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ORIGINAL ARTICLE

Quantification of free total plasma DNA and minimal residual disease detection in the plasma of children with acute lymphoblastic leukemia

Arne Kristian Schwarz · Martin Stanulla · Gunnar Cario · Thomas Flohr · Rosemary Sutton · Anja Möricke · Philippe Anker · Maurice Stroun · Karl Welte · Claus R. Bartram · Martin Schrappe · André Schrauder

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Abstract The analysis of total plasma DNA and the monitoring of leukemic clone-specific immunoglobulin and/or T-cell receptor gene rearrangements for the evaluation of minimal residual disease (MRD) in the plasma may be useful tools for prognostic purposes or for early detection of subclinical disease recurrence in children with acute lymphoblastic leukemia (ALL). The aim of this paper is to establish reference ranges for total plasma DNA concentrations and to test the feasibility of MRD measurements employing plasma DNA from children with ALL by using real-time quantitative (RQ)-PCR. Despite wide inter-individual variation, the median concentrations of total plasma DNA for 12 healthy donors (57 ng/ml), 21 children with ALL after day 4 of treatment initiation (62 ng/ml) and 13 children with other malignancies (76 ng/ml) were similar. However, ALL patients had significantly higher concentrations at diagnosis (277 ng/ml) and on treatment day 3 (248 ng/ml) before returning to normal afterwards. Early plasma DNA MRD kinetics could be established for 15 ALL patients and showed good concordance with bone marrow MRD. Plasma DNA was higher in children with ALL at diagnosis but returned to normal within the first four treatment days. Despite low concentrations of DNA, it is feasible to measure MRD kinetics in plasma DNA during ALL induction therapy by adapted real-time PCR methodologies.

Keywords Acute lymphoblastic leukemia · Childhood · Minimal residual disease · Ig/TCR gene rearrangements · Plasma DNA

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Introduction

Over the past 40 years, a number of studies have been published on the presence of free circulating nucleic acids in the blood plasma of humans. In this context, various malignancies have been associated with increased concentrations of free plasma DNA as compared to levels in healthy controls [1–3]. It has therefore been proposed that analyses of free plasma DNA could be a useful tool for

prognosis or the early diagnosis to detect subclinical disease recurrence [4]. Since the introduction of PCR-based techniques in the late 1980s, a large number of tumor-specific alterations have been identified in free plasma DNA, such as K-ras mutations in patients with colorectal cancer [5], microsatellite alterations in patients with small-cell lung cancer [6], head and neck cancer [7], clear cell renal carcinoma [8] and melanoma [9], and finally aberrant promotor hypermethylation of tumor suppressor genes in patients with non-small-cell lung cancer [10], liver [11], and breast cancer [12]. Whereas the majority of these studies examined patients with solid tumors, it has also been suggested that free plasma DNA can be used for the detection of molecular abnormalities in hematological malignancies [13–15].

In acute lymphoblastic leukemia (ALL), the detection of minimal residual disease (MRD) using leukemic clone-specific immunoglobulin (Ig) and T-cell receptor (TCR) rearrangements as PCR targets provides important prognostic information on the in vivo effectiveness of early treatment [16–20]. Up to now, most large-scale MRD studies have been performed using bone marrow (BM) aspirates. Due to a lack of study, there is no information available on both the DNA concentration in the plasma of healthy children as well as the abundance of tumor-specific plasma DNA in children with hematological malignancies (e.g., ALL) or solid tumors. As such information could potentially provide a basis for the use of plasma DNA for MRD measurements in children with malignant neoplastic diseases, we aimed to establish reference ranges for the total concentration of free plasma DNA in children and to evaluate the feasibility of MRD measurement in the early course of childhood ALL by means of plasma DNA.

Materials and methods

Patients

Peripheral blood (PB) samples were obtained from 21 children with ALL at diagnosis and during the 12-week induction therapy, 13 children with other malignancies during therapy and 12 healthy children (Table 1). All samples were collected under protocols approved by the Institutional Review Board of the Medizinische Hochschule Hannover and after informed consent was given by the parents and/or patients.

Sample processing

As previous studies have demonstrated that EDTA prevents DNA degradation [14] and that coagulation-associated factors may influence the concentration of cell-free DNA in serum [21], we systematically used EDTA tubes to collect PB samples and exclusively worked with PB plasma.

To determine the possible influence of the storage period on the total concentration of DNA being measured in the plasma, we performed pilot experiments in which the plasma was separated from blood cells at 6, 12, 24, 36, and 48 h after collection. The plasma DNA concentrations were stable for up to 24 h of storage, but increased after longer time intervals. These results were in accordance with previous experiments performed by Anker et al. [22]. As a consequence, all plasma samples analyzed in the present study were separated from PB within 24 h by centrifugation (2×10 min at 3,000×*g*). Leukocytes were isolated by Ficoll-Paque™ Plus density centrifugation (Amersham Biosciences, Piscataway, NJ). The obtained plasma samples

Table 1 Characteristics of individuals included in plasma DNA analysis

	No. of patients	No. of samples	Gender		Age, year (range)
			Male	Female	
ALL	21	150	8	13	5 (2–16)
Other malignancies					
AML	2	2	1	1	13
Anaplastic astrocytoma 1	1	1		1	11
Hepatoblastoma	1	2	1		7
Medulloblastoma	1	1	1		8
Nephroblastoma	3	5	3		9 (6–12)
Neuroblastoma	1	1	1		11
NHL	3	4	1	1	9 (6–12)
Rhabdomyosarcoma	1	3	1		8
Total	13	20	10	3	9 (6–13)
Healthy controls	12	12	10	2	8.5 (1–17)

AML Acute myelogenous leukemia, *NHL* non-Hodgkin lymphoma

were then further processed using the QIAamp® UltraSens Virus Kit to isolate plasma DNA. For the extraction of cellular DNA (e.g., from leukocytes), we employed the QIAamp® Blood Midi Kit (Qiagen, Hilden, Germany).

β-Actin real-time quantitative PCR (RQ-PCR)

To determine the total amount of plasma DNA, we established a β-actin (housekeeping gene) RQ-PCR with primers β-Actin F (TCACCCACACTGTGCCCATC TACGA) and β-Actin R (CAGCGGAACCGCTCATTGC CAATGG), using the LightCycler™ system (Roche Diagnostics, Mannheim, Germany). Each PCR was conducted using a buffy coat DNA dilution series (tenfold steps; range, 15 ng/μl to 1.5 pg/μl), a standard DNA as external control (150 pg/μl; LightCycler™ Control Kit DNA), two negative controls (H_2O), and the plasma DNA of patients (all in duplicate). All reactions were performed with LightCycler™ FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). The running protocol was programmed on the LightCycler™ software, version 3.5. The first step was an initial denaturation where the reaction was incubated for 10 min at 95°C. In the second step, DNA was amplified for 50 cycles of 1 s at 95°C, 10 s at 68°C–60°C, and 10 s at 72°C. Melting curve analyses were performed subsequently to verify the amplification specificity. A final cycle consisted of 1 s at 95°C, 10 s at 64°C, and 1 s at 95°C, followed by one cycle of 15 s at 40°C.

Allele-specific RQ-PCR for leukemic MRD analysis

Identification and sequencing of patient-specific gene rearrangements was carried out as part of routine MRD analysis for the clinical trial ALL—Berlin–Frankfurt–Münster 2000 (BFM 2000), at the Institute of Human Genetics, University of Heidelberg, Germany. Briefly, genomic DNA from ALL samples at diagnosis was screened by PCR amplification using the BIOMED-1 primer sets for immunoglobulin kappa, *IGK*, deleting element (Kde) gene rearrangements (V-Kde, intron-Kde), complete and incomplete TCR delta, *TCRD* (V-(D)-J1, D2-J1, V2-D3, D2-D3), and TCR gamma, *TCRG* (V-J1.3/2.3, V-J1.1/2.1) rearrangements. Complete and incomplete *IGH* rearrangements (V_H-(D_H)-J_H, D_H-J_H) were identified using five V_H and six D_H family primers in combination with one J_H consensus primer. As an extra PCR target for T-ALL patients, primer sets to detect the *SIL-TAL1/2* deletion were included in the PCR screening protocol utilizing breakpoint-spanning *SIL* gene forward and *TAL* gene reverse oligonucleotides. If no DNA rearrangements were identified for *IGH*, *IGK*-Kde, *TCRG, TCRD,* and *SIL-TAL* deletion, in a subset of patients alternative targets were added such as complete and incomplete *TCRB* (V-(D)-J, D-J), *IGK* (V-J), and *IGL* (V-J) using the respective BIOMED-2 multiplex PCR primer sets. PCR products obtained from Ig and TCR gene rearrangements were further examined by heteroduplex or gene scanning analysis to discriminate between amplifications derived from monoclonal or polyclonal lymphoid cell populations. Biclonal or biallelic PCR products were separated either by cutting out amplicons from the polyacrylamide gel or by DNA cloning. Junctional regions of monoclonal PCR products were sequenced. Thereafter, patient-specific junctional region sequences of potential MRD PCR targets were identified using the IMGT (http://www.imgt.cines.fr), Vbase (http://www.vbase.mrc-cpe.cam.ac.uk), or IgBlast (www.ncbi.nlm.nih.gov/igblast/) databases and commonly distributed hardcopy sequences. At least one allele-specific oligonucleotide (ASO) primer was designed complementary to the junctional region sequence of each target either manually or using the Primer Express software (Applied Biosystems, Darmstadt, Germany).

Since the concentration of DNA in plasma was very low compared to the 500 ng used in all routine bone marrow analyses, all markers were re-evaluated for their effectiveness in detecting MRD at the low levels of DNA found in plasma. Each patient's BM mononuclear cell DNA from day 0 (150 pg/μl; diluted with AE buffer (Qiagen, Hilden, Germany) was used to prepare a tenfold dilution series in DNA isolated from the buffy coat fraction from normal individuals. The dilution series was used to test all the RQ-PCR assays for markers available for each patient with hybridization temperatures adapted according to the primer-annealing temperatures. The assays used LightCycler™ FastStart DNA Master SYBR Green I on LightCycler™ equipment and all included two negative controls (H_2O) and three buffy coat samples for defining the non-specific background signals.

We established criteria to distinguish between an informative and a non-informative marker, with the melting curve analysis being the determining factor. A marker that showed a clear distinction between buffy coat and negative control on the one hand and the patient-specific DNA (BM, day 0) on the other hand, was assessed as informative (Fig. 1).

MRD assessment in plasma and peripheral leukocytes

MRD detection in PB leukocytes was conducted as published by others [23–26]. Briefly, MRD PCR targets were tested for specificity and sensitivity to enable on-time monitoring of remission samples. RQ-PCR analysis was performed either by using single PCR amplification with sequence-specific TaqMan hydrolysis probes or by a two-round nested RQ-PCR including SYBR Green I

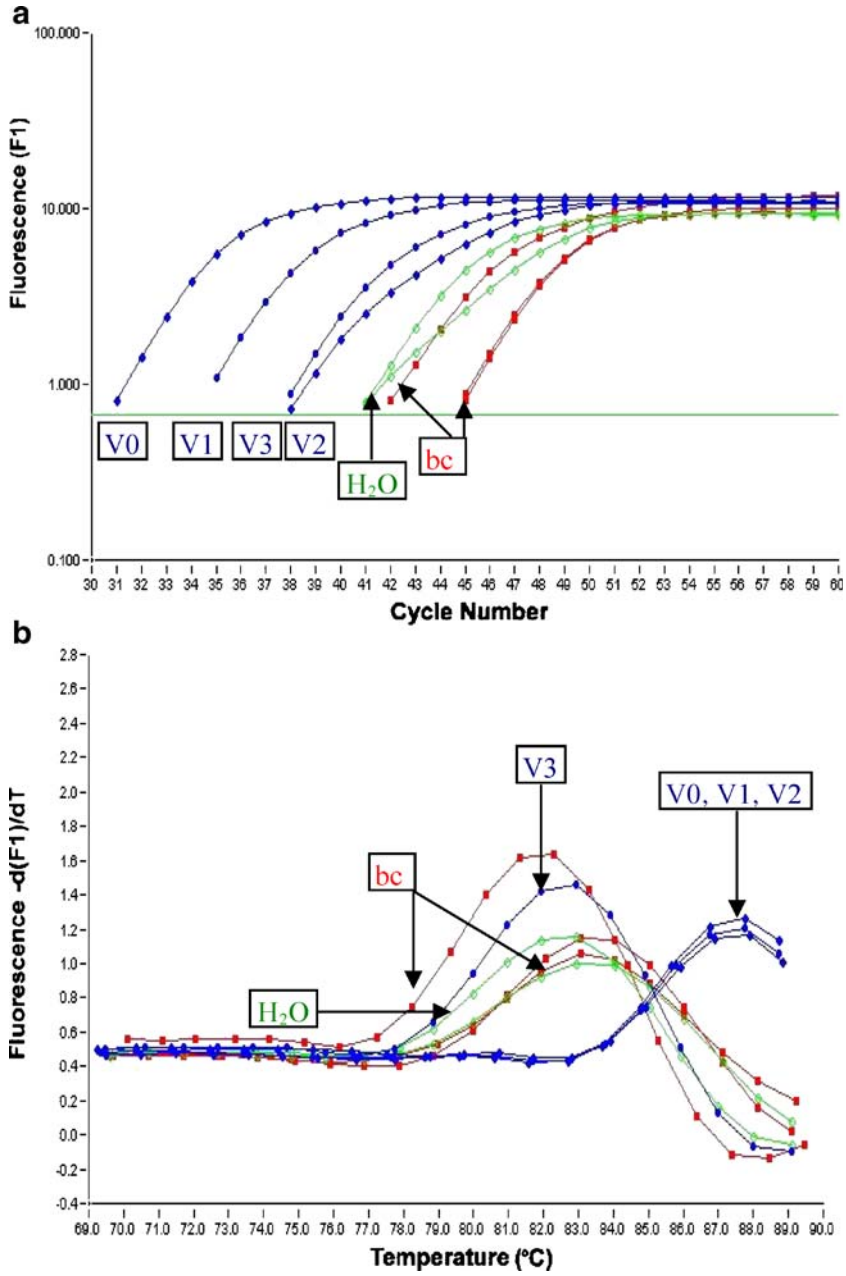


Fig. 1 Allele-specific RQ-PCR for testing patients markers. **a** Detection of amplification products by SYBR Green. *V0–V3* dilution series with day 0 BM, *H_2O*: negative control. *bc* buffy coat to define non-specific background signals (slope −2.47; r=−0.98). **b** Melting curve analysis. Showing a specific melting point for V0, V1, and V2 at 88°C, identifying the marker as informative. The amplification curve of V3 which arises between V1 and V2 (Figure 1A) should be disregarded for further analysis of this specific MRD-target as it is an unspecific amplification, shown by the melting curve analysis (Figure 1B)

detection. The nested RQ-PCR used a first-round consensus touchdown PCR (starting at 66°C) with a decreasing annealing temperature profile (−0.5°C per cycle) over 28 cycles and 12 additional cycles at 52°C, followed by the second-round RQ-PCR with a patient-specific ASO primer and an inner consensus primer. Tenfold serial dilutions of the diagnostic leukemic DNA sample were made in normal mononuclear cell DNA (pooled DNAs of six healthy donors) and used for RQ-PCR amplification in duplicate to generate a standard curve and to determine the sensitivity of the clone-specific Ig/TCR target. After receiving the follow-up samples, a freshly prepared dilution series was used to quantify the tumor load in triplicate in the patient's remission samples.

Since the assays for MRD detection in plasma were performed at very low concentrations of DNA (about 1 ng DNA per PCR), we were not able to perform quantitative measurements and defined positivity or negativity on the basis of melting curve analysis. Each sample was analyzed in duplicate and the results for the sample assessed qualitatively and reported as double negative, single, or double positive. Thus, the specificity of MRD assays on plasma DNA was based on the combination of allele-specific oligonucleotides (ASO) and target-specific melting curve analysis.

Results

Median plasma DNA concentration

The concentration of total free plasma DNA was determined in three sets of samples (I, II, and III). Set I included 150 PB plasma samples from 21 children with ALL at diagnosis and under treatment; Set II included 20 PB plasma samples from 13 children with other malignancies receiving treatment and Set III were 12 PB plasma samples from 12 healthy children (Table 1). The results revealed a wide spectrum of DNA concentrations, ranging from 2.85 to 12,260 ng/ml. Interestingly, the median concentrations between the three groups were highly comparable (I: 71 ng/ml, II: 76 ng/ml, and III: 57 ng/ml). When the ALL group was analyzed according to treatment day, it became apparent that initially, plasma DNA was elevated and upon treatment initiation rapidly returned to normal levels (Fig. 2a). Plasma DNA concentrations on days 0 and 3 were significantly higher in comparison to levels thereafter (*P* Mann–Whitney Test=0.001). This decrease paralleled the decline in PB leukocyte numbers (Fig. 2b) and we observed a previously unreported correlation between the concentration of plasma DNA from children with ALL and the number of PB leukocytes (Spearman coefficient of correlation=0.21, *P*=.001; data not shown). Two smaller peaks in DNA concentration were observed on day 33 and week 12.

Detection of MRD

MRD measurements were performed whenever less than or equal to five serial plasma samples were available which was the case for 15 of the 21 patients with ALL. Characteristics of these 21 patients are shown in Table 2. MRD was consistently detected until day 7 and MRD

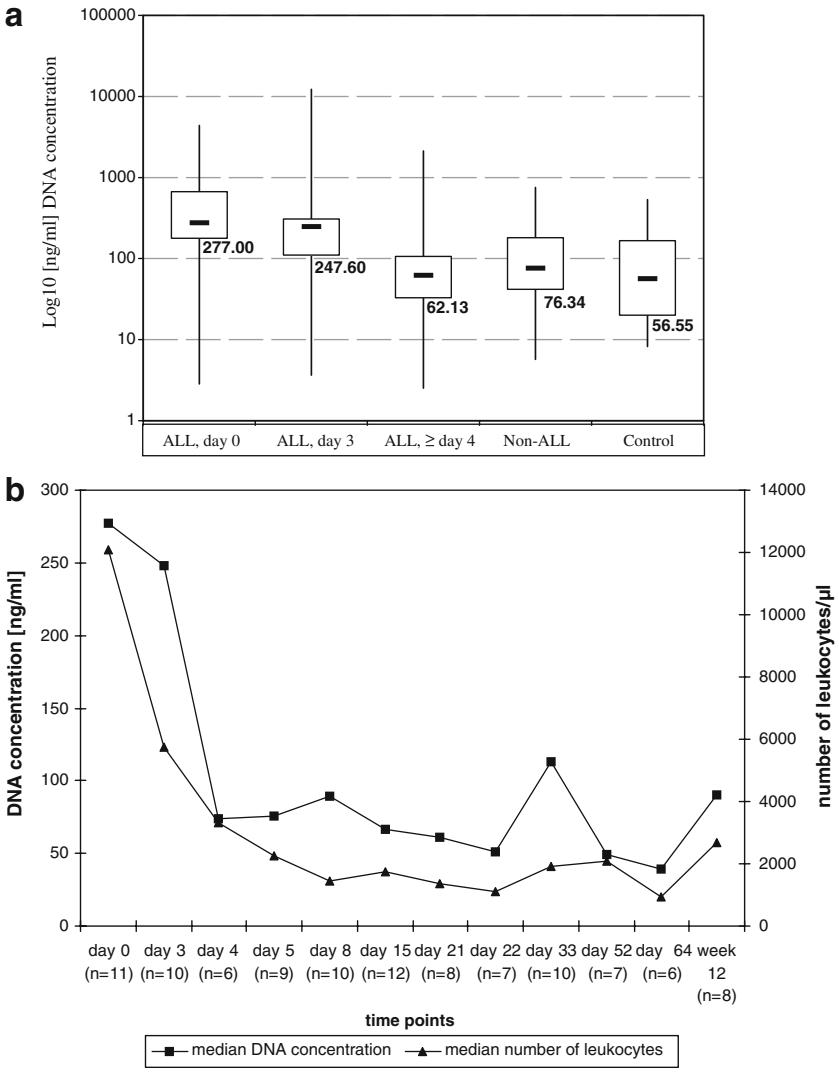


Fig. 2 Plasma DNA concentration in children with ALL, other malignancies and healthy controls. **a** Synopsis of the plasma DNA concentration in group I, patients with childhood ALL at *day 0*, *day 3*, and ≥*day 4* of treatment; group II, patients with other malignancies (*non-ALL*); and group III, healthy controls (*controls*). Each *box* represents the values between the 1st and 3rd quartile. The *bar* indicates the median DNA concentration with the absolute value being given next to each box. **b** The median plasma DNA concentration and median number of leukocytes in children with ALL over the course of therapy, reflecting a correlation between the plasma concentration and the number of leukocytes. For the correlation between median plasma DNA concentration and time points during ALL therapy, we only analyzed time points where greater than or equal to five samples had been collected

Table 2 Characteristics of patients with acute lymphoblastic leukemia included in the present study and evaluated for plasma DNA (all patients) and plasma DNA MRD monitoring (patients #7 to #15)

Patients	Age at diagnosis (years)	Gender	WBC at diagnosis	Immuno-phenotype	TEL/AML1	Prednisone response	MRD risk group	Number of MRD markers	MRD		Relapse
									Day 33	Day 78	
ALL patients											
#1	14.9	m	31,500	Common	Neg	Good	MR	4	10^{-2}	Neg	Bone marrow
#2	15.7	m	2,500	Common	Neg	Good	HR	2	10^{-2}	$10^{-2/-3}$	–
#3	4.3	f	3,300	common	Neg	Good	MR	2	10^{-4}	Neg	–
#4	4.3	f	15,000	Pre-B	Pos	Good	No markers	0	–	–	–
#5	3.2	f	41,000	Unknown	Neg	Good	SR	2	Neg	Neg	–
#6	15.5	f	38,600	T	Neg	Good	No markers	0	–	–	–
Patients with MRD monitoring in plasma DNA											
#7	12.7	f	5,400	Common	Neg	Good	HR	3	10^{-3}	10^{-3}	–
#8	8.6	f	7,100	T	Neg	Good	HR	2	10^{-2}	10^{-3}	–
#9	4.6	f	2,800	Common	Neg	Good	MR	3	10^{-5}	10^{-5}	–
#10	9.4	f	17,800	Common	Neg	Good	SR	4	Neg	Neg	–
#11	2.6	m	7,200	Common	Neg	Good	MR	2	10^{-4}	10^{-4}	–
#12	18.0	m	52,800	Pre-B	Neg	Good	SR	3	Neg	Neg	–
#13	5.2	m	76,800	Common	Pos	Poor	MR	2	10^{-3}	10^{-4}	–
#14	4.1	f	90,200	Common	Neg	Good	MR	3	10^{-3}	Neg	Bone marrow
#15	8.3	m	2,300	Common	Neg	Good	SR	3	Neg	Neg	–
#16	7.8	m	33,600	Common	Neg	Good	MR	4	10^{-4}	Neg	–
#17	4.1	m	18,300	Common	Pos	Good	SR	2	Neg	Neg	–
#18	13.4	f	125,000	Common	Neg	Poor	MR	3	10^{-4}	Neg	Bone marrow
#19	5.2	f	5,700	Common	Neg	Good	SR	3	Neg	Neg	Bone marrow
#20	15.2	F	3,000	Common	Neg	Good	MR	2	10^{-4}	Neg	–
#21	3.4	F	17,100	Common	Neg	Good	MR	2	10^{-3}	Neg	Bone marrow

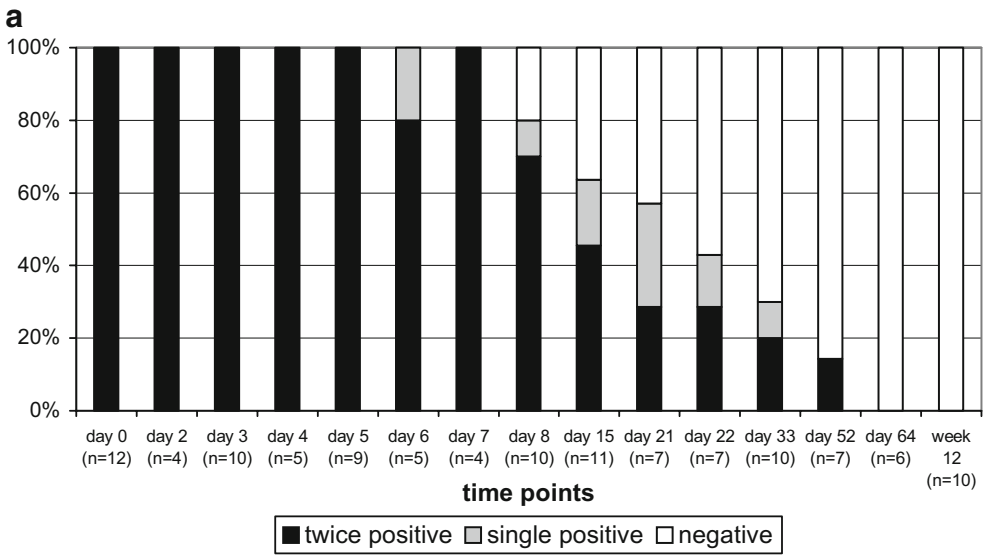
m Male, *f* female, *good* less than 1,000 leukemic blood blasts on treatment day 8, *poor* more than 1,000 leukemic blood blasts, *SR* negative at days 33 and 78, *HR* still 10^{-3} or more at day 78, *MR* all others, *TEL/AML1* fusion transcript positivity, none of the patients was positive for the BCR/ABL fusion transcript or an MLL aberration

kinetics could be described for all of the 15 patients. Figure 3a shows a steady decrease in the percentage of plasma MRD-positive patients during early therapy with samples for the majority of patients becoming MRD negative by day 22. All 15 patients were below the detection limits of MRD after day 52. The earliest MRD negative results were observed at day 8 in two out of ten patients tested.

When we compared the MRD measurements in plasma and leukocytes, we discovered a high concordance (86.7%) between the results. In 32 pairs, MRD was detected in both PB leukocytes and plasma, in eight pairs, MRD was solely detected in PB leukocytes, in two pairs, only the plasma sample was MRD-positive, and in 33 pairs, MRD was negative both in plasma and PB leukocytes. Similar to our observations in plasma analysis, there was a change from predominantly positive MRD signals to predominantly negative findings between day 21 and 22 in the leukocytes.

Discussion

The present study is the first approach to quantify the concentration of total plasma DNA in healthy children and children with malignant diseases by RQ-PCR. It is widely known that a substantial, if not predominant (depending on the course of therapy) portion of plasma DNA in cancer patients derives from tumor cells [27]. Higher plasma concentrations have been described in patients with large tumors or at an advanced stage of the disease [2, 3, 9, 28, 29] and it has been postulated that circulating plasma DNA in these disease states originates largely from tumor lysis/



b

PB Leukocytes

PB Plasma	+	-
+	32	2
-	83	3

concordance: 86.7%

Fig. 3 Detection of MRD in childhood ALL. **a** Synopsis of MRD in plasma over the course of therapy showing a steady decrease of percentage of MRD. We performed MRD measurements when greater than or equal to five serial plasma samples had been obtained. **b** Comparison of MRD in PB plasma vs. MRD in peripheral leukocytes as detected in 75 paired samples indicating a high concordance of 86.7%

necrosis and apoptosis. In 2001, Jahr et al. described a significant increase of plasma DNA after induction of apoptosis and necrosis in both, a cell culture and a murine model system, with the increase being more impressive in the necrosis model [30]. Similar findings were reported by Li et al. in 2003 in tumor cell lines and cell tissue cultures [31].

Our results reveal that DNA is abundant at high levels in the plasma of children with ALL at initial diagnosis and decreased rapidly in the first few days after the initiation of therapy to reach a level that is comparable to that of either children with other malignancies or to healthy children. Interestingly, small increases in plasma DNA concentrations were also observed during therapy intermission periods (day 33 and week 12) when active bone marrow regeneration occurs (Fig. 2b).

We were able to demonstrate higher plasma DNA levels in patients newly diagnosed with ALL. Since the amount of total PB plasma DNA in all analyzed patients decreased rapidly and reached levels comparable to those observed in healthy subjects by the fourth treatment day, the method does not seem to be broadly applicable for prognostic purposes in childhood ALL. Thus, we explored instead the applicability of MRD techniques in plasma. For this purpose, we successfully adapted the techniques used for MRD studies in bone marrow for the use with plasma DNA. The observed high initial concentration of both total DNA as well as leukemia-specific DNA in plasma before the induction of chemotherapy was predictable. It can most likely be explained by the higher turnover rate of leukemic cells in comparison to normal cells, which, combined with incomplete processing and degradation of cellular contents would lead to higher abundance of DNA in plasma [15]. In this context, Frickhofen et al. speculated that, normally, there is a steady state between DNA release from cells and degradation by plasma nucleases [14]. On treatment day 3, after significant tumor lysis, the levels of both total DNA and MRD are still very high in plasma so that we assume that the plasma nucleases are not able to cope with the amount of circulating DNA being released from leukemic cells in these first days. Subsequently, plasma concentrations were restored rapidly which is consistent with the view that the interference with DNA replication may shift the balance towards degradation of DNA. It is interesting that the rapid decline in number of leukocytes did not lead to a transient increase in the concentration of circulating plasma DNA (Fig. 2b) suggesting mechanisms other than tumor lysis may play a role here. Unfortunately, we can not make any interferences on the plasma DNA levels of the children with other malignancies (group II) at diagnosis as in this group we only had access to plasma samples under treatment.

Nevertheless, the origin of the baseline level of plasma DNA which is also present in normal individuals remains unanswered. One hypothesis is that DNA is spontaneously and actively released from intact cells, given the evidence

that there are marked differences between released DNA and cellular DNA which suggests that the extracellular DNA is not simply derived from dying cells [27, 32, 33]. This theory is supported by the correlation between the concentration of plasma DNA from children with ALL and the number of leukocytes shown in our study.

In summary, we were able to quantify plasma DNA in children using RQ-PCR and demonstrate that it is feasible to use plasma for the evaluation of DNA-based MRD kinetics during the early course of childhood ALL treatment. However, we were neither able to demonstrate a superior role of plasma DNA-based MRD analysis in comparison to conventional MRD analyses relying on DNA from BM or PB cells nor were we able to prove an added clinical value of this method in association with the already-established MRD techniques [34, 35].

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