood and (B) bone marrow samples in 70 patients. PB: blood; BM: bone marrow; NCN: normalized copy number.

by the same persons, according to standard operating procedures for RQ-based MDD measurement and analysis. However, there are still significant inter-laboratory differences and quantification of minimal disease in patients with ALCL can currently not be compared between laboratories.[12,13,17,33] This is exemplified by the comparison of the Japanese study group's data with our data. Both groups used the same therapy and the same RQ-PCR assay for quantification. Twenty per cent of patients showed >10 NCN *NPM-ALK* in our cohorts and 37% of patients had high copy numbers in the Japanese cohort even though the relapse rate in the Japanese cohort was somewhat lower. Accordingly, the relapse risk of patients with >10 NCN *NPM-ALK* was higher in our cohorts (65%) than in the Japanese cohort (40%). In order to use quantification of copy numbers for patient stratification or to follow the course of individual patients in multinational studies, the RQ-PCR method needs very strict protocol harmonization and quality control. The experiences from quantification of *BCR-ABL1* transcripts can partly guide this development.[34] The introduction of calibrators, specific conversion factors to the calibrators for each laboratory and calibrated reference material led to a high standardization of *BCR-ABL1* measurements.[19,35-37] In Philadelphia-positive acute leukemia the optimization and standardization process for RQ-PCR-based measurement of m-*BCR-ABL1* transcripts underscores the importance of standardization of all steps for quantitative PCR, including data interpretation and quality controls.[38] In the standardization process of MRD assessment of m-*BCR-ABL1* fusion gene transcripts organized by the Euro MRD consortium, the usage of a common primer and probe set as well as a centrally distributed plasmid standard curve had the greatest impact on overcoming inter-laboratory variability.[38]

Since the same primer/probe sets and the same RQ-PCR protocol were applied by the Japanese and BFM study

Table 2. Normalized copy numbers of *NPM-ALK* measured by digital polymerase chain reaction analysis in a 10-fold serial dilution of a *NPM-ALK* anaplastic large cell lymphoma cell line in 10^6 mononuclear cells.

Dilution	ALK$^+$ cells in 10^6 MNC	Copies of *NPM-ALK*/10^4 *ABL1*
10^{-1}	100,000	9222
10^{-2}	10,000	974
10^{-3}	1,000	93
10^{-4}	100	8
10^{-5}	10	0.4
0	0	0

MNC: mononuclear cells.

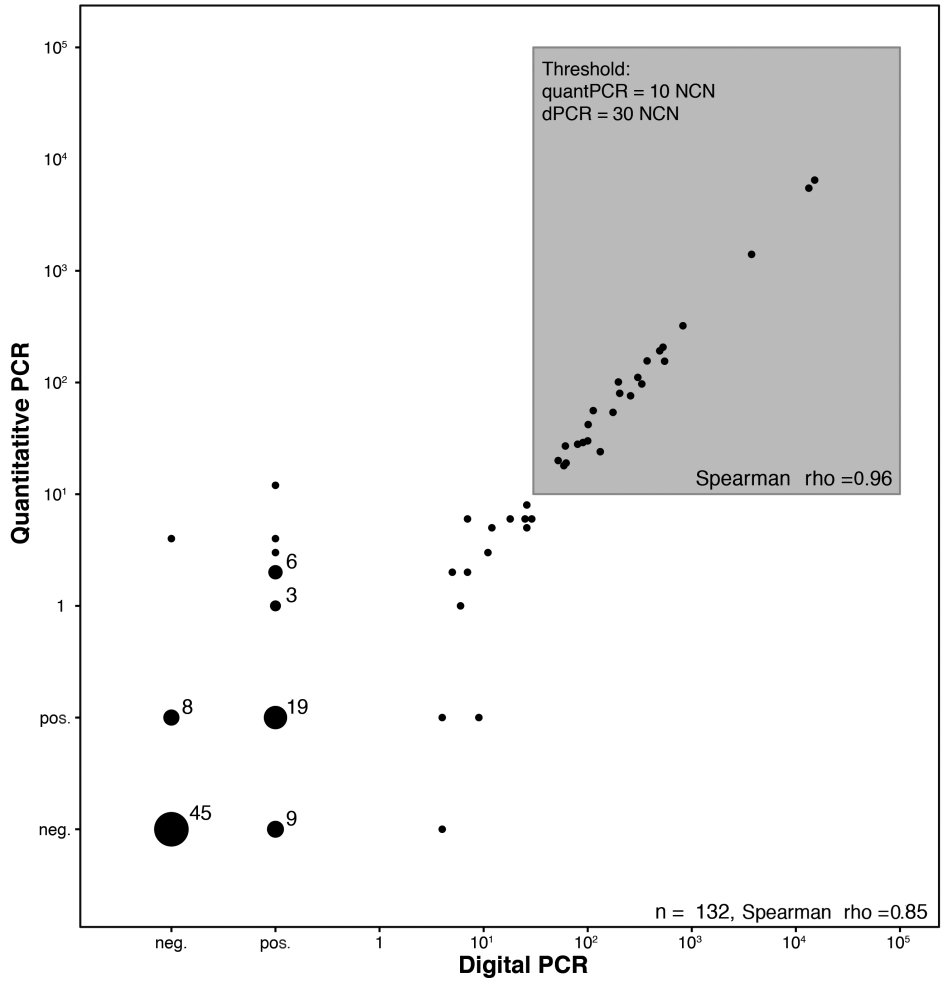

Figure 3. Comparison of *NPM-ALK* copy numbers measured by quantitative real-time polymerase chain reaction and digital polymerase chain reaction. Normalized copy numbers of *NPM/ALK*/10^4 copy numbers of *ABL1* measured in 132 blood and bone marrow samples. quantPCR: quantitative real-time polymerase chain reaction; dPCR: digital polymerase chain reaction; NCN: normalized copy number.

groups, the differences in results might be related to the use of standard curves that were not centrally distributed and to the fact that the cut-off at 10 NCN *NPM-ALK* is close to the detection limit of the assay. The latter point is an unchangeable limitation to inter-laboratory comparability for quantification of *NPM-ALK* transcripts and a major difference from MRD quantification in leukemia.

In order to overcome some of the technical problems inherent to RQ-PCR we developed a dPCR method for the quantification of *NPM-ALK* transcripts and compared the results obtained with this method to those obtained with RQ-PCR in a large cohort of patients. Using dPCR with a cut-off at 30 NCN *NPM-ALK* for quantitative measurements of MDD in blood and BM in the presented study allowed measurements near to the detection limit without needing standard curve calibration. The dPCR assay might be more suitable for quantitative measurements of *NPM-ALK* in a multinational setting, because it overcomes several limitations of the RQ-PCR assay. First, it is independent of a calibration curve, thereby excluding the impact of

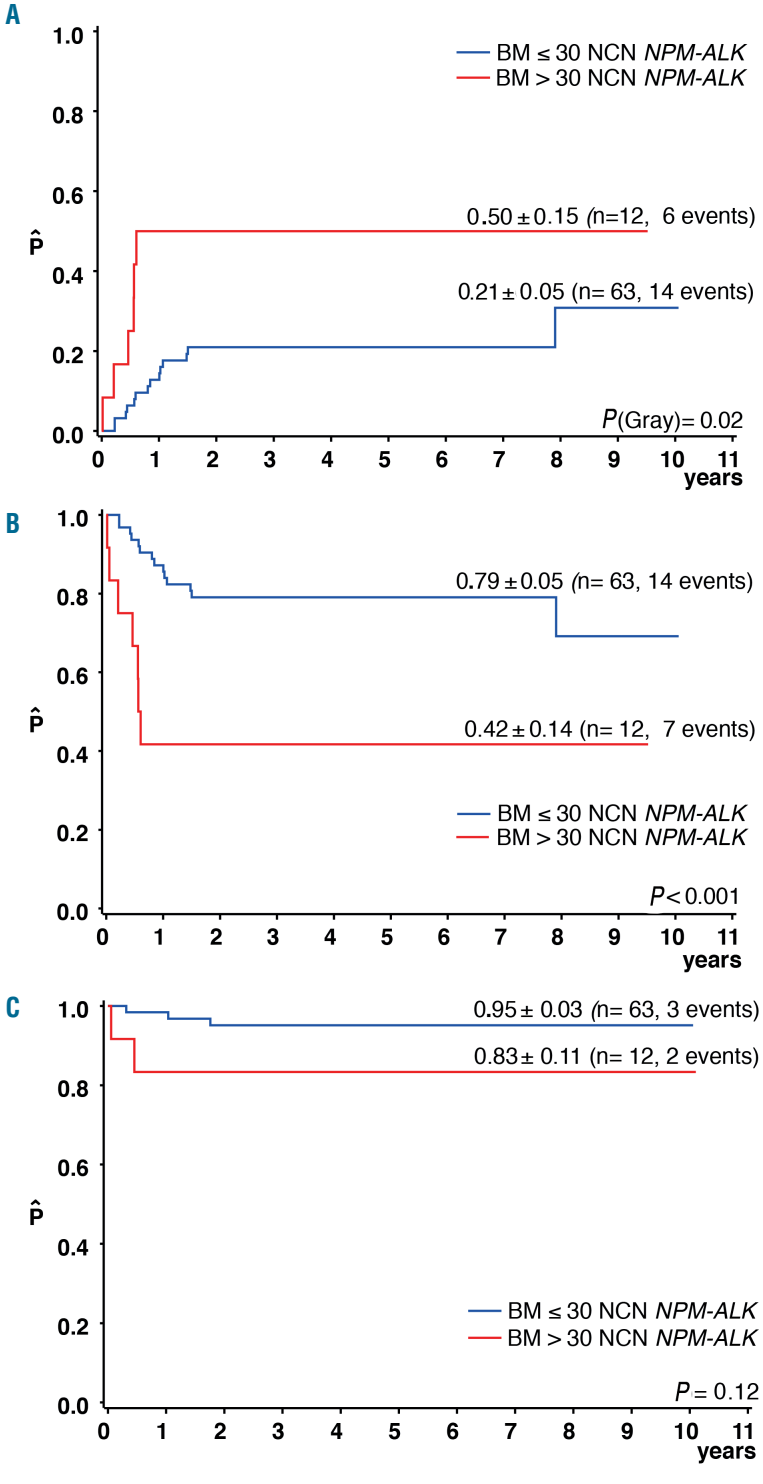

Figure 4. Outcome according to *NPM-ALK* copy numbers measured by digital polymerase chain reaction in bone marrow. (A) Cumulative incidence of relapse, (B) event-free survival and (C) overall survival at 3 years according to a cut-off of 30 normalized copy numbers of *NPM/ALK*/10^4 copy numbers of *ABL1*. BM: bone marrow, NCN: normalized copy numbers.

standard curve differences for inter-laboratory comparisons. Second, partitioning of target molecules leads to a more precise detection especially of rare events so that the assay is able to detect low copy numbers more accurately than RQ-PCR.[39,40] To exclude that templates are not amplifiable during the dPCR reaction it is still unavoidable to verify the quality of a given cDNA by parallel estimation of a reference gene. Furthermore, for measurement of clinical samples, appropriate positive and negative controls must be included in order to control for the overall performance of a given dPCR experiment.

The applicability of dPCR for MRD measurement has already been shown for several hematologic malignancies. The accuracy of dPCR and high concordance with RQ-PCR was demonstrated for DNA-based MRD measurements of the *BCL2/IGH* rearrangement in blood and BM from patients with low stage follicular lymphoma[41] and immunoglobulin/T-cell receptor rearrangements in patients with acute lymphoblastic leukemia.[42] dPCR was shown to be reliable for quantifying *BCR-ABL1* transcripts for MRD monitoring in chronic myeloid leukemia and Philadelphia-positive acute lymphoblastic leukemia.[43-46] Altogether, dPCR is a valuable tool for highly reproducible quantification of minimal disease at both DNA and RNA levels in patients' samples without requiring standard curves. In addition, for MDD and MRD in ALCL, it might have the advantage over RQ-PCR that the quantification is more accurate at lower copy numbers. Stringent protocol standardization and quality control are needed for this technique, as well.[47,48]

In summary, our data validate that quantification of *NPM-ALK* transcripts by RQ-PCR using a cut-off of 10 NCN identifies very high-risk patients if performed in one laboratory. Quantification of MRD is indicated to follow the course of disease and response to treatment modules in MDD-positive or relapsed ALCL patients. In a rare disease such as ALCL, with planned and ongoing international trials, both methods for transcript quantification require inter-laboratory comparability of measurements. Since harmonization is difficult and expensive with RQ-PCR, we developed and validated a dPCR assay enabling reliable quantification of *NPM-ALK* transcripts at very low copy numbers without the need for standard calibration curves.

Table 3. Concordance of patients' stratification according to quantity of *NPM-ALK* transcripts determined using digital polymerase chain reaction and quantitative real-time polymerase chain reaction in (A) bone marrow and (B) blood.

(A)

		dPCR	
		≤30 NCN	>30 NCN
RQ-PCR	≤10 NCN	63	0
	>10 NCN	0	12

(B)

		dPCR	
		≤30 NCN	>30 NCN
RQ-PCR	≤10 NCN	44	0
	>10 NCN	1	12

The cut-off for the digital polymerase chain reaction was 30 normalized copy numbers (NCN), while that for quantitative real-time polymerase chain reaction was 10 NCN. dPCR: digital polymerase chain reaction; RQ-PCR: quantitative real time polymerase chain reaction.

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