Title

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Plasma cell-free DNA in patients with acute promyelocytic leukemia treated with arsenic trioxide

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Abstract

Background: Cell-free DNA (cfDNA) is free DNA found in circulating blood that originates from apoptosis or necrosis, and elevated cfDNA concentrations have been reported in cancers and other diseases.

Methods: In this study, the concentrations and fragment distributions of plasma cfDNA were preliminary investigated in elderly ($n = 1$) and pediatric ($n = 1$) patients with acute promyelocytic leukemia (APL) treated with arsenic trioxide (ATO).

Results: A slight increase in cfDNA concentrations was observed in the APL patients compared with healthy controls. The change in plasma cfDNA concentrations corresponded to the change in plasma arsenic concentrations during ATO treatment. The fragment distribution pattern did not differ before and during treatment. Three ladder fragments were observed in part of the cfDNA in the second consolidation therapy in an elderly APL patient and the first consolidation therapy of a pediatric APL patient, while two fragments were observed in all other treatment periods. Moreover, APL-related gene mutations were successfully genotyped from plasma cfDNA by using polymerase chain reaction-based methods and these results are consistent with those from leukocytes.

Conclusion: This study is the first to report the concentrations and fragment patterns of cfDNA from APL patients treated with ATO. The results suggested that plasma cfDNA concentration in APL patients increased with ATO treatment and that cfDNA is released

mainly via neutrophil extracellular traps (and/or necrosis) in addition to apoptosis. To

confirm whether cfDNA concentrations and fragment patterns can be used as a

biomarker for APL treated with ATO, further accumulative data are needed.

Introduction

Circulating cell-free DNA (cfDNA) is free DNA in blood plasma that originates from apoptosis or necrosis of all cell types [1–3]. cfDNA has been reported to be relevant in cancer, diabetes, stroke, systemic lupus erythematosus, trauma, rheumatoid arthritis, and myocardial infarction [4, 5]. We previously reported elevated levels and three DNA ladder fragments of plasma cfDNA in cardiac disease patients [6]. In addition, we observed considerably higher plasma cfDNA levels in postmortem subjects than in living subjects [7]. Recently, plasma cfDNA has been used as a liquid biopsy, which enables early diagnosis, treatment guidance, prognostic information, and tumor monitoring with minimal invasion [6, 8]. Furthermore, cfDNA is a potential biomarker for hematological diseases [8, 9].

Patients with acute promyelocytic leukemia (APL) have coagulopathy, and their bone marrow morphology shows microgranular APL and t(15; 17) chromosomal translocations. Intractable APL can be effectively treated with arsenic trioxide (ATO) [10–12], and a recent study reported that ATO induces high rates of complete hematologic remission and molecular remission, followed by a long relapse-free survival [13]. We have previously reported the urinary arsenicals and 8-hydroxy-2′-deoxyguanosine concentrations of APL patients following ATO treatment [11, 12]. Previous studies reported cfDNA in patients with hematologic malignancy [14–16]. However, to our knowledge,

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there are no reports on the plasma cfDNA concentration and fragment pattern of APL patients treated with ATO. Therefore, in this study, we preliminarily investigated the plasma cfDNA concentrations and fragment patterns of two APL patients, one elderly and one pediatric, in order to evaluate the potential of cfDNA as a new biomarker for APL treated with ATO.

Materials and methods

Plasma samples of APL patients

An elderly Japanese man aged 85 years and a Japanese girl aged 4 years were diagnosed as having APL [9, 10]. Their bone marrow morphology showed microgranular APL, and chromosomal translocation t (15; 17) and PML-RARA fusion gene were observed. They were treated with ATO (Trisenox®; Nippon Shinyaku, Kyoto, Japan) at Shimane University Hospital after giving informed consent. ATO was intravenously administered at a dose of 0.15 mg/kg/day for 6 weeks for the induction therapy. Two courses of consolidation therapy (0.15 mg/kg/day, 3 days a week for 5 weeks) were administered with a 6-week interval after a 4-week withdrawal period. Plasma samples from the older patient were collected during the two consolidation therapies while those of the pediatric patient were collected during the induction therapy. Plasma control specimens

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were obtained from healthy Japanese volunteers (n = 76; mean age ± standard deviation,

36.8 ± 10.9 years). Written informed consent was obtained from the elderly patient,

from the parents of the pediatric patient, and from the healthy volunteers. This study

was approved by the Human Ethics Committee of Shimane University School of

Medicine.

Extraction of cfDNA

Plasma cfDNA was extracted from 1 mL plasma by using the Maxwell® RSC ccfDNA

Plasma Kit (Promega Corp., Madison, WI) with the Maxwell® RSC Instrument

(Promega Corp.) in accordance with our previous studies [6, 7]. The cfDNA (2 μL) was

plated on Thermo Scientific μDrop™ plates and concentrations were determined with a

Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Inc.,

Waltham, MA) by measuring spectrophotometric absorbances at 260 nm, 280 nm, and

320 nm.

Mutation analysis

Using cfDNA from APL patients as shown in Table 1 and in accordance with previous

studies [17–21], mutations in FMS-like tyrosine kinase 3-internal tandem duplication (*FLT3-*

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ITD), GATA-binding protein 2 gene (*GATA2*), Janus-associated kinase 2 gene (*JAK2*), Wilms' Tumor 1 (*WT1*), Kirsten-ras (*KRAS)*, and neuroblastoma-ras (*NRAS)* were assayed using the allele-specific primer-polymerase chain reaction (PCR) method, amplification-refractory mutation system (ARMS)-PCR, and PCR-restriction fragment length polymorphism (RFLP). cfDNA was amplified in a 20-µL reaction mixture by using a thermal cycler (T100 Thermal Cycler; Bio-Rad Laboratories, Inc., Hercules, CA). The reaction mixture contained forward and reverse primers and Green Master Mix (GoTaq®; Promega Corp.).

Microchip electrophoresis

Plasma cfDNA was electrophoresed using an MCE-202 MultiNA automated microchip-based electrophoresis system equipped with MultiNA Viewer software (Shimadzu Corp., Kyoto, Japan) in accordance with our previous studies [6, 7]. cfDNA samples were run with separation buffer reagents and DNA marker reagent (DNA-1000 kit; Shimadzu), and a 100-bp DNA ladder (Takara Bio, Inc., Shiga, Japan) was used as a marker. Amplified cfDNA samples for gene mutations were run with the separation buffer reagents and DNA marker reagent (DNA-500 kit; Shimadzu), and 25 bp DNA Step Ladder (Promega Corp.) was used as a marker.

Total arsenic analysis

The total arsenic concentration in plasma was determined by high-performance liquid chromatography (LC10A Series, Shimadzu) coupled with inductively coupled plasma mass spectrometry (HP-4500; Hewlett-Packard, Avondale, PA) in accordance with our previous reports [11, 12]. Plasma was filtered and analyzed without dilution. The concentrations of total arsenic in plasma are expressed as μg/mL.

Statistical analysis

The differences in cfDNA concentrations between the control group and APL patients were analyzed with Dunnett's test. In addition, the correlation between arsenic and the cfDNA concentration was analyzed with Spearman's rank correlation test. These analyses were performed by using Bell Curve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results and discussion

cfDNA concentrations

cfDNA levels in the cerebrospinal fluid from leptomeningeal metastasis patients with

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hematologic malignancies have been reported to be about 10 times higher than those of the controls [15]. We previously reported elevated cfDNA concentrations in patients with cardiac disease (9-fold) compared with control subjects: among cardiac diseases, cfDNA concentrations in myocardial infarction and cardiac angina were significantly elevated [6]. The cfDNA concentrations of our two APL patients treated with ATO were compared at all time points with those of the control subjects (Fig. 1). Although the difference was small, the cfDNA concentration in the APL patients was significantly higher than that of the control subjects (2-fold).

Previous work showed elevated plasma cfDNA levels during the first 3 weeks of induction therapy, with the highest levels observed at week 1 in acute lymphoblastic leukemia patients [16]. In this study, time course changes in plasma cfDNA levels in APL patients during the ATO therapy were investigated (Figs. 2 and 3). The plasma cfDNA concentration in the elderly APL patient increased with treatment duration during the first consolidation therapy and the plasma cfDNA concentration fluctuated during the second consolidation therapy (Fig. 2). Similar to the second consolidation therapy of the elderly patient, the plasma cfDNA concentration in the pediatric APL patient fluctuated during the induction therapy (Fig. 3). The discrepancy between the results shown in Fig. 2a and those in Fig. 2b and Fig. 3 is related to the plasma total arsenic concentration,

which showed a tendency similar to that of the cfDNA concentration (data not shown). Although no correlations were observed between plasma total arsenic and cfDNA concentrations in the second consolidation therapy in the elderly patient or the first consolidation therapy in the pediatric patient, significant positive correlations were observed between the plasma total arsenic concentration and cfDNA concentration during the first consolidation therapy in the elderly APL patient (Fig. 4; Spearman's rank correlation, $r = 1.000$, $n = 5$).

The formation of neutrophil extracellular traps (NETs) is a cell death pathway distinct from apoptosis that is called NETosis, and cfDNA is also presumed to be released via NETosis, in addition to apoptosis [22, 23]. Plasma cfDNA is used as a marker of NET formation [24]. We recently reported that cfDNA is chiefly released by neutrophils via NETosis, in addition to necrosis, in cardiac disease patients [25]. A previous *in vitro* study demonstrated that APL cells from patients undergo NETosis (ETosis) through autophagy induced by ATO treatment [26]. Disseminated intravascular coagulation (DIC) is highly prevalent in APL (up to 90% of cases satisfy the criteria for DIC) and APL-associated DIC is dominated by bleeding[27]. Although no studies have investigated the relationship between DIC and NETosis in APL, NETosis may provide additional ways of modifying the course of DIC in patients with acute leukemia [27].

Fragment distribution of cfDNA

Evaluation of cfDNA fragmentation is a relatively new application, and cfDNA

analysis has the potential to help identify cancers and cancer type [28]. We have previously

reported the presence of three ladder fragments—150–200 bp, 300–400 bp, and 500–600

bp—in plasma cfDNA in almost all cardiac disease patients and that the 150–200

bp/500–600 bp ratio was higher in myocardial infarction patients than in other cardiac

disease patients. Here, we subjected plasma cfDNA from APL patients to microchip

electrophoresis, and gel-like images are shown in Fig. 5. Three ladder fragments—150–

200 bp, 300–400 bp, and 500–600 bp—were observed on day 7 and day 21 of the second

consolidation therapy in the elderly patient (Fig. 5a) and on day 3, day 5, day 21, and

day 40 of the induction therapy in the pediatric patient (Fig. 5b). In all other treatment

periods, two fragments (150–200 bp and 300–400 bp) were observed, whereas 500–600

bp fragments were rarely observed (Fig. 5). The bands detected on days 6 and 18 of the

second consolidation therapy in the elderly patient were weak (Fig.5a). In contrast, the

cfDNA concentrations of these samples were high (Fig.2a). This discrepancy may due to

the fact that the cfDNA on days 6 and 18 were released mainly from NETosis (and/or

necrosis), not by apoptosis. The three ladder fragments of cfDNA are characteristic of

apoptotic cell death [29] but apoptotic DNA fragments might be shorter (about 140–200

bp) [30]. In contrast, long DNA fragments are released from neutrophils [31], and necrotic

cells generate mainly cfDNA fragments larger than 10,000 bp [32]. Conventional methods

cannot observe fragments over 1,000 bp, and thus no cfDNA fragments released from

NETosis (and/or necrosis) were observed.

Plasma sample before treatment is available only for the pediatric patient and two

bands were observed in this sample (Fig.2b). There was no difference between the ladder

pattern before and during treatment. As mentioned above, three ladder fragments were

observed in part of the cfDNA in the second consolidation therapy in the elderly patient

as well as the first consolidation therapy in the pediatric patient. This suggests that the

fragment pattern of cfDNA does not reflect the treatment period or the cfDNA

concentrations. This study is preliminary and thus further research with larger numbers

of samples is needed to confirm whether cfDNA can in fact be used as a biomarker for

APL treated with ATO.

Mutation analysis

Recent studies reported gene mutations in *FLT3-ITD*, *GATA*, *JAK2*, *WT1*, *KRAS*,

and *NRAS* in APL patients [33–35]. In this study, gene mutations in these genes were

assayed using cfDNA from APL patients (Table 1). Genotyping using cfDNA from

APL patients was successful. Both patients were negative for the *FLT3-ITD* mutation

and heterozygous for *GATA* rs273164. The elderly patient was heterozygous for *JAK2*

rs10974944 and the pediatric patient was homozygous for the wild-type. As for *WT1*

rs16754, the elderly patient was homozygous for the mutant allele and the pediatric

patient was homozygous for the wild-type allele. Both patients were heterozygous for

the *KRAS* mutation and homozygous for the *NRAS* mutation. To confirm that the

genotypes obtained from cfDNA are consistent with those obtained from DNA in

leukocytes, gene mutation analysis was also performed using DNA extracted from whole

blood. All of the mutation analysis results for cfDNA were consistent with those of DNA

obtained from leukocytes (data not shown).

Conclusion

In this study, plasma concentrations of cfDNA and its fragment distribution were

preliminarily investigated in elderly and pediatric APL patients during ATO treatment.

The cfDNA concentrations were significantly higher in APL patients than in controls. The

change in plasma cfDNA concentration during ATO therapy corresponded to the change

in the plasma total arsenic concentrations. No difference was observed in fragment

distribution pattern before and during treatment. Three ladder fragments (150–200 bp, 300–400 bp, and 500–600 bp) were observed in part of the cfDNA in the second consolidation therapy in the elderly patient as well as the first consolidation therapy in the pediatric patient, while two fragments (150–200 bp and 300–400 bp) were observed in all other treatment periods. Moreover, the band densities did not correspond to the cfDNA concentrations. These results suggested that plasma cfDNA in APL patients may be released mainly via NETosis (and/or necrosis) in response to ATO treatment in addition to apoptosis. Using cfDNA, APL-related mutations could be genotyped, which were consistent with those of leucocytes. This study is the first to report the concentrations and fragment patterns of the cfDNA from APL patients treated with ATO. To confirm whether cfDNA concentrations and fragment patterns can be used as a biomarker for APL patients treated with ATO, further accumulative data are needed. cfDNA analysis may become part of clinical practice. The results of this study provide fundamental information on the cfDNA of APL patients treated with ATO.

Declarations of conflicts of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical approval

The study, including usage of plasma derived from patients and control subjects, was reviewed and approved by the Human Ethics Committee of Shimane University School of Medicine and was conducted in accordance with the principles of the Declaration of Helsinki.

Guarantor

Contributorship

JF performed the formal analysis and wrote the original draft. NN reviewed and edited the article. HT contributed to the sample collection. All the authors approved the final version of the article for submission.

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Table 1. *FLT3-ITD* mutation, *GATA2* rs2713604, *JAK2* rs10974944, *WT1* rs16754, *KRAS*, and *NRAS* genotypes in APL patients

Gene mutation	Method	Restriction enzyme	Fragment size	Genotype Elderly patient	Pediatric patient
FLT3-ITD mutation	ASP-PCR [16]	-	Mutation$^+$: more than 329 bp Mutation$^-$: 329 bp	FLT3-ITD$^-$	
GATA2 rs2713604 (G > A)	PCR-RFLP [17]	*Ava I*	G allele: 227 bp A allele: 157 and 70 bp	AG	
JAK2 rs10974944 (C > G)	PCR-RFLP [18]	*Mbo I*	C allele: 176, 37, 23 and 7 bp G allele: 213, 23 and 7 bp	CG	CC
WT1 rs16754 (A > G)	ARMS-PCR [19]	-	A allele: 240, 604 bp G allele: 400, 604 bp	GG	AA
KRAS (codon 12 mutation)	PCR-RFLP [20]	*Mva I*	WT allele: 113, 29, 15 bp Mutant allele: 142, 15 bp	Heterozygous mutation	
NRAS (codon 12 mutation)			WT allele: 41, 23, 19 bp Mutant allele: 60, 23 bp	Homozygous mutation	

FLT3-ITD: FMS-like tyrosine kinase 3-internal tandem duplication

GATA2: GATA-binding protein 2

JAK2: Janus-associated kinase 2 gene

WT1: Wilms' Tumor 1

KRAS: Kirsten-ras

NRAS: Neuroblastoma-ras

ASP-PCR: allele-specific primer-polymerase chain reaction

PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism

ARMS-PCR: amplification-refractory mutation system-polymerase chain reaction
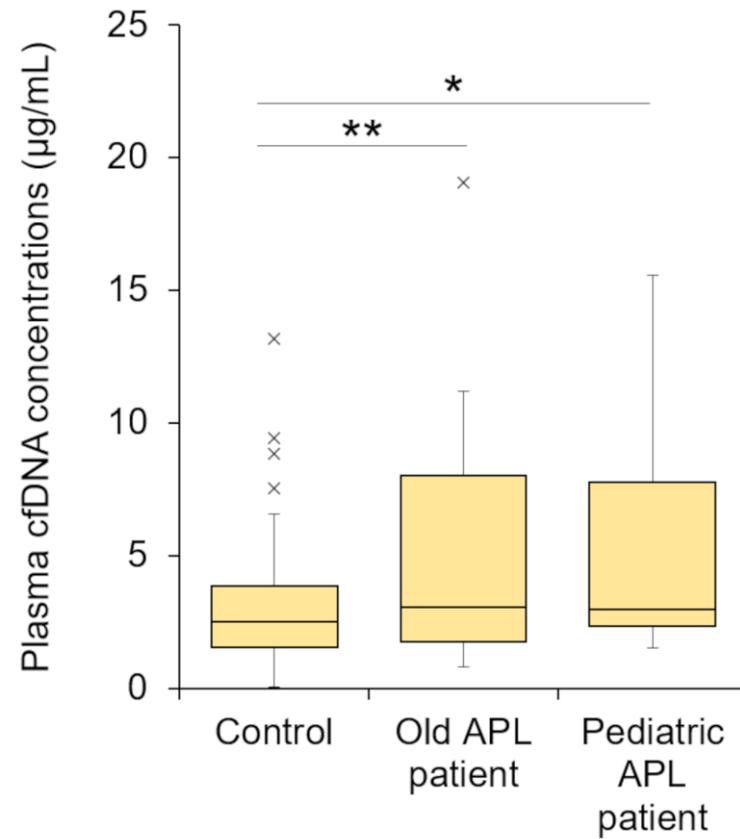
Figure. 1 Box plots of the plasma cfDNA concentrations in plasma from healthy control subjects and APL patients. *p < 0.05, **p < 0.01 when compared with the control group using Dunnett's test.
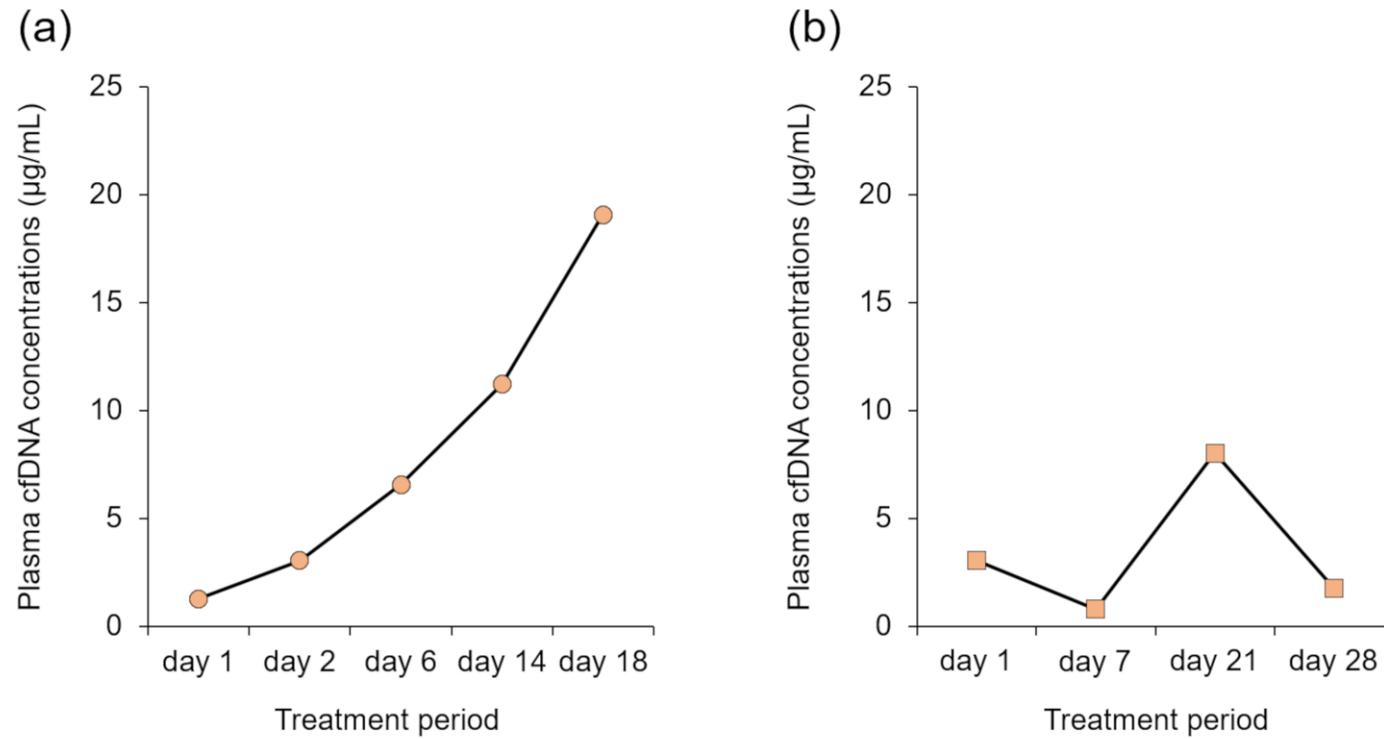
Figure. 2 Time course change in the plasma cfDNA concentrations of the elderly patient with acute promyelocytic leukemia during the (a) first consolidation therapies and (b) second consolidation therapies with arsenic trioxide.
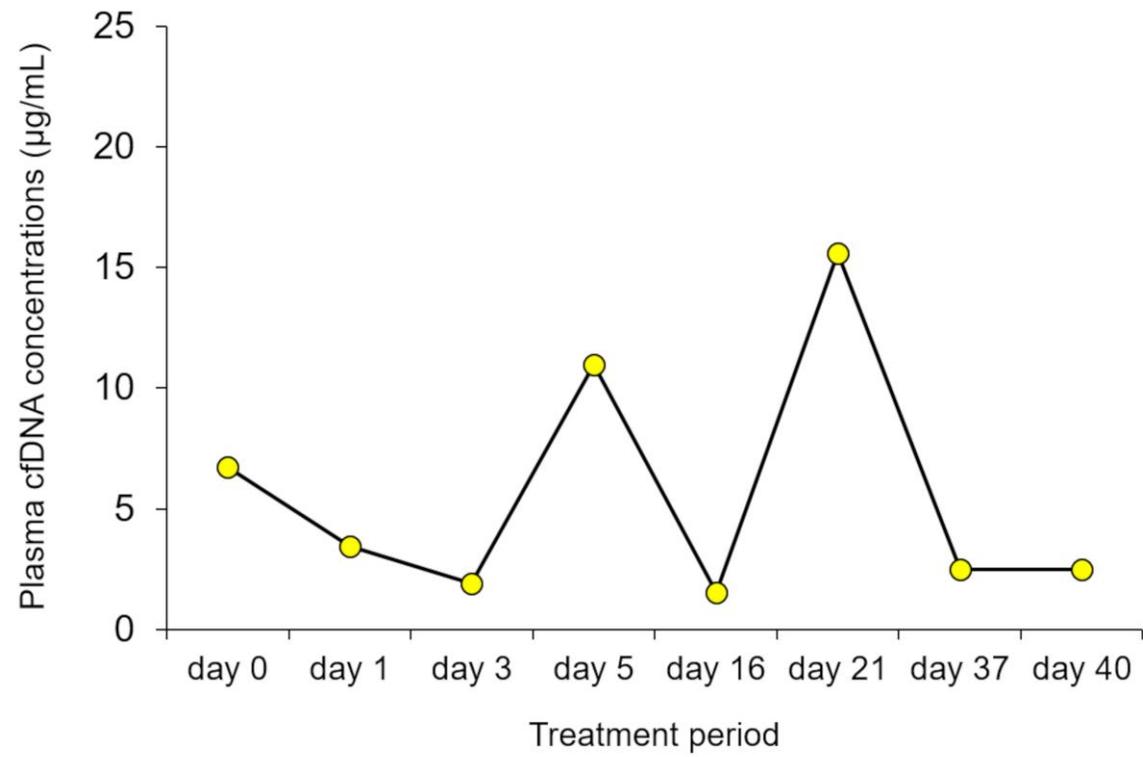
Figure. 3 Time course change in the plasma cfDNA concentrations of the pediatric patient with acute promyelocytic leukemia during the induction stage of arsenic trioxide therapy.
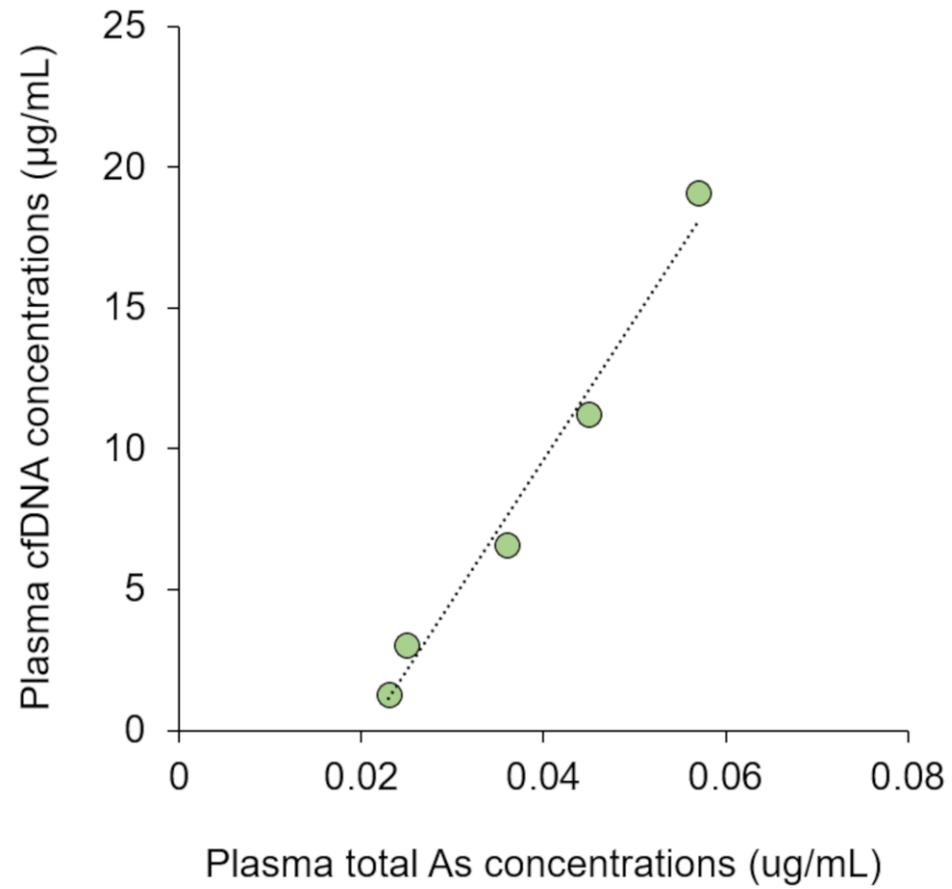
Figure. 4 Relationship between plasma total arsenic and plasma cfDNA concentrations in the elderly acute promyelocytic leukemia patient during the first consolidation therapy with arsenic trioxide.
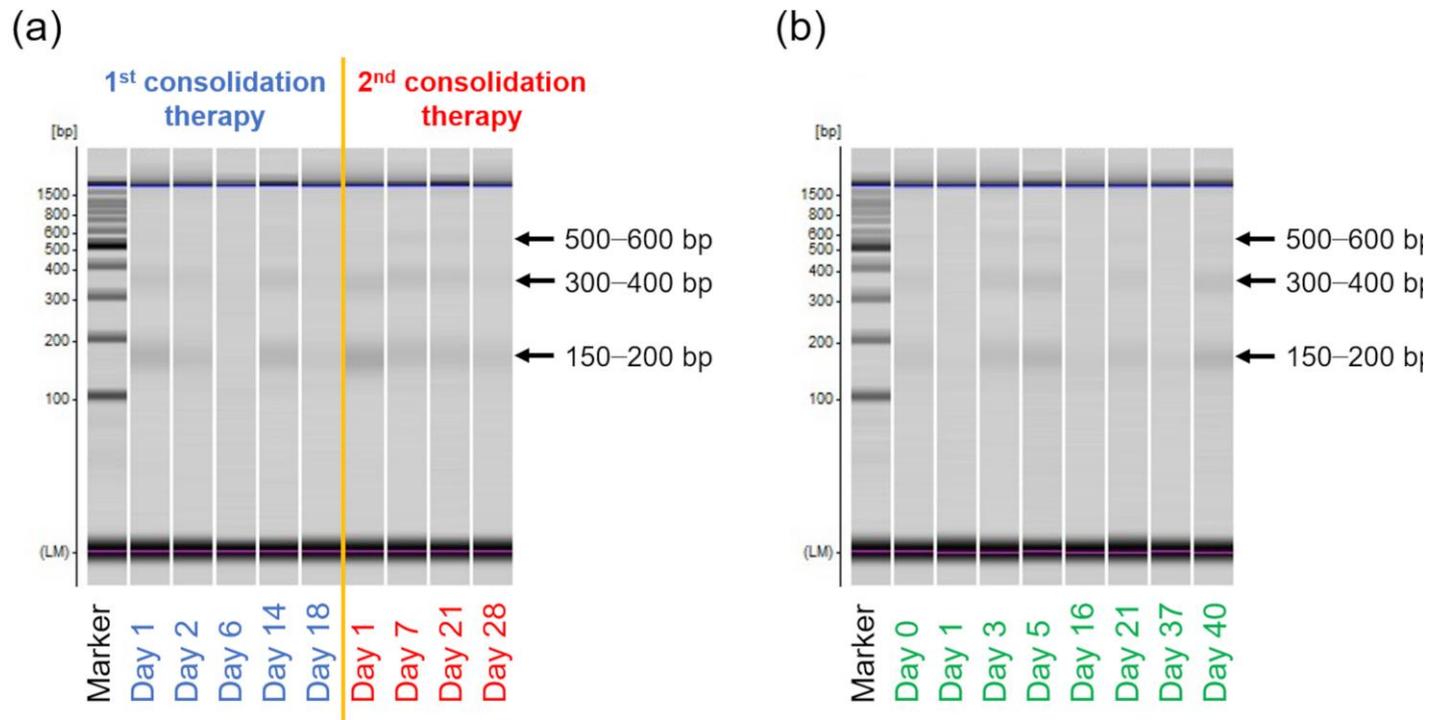
Figure. 5 Gel-like image of plasma cfDNA from (a) elderly and (b) pediatric acute promyelocytic leukemia patients treated with arsenic trioxide. Marker: 100-bp ladder marker.