

# Peripheral blood cell-free DNA is an alternative tumor DNA source reflecting disease status in myelodysplastic syndromes

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## Key words

Biomarkers, genetic mutations, myelodysplastic syndromes, peripheral blood cell-free DNA, targeted sequencing

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Myelodysplastic syndromes (MDS) are a group of hematopoietic stem cell disorders characterized by peripheral cytopenia, dysplasia and a risk of progression to acute myeloid leukemia (AML).<sup>(1)</sup> Recently, several somatic mutations in genes that affect DNA methylation (e.g. *DNMT3A*, *TET2* and *IDH1/2*), chromatin modification (e.g. *EZH2* and *ASXL1*), RNA splicing (e.g. *SF3B1*, *U2AF1*, *SRSF2* and *ZRSR2*), cohesion complexes (e.g. *STAG2*, *SMC3* and *RAD21*) and others<sup>(2–8)</sup> have been identified in more than 90% of MDS patients.<sup>(9,10)</sup> Some of these genetic abnormalities are closely related to specific phenotypes, such as ring sideroblasts,<sup>(6)</sup> hypo-lobulated granulocytes<sup>(11,12)</sup> and micro-megakaryocytes<sup>(13)</sup> as well as prognosis.<sup>(9,10,14)</sup> In AML, several specific genetic mutations act as initiating and/or driver mutations and result in disease onset and progression via a growth advantage and clonal expansion of leukemic stem cells.<sup>(15–18)</sup> Similar molecular mechanisms may also occur in MDS.<sup>(19–21)</sup> Researchers have speculated that accumulation of specific genetic abnormalities, such as mutations in *BCOR*,<sup>(22)</sup>

Genetic alterations in myelodysplastic syndromes (MDS) are critical for pathogenesis. We previously showed that peripheral blood cell-free DNA (PBcfDNA) may be more sensitive for genetic/epigenetic analyses than whole bone marrow (BM) cells and mononuclear cells in peripheral blood (PB). Here we analyzed the detailed features of PBcfDNA and its utility in genetic analyses in MDS. The plasma-PBcfDNA concentration in MDS and related diseases ( $N = 33$ ) was significantly higher than that in healthy donors ( $N = 14$ ;  $P = 0.041$ ) and in International Prognostic Scoring System higher-risk groups than that in lower-risk groups ( $P = 0.034$ ). The concentration of plasma-/serum-PBcfDNA was significantly correlated with the serum lactate dehydrogenase level (both  $P < 0.0001$ ) and the blast cell count in PB ( $P = 0.034$  and  $0.025$ , respectively). One nanogram of PBcfDNA was sufficient for one assay of Sanger sequencing using optimized primer sets to amplify approximately 160-bp PCR products. PBcfDNA (approximately 50 ng) can also be utilized for targeted sequencing. Almost all mutations detected in BM-DNA were also detected using corresponding PBcfDNA. Analyses using serially harvested PBcfDNA from an RAEB-2 patient showed that the somatic mutations and a single nucleotide polymorphism that were detected before allogeneic transplantation were undetectable after transplantation, indicating that PBcfDNA likely comes from MDS clones that reflect the disease status. PBcfDNA may be a safer and easier alternative to obtain tumor DNA in MDS.

*SETBP1*,<sup>(23)</sup> *ASXL1*,<sup>(10)</sup> *STAG2* and *SMC3*,<sup>(24)</sup> may be critical for relapse, progression and acquisition of drug resistance.<sup>(25)</sup>

Thus, genetic analyses using serially harvested samples at various time points during the clinical course have become more important not only for diagnosis but also for recognizing the disease status and selecting the optimal molecular targeted therapies. Genomic DNA from bone marrow (BM) mononuclear cells (MNC) obtained by BM aspiration is usually used for genetic analyses. However, repeated BM aspirations should be avoided as much as possible because they are difficult for patients.

Recently, peripheral blood circulating cell-free DNA (PBcfDNA), which is fragmented DNA detected in plasma and serum, has received attention as an alternative DNA source that originates from malignant tumors. Utilization of PBcfDNA for genetic analyses has been reported mainly for solid tumors, such as breast,<sup>(26–28)</sup> colorectal<sup>(29)</sup> and lung cancer,<sup>(30)</sup> especially in advanced stage patients. To date, few studies on PBcfDNA in MDS have been published.<sup>(31,32)</sup> Previously, we

reported the usefulness of PBcfDNA for detecting genetic and epigenetic abnormalities in a limited number of MDS cases. We showed that genetic mutations detected in BM CD34(+)/CD38(−) blast cells accumulate in PBcfDNA and that epigenetic changes after using azacitidine, a DNA methyltransferase inhibitor, can be detected more sensitively in serially harvested PBcfDNA than PBMNC.<sup>(31)</sup> However, detailed information about the clinical and molecular features of PBcfDNA has not been reported.

In this manuscript, we report the molecular characteristics of PBcfDNA from MDS patients and healthy donors, and the correlation of PBcfDNA with laboratory data and clinical features. We also show that PBcfDNA can be utilized as a biomarker of the disease status and as an alternative source of tumor DNA instead of BMMNC for genetic analyses.

## Materials and Methods

**Patients' characteristics.** After obtaining appropriate informed consent, the study population included 33 patients with MDS-related diseases (MDS;  $N = 24$ , MDS/MPN;  $N = 2$ , and MDS/tAML;  $N = 7$ ) diagnosed at Nagoya University Hospital. Diagnosis and prognostic stratification were assigned according to the World Health Organization classification<sup>(33)</sup> and International Prognostic Scoring System (IPSS),<sup>(34)</sup> respectively. Patient characteristics are summarized in Table S1.

**Collection of plasma, serum and mononuclear cells from peripheral blood.** Bone marrow and PB samples were harvested from these patients at or close to the same disease period. In one patient with MDS refractory anemia with excess blasts-2 (RAEB-2) at diagnosis, BM and PB samples were serially collected during the clinical course, and a total of 34 BM and PB paired samples were analyzed. Plasma samples were collected in all 33 patients, and serum samples were harvested from 28 patients. Plasma from 14 healthy volunteer donors was also harvested after obtaining appropriate informed consent. Blood collection tubes with EDTA-2Na were mainly utilized for plasma collection. For serum, tubes with a separating agent were used. Collected blood samples were stored at 4°C until further use. After centrifugation for 10 min at 430 g for plasma and 1710 g for serum, plasma and serum were aliquoted into 1.7-mL tubes and stored at −80°C.

Mononuclear cells from PB (PBMNC) and BM were collected using Ficoll-paque (GE Healthcare, Uppsala, Sweden) as indicated previously.<sup>(35)</sup> CD3-positive T cells were purified from PBMNC or BMMNC using CD3 MicroBeads/human (Miltenyi Biotec, Bergisch Gladbach, Germany) and Dynabeads Human T-activator CD3/CD28 (Invitrogen, Carlsbad, CA, USA).<sup>(36)</sup> DNA from buccal mucosa was obtained for use as germline control DNA.

**Extraction of PBcfDNA and DNA from peripheral blood/bone marrow mononuclear cells.** Genomic DNA from PBMNC and BMMNC was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). PBcfDNA from plasma (plasma-PBcfDNA) and serum (serum-PBcfDNA) was extracted using the QIAamp MinElute Virus Vacuum Kit (Qiagen) or QIAamp Circulating Nucleic Acid Kit (Qiagen). For germline control DNA, genomic DNA was extracted from CD3<sup>+</sup> T cells and/or buccal mucosa using the QIAamp DNA Blood Mini Kit.

**Confirmation of PBcfDNA concentration.** Plasma-/serum-PBcfDNA extracted from 45  $\mu$ L plasma and/or serum was visualized with 1% agarose gel electrophoresis. Fragmented bands of PBcfDNAs were quantitated by using DNA analysis

software (CS Analyzer ver. 3.0; ATTO, Tokyo, Japan), and the size and total amount of PBcfDNA were calculated. In some cases, the concentration and quality of PBcfDNA were also analyzed by BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). The concentration of PBcfDNA was compared with the patients' clinical data.

**Detection of genetic mutations.** Using genomic DNA from BMMNC (BM-DNA), PBMNC (PBMNC-DNA) and PBcfDNA, the nucleotide sequences of *IDH2* (exon 4), *SETBP1* (exon 4), *U2AF1* (exon 2 and 6), *SRSF2* (exon 2) and *NRAS* (exon 2 and 3) were determined by PCR followed by Sanger sequencing. *TET2* mutation (exons 3 to 11) was analyzed in patients showing 4q uni-parental disomy in single nucleotide polymorphism (SNP) array analysis (unique patient number [UPN] #3 and #6) as indicated previously.<sup>(31)</sup> All mutations were determined to be somatic mutations following comparison with the corresponding germline control DNA. The PCR primers utilized are shown in Table S2. Targeted sequencing was performed for two patients (UPN #2-2 and #33) using the TruSight Myeloid Sequencing Panel (Illumina, San Diego, CA, USA). Detection of *FLT3* internal tandem duplication (*FLT3-ITD*) by PCR was carried out as reported previously.<sup>(37)</sup>

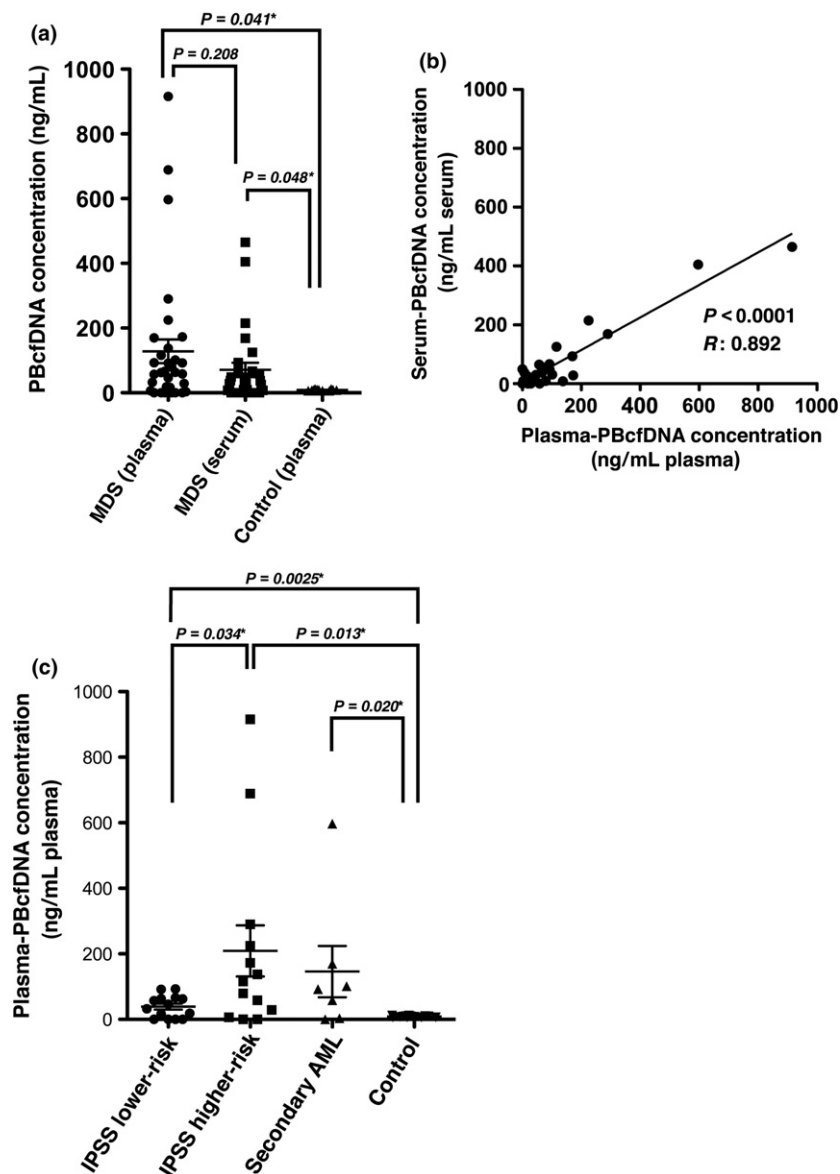
**Statistical analyses.** Statistical analysis with the unpaired *t*-test and nonlinear regression were performed with Prism version 5 software (Graph Pad Software, La Jolla, CA, USA). *P*-values < 0.05 were considered statistically significant.

## Results

**Characteristics of PBcfDNA in myelodysplastic syndromes and healthy donors.** To determine the characteristics of PBcfDNA in MDS, plasma-/serum-PBcfDNA from a clinically well-characterized cohort of MDS patients was analyzed by agarose gel electrophoresis, and the concentration was measured by using gel imaging software. All plasma-PBcfDNA (MDS;  $N = 34$ , healthy donors;  $N = 14$ ) and serum-PBcfDNA (MDS;  $N = 29$ ) samples were successfully visualized in 1% agarose gels (Fig. S1a). As indicated previously,<sup>(31)</sup> PBcfDNA was detected as a ladder pattern of 160–180 bp following 1% agarose gel electrophoresis and analysis using BioAnalyzer, a capillary electrophoresis system (Fig. S1b). Considering the fragmented size of PBcfDNA, PBcfDNA may exist in nucleosomal structures. The red arrows in Figure S1(a) may correspond to DNA fragments from mono-nucleosomes to penta-nucleosomes. Note that large variations in PBcfDNA concentrations were detected among patients (Fig. S1a).

**Comparison of the PBcfDNA concentrations from plasma and serum.** PBcfDNA concentrations (ng/mL of plasma or serum) are depicted in Figure 1(a). The median plasma-PBcfDNA and serum-PBcfDNA concentrations were 60.7 ng/mL (range; 0–915.9) and 30.7 ng/mL (range; 0–465.2), respectively. The plasma-/serum-PBcfDNA concentration from MDS patients was significantly higher than the plasma-PBcfDNA concentration from healthy donors ( $P = 0.041$  and  $0.048$ , respectively). The plasma-PBcfDNA and serum-PBcfDNA concentrations were significantly correlated ( $r: 0.892$ ,  $P < 0.0001$ ) (Fig. 1b).

**The PBcfDNA concentration is significantly higher in higher-risk International Prognostic Scoring System patients than in lower-risk International Prognostic Scoring System patients.** International Prognostic Scoring System scores<sup>(34)</sup> in this cohort are shown in Table S1. Of the 33 MDS patients, 14 and 12 cases were stratified into lower (low and intermediate-1) and higher (intermediate-2 and high) IPSS scores, respectively. Seven cases showed transformation to secondary AML at the time of



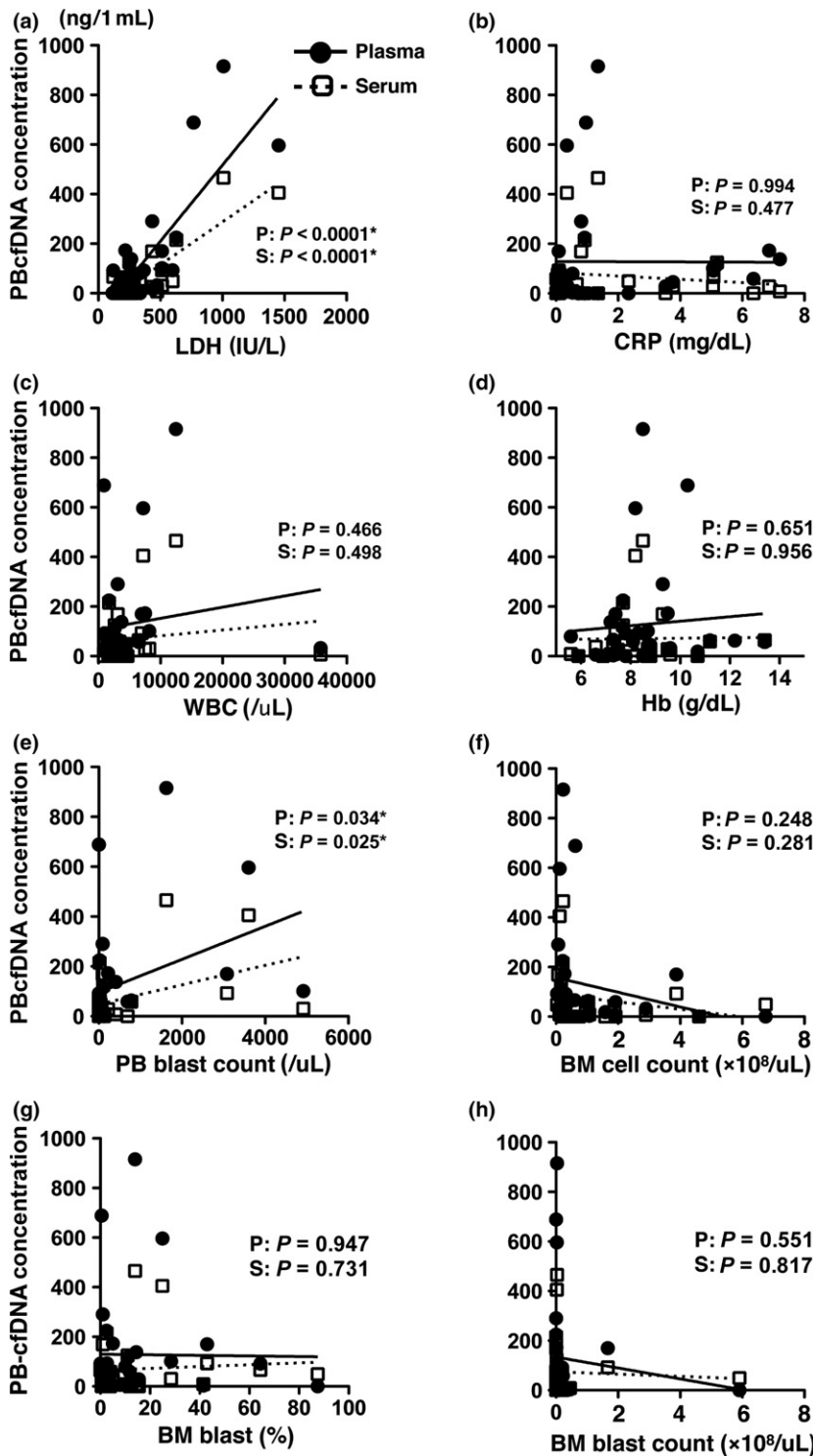
**Fig. 1.** Plasma-PBcfDNA and serum-PBcfDNA concentration in myelodysplastic syndromes (MDS), and correlation of the PBcfDNA concentration with the International Prognostic Scoring System (IPSS) risk group. (a) Plasma-/serum-PBcfDNA from MDS patients ( $N = 33$ ) and healthy donors (plasma) ( $N = 14$ ) was analyzed. The plasma-PBcfDNA and serum-PBcfDNA concentrations in MDS were significantly higher than those in healthy donors. The  $P$ -value marked with an asterisk indicates a significant difference with the unpaired  $t$ -test. (b) Comparison of the plasma-/serum-PBcfDNA concentration shows a significant linear correlation following linear regression analysis. (c) The plasma-PBcfDNA concentrations in the lower-/higher-risk IPSS score groups and secondary AML were compared. Plasma-PBcfDNA from the higher-risk IPSS group was significantly higher than that in the lower-risk IPSS group, and the concentration in both the lower-risk and higher-risk IPSS groups was significantly higher than that in normal donors. Asterisks indicate a significant difference in the unpaired  $t$ -test.

PB sample collection. The concentrations of plasma-PBcfDNA were significantly higher in the higher-risk IPSS score group compared to the lower-risk IPSS score group (Fig. 1c,  $P = 0.034$ ). The plasma-PBcfDNA concentration in secondary AML was not significantly higher than that in MDS lower-risk and higher-risk IPSS groups ( $P = 0.069$  and  $0.61$ , respectively). The concentrations of serum-PBcfDNA from the lower-risk and higher-risk IPSS groups were not significantly different ( $P = 0.121$ ) (data not shown).

**Correlation of PBcfDNA concentration with laboratory data.** To determine the biochemical characteristics of PBcfDNA, we compared the PBcfDNA concentrations with laboratory data (Fig. 2). The plasma-PBcfDNA and serum-PBcfDNA concentrations were significantly correlated with the serum lactate dehydrogenase (LDH) level (plasma:  $P < 0.0001$ , serum:  $P < 0.0001$ ) and the PB blast count (plasma:  $P = 0.034$ , serum:  $P = 0.025$ ). However, we found no significant correlations between the PBcfDNA concentration and the following PB laboratory data: C-reactive protein (CRP) (plasma:  $P = 0.994$ , serum:  $P = 0.477$ ), white blood cell counts (WBC) (plasma:  $P = 0.4656$ , serum:  $P = 0.498$ ), and hemoglobin

(Hb) (plasma:  $P = 0.651$ , serum:  $P = 0.956$ ). We also found no correlation between the PBcfDNA concentration and the following laboratory data using BM samples: BM cell count (plasma:  $P = 0.248$ , serum:  $P = 0.281$ ), BM blast percentage (plasma:  $P = 0.947$ , serum:  $P = 0.731$ ) and BM blast count (plasma:  $P = 0.5510$ , serum:  $P = 0.8172$ ). These data suggest that the PBcfDNA originates from MDS clones that were likely more fragile than normal blood cells, and the PBcfDNA concentration may be correlated with the disease status and reflects the LDH level and PB blast cell counts, as well as IPSS risk stratification.

If the fragility of MDS clones is closely related to the PBcfDNA concentration, the strategy for sample preparation in laboratories is quite critical. To investigate the possibility that genomic DNA may be released from MDS clones in sample tubes after sample collection and storage in refrigerators, plasma from whole blood samples of a patient that had been stored in a refrigerator for different time periods, from 15 min to 24 h after blood collection, was prepared, and then PBcfDNA extraction was performed. No significant elevation of PBcfDNA concentration was observed in this case, even



**Fig. 2.** Correlation of the plasma-PBcfDNA and serum-PBcfDNA concentration with laboratory data. Correlation of the plasma-PBcfDNA and serum-PBcfDNA concentrations with laboratory data was analyzed. (a) Serum lactate dehydrogenase (LDH), (b) C-reactive protein (CRP), (c) white blood cell counts (WBC), (d) hemoglobin (Hb), (e) peripheral blood (PB) blast count, (f) bone marrow (BM) cell count, (g) BM blast percentage and (h) BM blast count. The PBcfDNA concentration was significantly correlated with the LDH level and the PB blast count. The *P*-value was confirmed in linear regression analysis, and the asterisks indicate significant data. Black circles, plasma-PBcfDNA; white squares, serum-PBcfDNA.

after preservation for 24 h in a refrigerator (Fig. S2). These data suggest that the PBcfDNA concentration may depend on ineffective hematopoiesis resulting from increasing apoptosis in a patient's body rather than release from cells in sample collection tubes.

**PCR amplification using PBcfDNA for Sanger sequencing.** For mutation analyses using the Sanger sequencing strategy, we first optimized the PCR, because genomic DNA in PBcfDNA is fragmented and is quite different from total genomic DNA

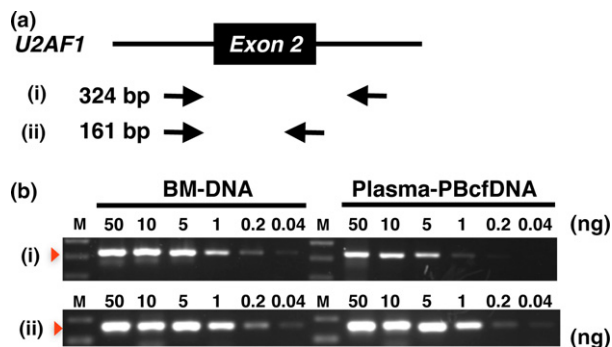
extracted from whole cells. Two different primer pairs were used to amplify *U2AF1* exon 2, resulting in PCR products of different sizes (primer set (i): 324 bp and (ii): 161 bp (Fig. 3a). When using BM-DNA, the PCR amplification efficacy was similar between primer sets (i) and (ii) (Fig. 3b; BM-DNA), even when using a lower concentration (0.2 ng) of template DNA. When using PBcfDNA as a PCR template, the amplification efficacy was significantly higher when using primer set b) than a) (Fig. 3b; PBcfDNA). The difference was

obvious when using a lower concentration (1 ng or less). Considering these data, we designed all primer sets to amplify approximately 160 bp for Sanger sequencing of the target genes (Table S2).

**Detection of genetic mutations with Sanger sequencing and targeted sequencing strategies in BM-DNA and PBcfDNA.** To confirm whether the genetic mutations detected in BM-DNA are also detectable in PBcfDNA, Sanger sequencing was performed for *IDH2*, *U2AF1* and *SRSF2* using BM-DNA for all samples. For 2 patients (UPN #2 and 6) that showed 4q uniparental disomy in SNP array analysis, *TET2* mutation was analyzed with Sanger sequencing. The FLT3-ITD mutation was also analyzed by PCR using BM-DNA for all samples. In patients in whom some genetic mutations were detected in BM-DNA, genetic mutations were also analyzed in PBcfDNA. Targeted sequencing, a next-generation sequencing strategy, was performed in two patients (UPN #2-2 and #33) using both BM-DNA and PBcfDNA, and the concordance was confirmed.

The genetic mutations detected in BM-DNA and PBcfDNA from the 33 MDS patients are summarized in Table 1. Almost all genetic mutations detected by Sanger and targeted sequencing in BM-DNA were also detected in the corresponding PBcfDNA. Representative data from Sanger sequencing are shown in Figure S3. In one case (UPN #7), a guanine (G) to cytosine (C) mutation resulting in an R140Q substitution in *IDH2* was detected in BM-DNA and three serially harvested plasma-PBcfDNA samples, but was not detected in serum-PBcfDNA (Table 1 and Fig. S4). FLT3-ITD was detected in one patient (UPN #7) in BM-DNA, but was not detected in plasma-/serum-PBcfDNA in the same patient (PCR data not shown). These data suggest that most genetic mutations detected in BM-DNA are also detectable in plasma-/serum-PBcfDNA, and that plasma-PBcfDNA may be more suitable for mutational analysis than serum-PBcfDNA. To detect FLT-ITD in PBcfDNA, optimization of the PCR conditions may be necessary because of the larger size (>180 bp) of the PCR product.

**Utilization of serially harvested PBcfDNA to detect disease status.** To determine the usefulness of PBcfDNA for confirming disease status, such as disease progression and/or remission, PBcfDNA was harvested serially from several patients. The clinical course of a representative patient is shown in Figure 4.



**Fig. 3.** Utilization of PBcfDNA for PCR amplification. (a) Schematic representation of exon 2 and the adjacent introns of *U2AF1*. Two primer sets for PCR, (i) and (ii), and the size of those products are indicated. Black arrows indicate the primers. (b) Semi-quantitative PCR was performed using (i) and (ii). BM-DNA and plasma-PBcfDNA were used as template DNA. The template DNA concentration in each reaction is also indicated. Note that amplification efficiencies were obviously higher with primer set (ii) than with (i) when using PBcfDNA as the PCR template.

The patient was a 29-year-old man with MDS-RAEB-2, in whom a *U2AF1* mutation was detected (TCT to TAT substitution in exon 2, resulting in S34Y) (Fig. 4a). After 5-Aza treatment for 7 days, cord blood transplantation (CBT) was performed after a myeloablative conditioning regimen, and complete remission (CR) was obtained. PB and BM samples were obtained at time points a, b and c, as indicated (Fig. 4a). A chimeric screening test using BM cells to confirm the engraftment efficiency was carried out at time point c, and the complete chimera (donor type) was confirmed.

*U2AF1* and *SETBP1* mutation were detected in BM-DNA, PBMNC-DNA and plasma-/serum-PBcfDNA before CBT (Fig. 4b, time points a and b, and Fig. 4c, time points a and b). The PBcfDNA concentration was elevated at time point b, showing clinically refractory disease with tumor fever and LDH elevation. The frequency of the *U2AF1* mutation in plasma-PBcfDNA at time point b was also increased (Fig. 4b, time points a and b). However, the blast percentage in BM and PB and the mutation frequency in PBMNC-DNA at time point b decreased compared to time point a. After CBT at time c, *U2AF1* and *SETBP1* mutations were not detected in any of the DNA samples (Fig. 4b, time point c, and Fig. 4c, time point c). These data suggest that the concentration of PBcfDNA and the existing frequency of genetic mutations may correlate more accurately with disease status than the blast percentage in PB and BM and reflect the tumor burden in the patient's entire body.

Interestingly, an SNP in *CDKN2A* confirmed in all samples harvested before CBT was not detected after CBT in BM-DNA, PBMNC-DNA or plasma-PBcfDNA (Fig. 4d). This phenomenon clearly indicated that PBcfDNA is mainly derived from allogeneic transplanted donor blood cells, not germline cells from non-hematopoietic organs in the host body.

## Discussion

For quantification of PBcfDNA, we performed agarose gel electrophoresis followed by semi-quantification with gel imaging software. We also used BioAnalyzer (Agilent Technologies), which is a capillary electrophoresis system, and NanoVue (GE Healthcare), which is a micro-volume UV-Vis spectrophotometer. The lower limits of sensitivity are 2 and 0.1 ng/ $\mu$ L, respectively, according to the manufacturers' instructions. Unexpectedly, these two strategies were not useful for accurately detecting the DNA, probably because of the low concentration and/or fragmentation of DNA. Agarose gel electrophoresis followed by quantification using imaging software is a conventional strategy for confirming the concentration and DNA fragmentation pattern, even with low concentrations. However, it takes time, and the background is sometimes problematic. Further improvement of strategies for measuring the DNA concentration is still needed.

The PBcfDNA concentration varied among the MDS patients (Figs 1a,S1a) and, importantly, the concentration was significantly lower in healthy donors. The PBcfDNA concentration was significantly correlated with the serum LDH level and the PB blast count (Fig. 2a,e), and was significantly higher in the higher-risk IPSS score group than in the lower-risk IPSS score group (Fig. 1c). Furthermore, the PBcfDNA concentration in serially harvested samples at different time points of the disease status in a patient appeared to be closely related to disease progression and/or regression (Fig. 4a–c). Importantly, MDS-specific genetic mutations seen in BM cells were also confirmed in PBcfDNA. Our previous data indicated that the

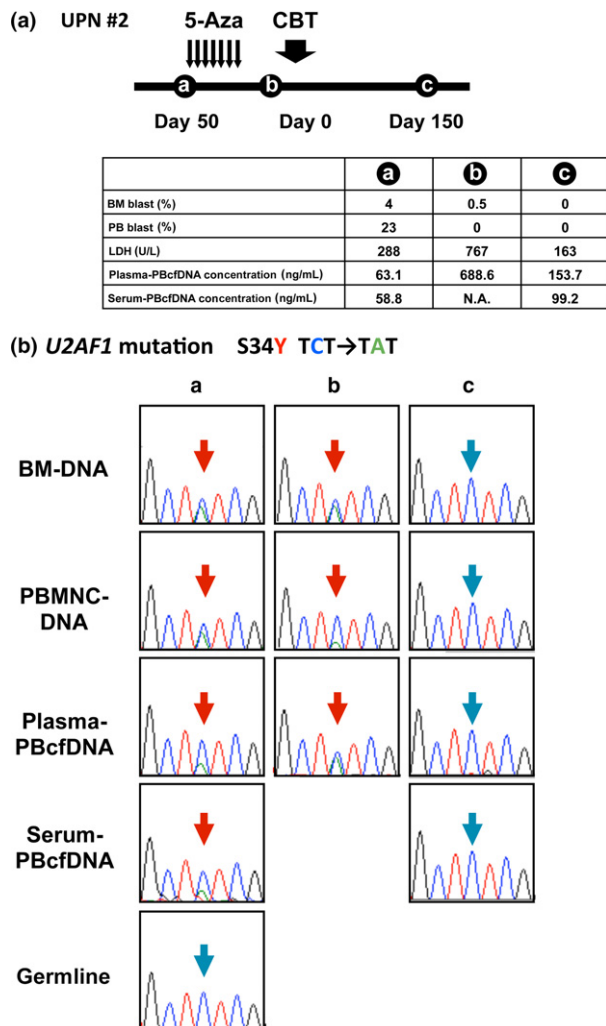
Table 1. Comparison of genetic mutations detected in BMDNA and PBcfDNA

UPN #	DNA source	PBcfDNA amount for sequencing	TET2	IDH2	SETBP1	U2AF1	SRSF2	NRAS	TP53	FLT3
1	BM			WT	WT	WT	WT	WT		WT
2-1†	BM			WT	D868N I871T	S34Y	WT	WT		WT
	Plasma	1 ng			D868N I871T	S34Y				
	Serum	1 ng			D868N I871T	S34Y				
2-2‡	BM	50 ng¶	WT	WT	D868N I871T	S34Y	WT	WT	WT	WT
	Plasma	50 ng¶	WT	WT	D868N I871T	S34Y	WT	WT	WT	WT
3	BM		Y1245X	WT	WT	WT	P95H	WT		WT
	Plasma	1 ng	Y1245X				P95H			
	Serum	1 ng	Y1245X				P95H			
4	BM			WT	WT	WT	WT	WT		WT
5	BM			WT	WT	WT	WT	WT		WT
6	BM		K513X	WT	WT	WT	P95R	Y64N		WT
	Plasma	1 ng	K513X				P95R	Y64N		
	Serum	1 ng	K513X				P95R	Y64N		
7	BM			R140Q	WT	WT	WT	WT		ITD
	Plasma§	0.0 ng††		R140Q						‡
	Plasma§	0.0 ng††		R140Q						‡
	Plasma§	1 ng		R140Q						‡
	Serum	1 ng		WT						‡
8	BM			WT	WT	WT	WT	WT		WT
9	BM			WT	WT	WT	WT	WT		WT
10	BM			WT	WT	WT	WT	WT		WT
11	BM			WT	WT	WT	WT	WT		WT
12	BM			WT	WT	WT	WT	WT		WT
13	BM			R140Q	WT	WT	WT	WT		WT
	Plasma	1 ng		R140Q						
	Serum	1 ng		R140Q						
14	BM			WT	WT	WT	WT	WT		WT
15	BM			WT	WT	WT	WT	WT		WT
16	BM			WT	WT	WT	WT	WT		WT
17	BM			WT	WT	WT	WT	WT		WT
18	BM			WT	WT	WT	WT	WT		WT
19	BM			WT	WT	WT	WT	WT		WT
	Plasma	0.0 ng††				WT				
	Serum	0.0 ng††				WT				
20	BM			WT	WT	WT	WT	WT		WT
21	BM			WT	WT	WT	WT	WT		WT
22	BM			WT	WT	WT	WT	WT		WT
23	BM			R140Q	WT	Q157R	WT	WT		WT
	Plasma	0.0 ng††		R140Q		Q157R				
24	BM			WT	WT	WT	WT	WT		WT
25	BM			WT	WT	WT	WT	WT		WT
27	BM			WT	WT	WT	WT	WT		WT
28	BM			WT	WT	WT	WT	WT		WT
29	BM			WT	WT	WT	WT	WT		WT
31	BM			WT	WT	WT	WT	WT		WT
32	BM			WT	WT	WT	WT	WT		WT
33 7)	BM	50 ng¶	WT	WT	WT	WT	WT	WT	R175H	WT
	Plasma	50 ng¶	WT	WT	WT	WT	WT	WT	R175H	WT

†Harvested at diagnosis as indicated in Figure 4(a). ‡Harvested just before cord blood transplantation without obtaining remission, as indicated in Figure 4(a). §Serially harvested samples during disease progression. ¶Analyzed in targeted sequencing method. ††DNA concentration was below the lower limitation in agarose gel electrophoresis method. ‡‡Band for the tandem duplication was not amplified correctly. KDM6A T726K, GATA2 A164T, STAG2 C527F detected in both BM-DNA and PBcfDNA samples were determined as single nuclear polymorphisms (SNP). BM, bone marrow; plasma, PBcfDNA from plasma; serum, PBcfDNA from serum; WT, wild type; ■ detected mutations; ■ not analyzed.

ratio of the mutated allele in BM CD34(+)/CD38(-) blast cells is much higher than that in CD34(+)/CD38(+) and CD34(-) cells, and the mutant ratio in PBcfDNA was almost equivalent to or much higher than that in CD34(+)/CD38(-) cells.<sup>(31)</sup> These data strongly suggest that PBcfDNA is mainly derived

from MDS clones rather than normal clones and normal tissue. This phenomenon may suggest that genomic DNA release occurs much more readily from MDS clones than from normal hematologic cells. MDS clones are more fragile in the patients' bodies than normal clones due to ineffective



**Fig. 4.** Mutational analyses using serially harvested PBcfDNA samples at different time points of disease status. (a) The clinical course of the MDS-RAEB2 patient (UPN #2) is indicated. After one course of 5-Aza, cord blood transplantation (CBT) was performed at Day 0, and complete remission (CR) was obtained. Peripheral blood (PB) and bone marrow (BM) samples were obtained at time points a, b and c. PB and BM blast percentage, serum lactate dehydrogenase (LDH) level and PBcfDNA concentration are also indicated. At time point b, disease progression was clinically observed. (b) *U2AF1* and (c) *SETBP1* mutations were confirmed by Sanger sequencing of BM-DNA, PBMNC-DNA and plasma-/serum-PBcfDNA. Note that those two mutations were not detected at time point c. (d) An SNP in *CDKN2A* detected by Sanger sequencing in BM-DNA, PBMNC-DNA, plasma-/serum-PBcfDNA and germline cells at time point b was not confirmed after CBT at time point c. Blue arrows, wild-type sequence; red arrows, mutated sites. N.A., sample not available.

hematopoiesis, aberrant membrane structure, and accelerated apoptosis.<sup>(34)</sup>

In contrast, the plasma-PBcfDNA concentration in secondary AML was significantly higher than that in normal controls, but was not significantly higher than that in lower-risk and higher-risk MDS patients. This phenomenon suggests that the DNA release from tumor cells in secondary AML cannot be estimated simply by PB blast cell count but that fragility also impacts DNA release. Further accumulation of patients and detailed analyses of the backgrounds of secondary AML in each patient may be required.

The serum-PBcfDNA concentration tended to be lower compared to plasma-PBcfDNA (Fig. 1a), and the difference in

concentration between lower-risk and higher-risk MDS patients was not significant when using serum-PBcfDNA (data not shown). One possible reason for this phenomenon is that a significant amount of PBcfDNA can be trapped in blood clots, decreasing the precision for determination of the PBcfDNA concentration.

An SNP in *CDKN2A* (Fig. 4d), which was confirmed in recipient PBcfDNA before CBT, was not detected in PBcfDNA after CBT with CR. This finding suggests that a large proportion of PBcfDNA in a healthy person originates from blood cells, rather than from other tissues such as the liver, intestine and others. Moreover, the PBcfDNA concentration was still high in the condition of CR after CBT, as shown in Figure 4(a). This phenomenon indicated that the PBcfDNA concentration may not reflect only the pathologic state but also certain aspects of the physiological state such as proliferation of hematopoietic cells after engraftment following stem cell transplantation. Further accumulation of samples from patients who have undergone stem cell transplantation is also required to investigate this possibility.

As shown in Figure 2(f–h), we found no correlations between the PBcfDNA concentration and BM data, including BM cell count, BM blast percentage and BM blast count. As we experienced in the clinical setting, results of BM aspiration, such as cellular density, are sometimes different according to the location of BM aspiration and because the aspiration skills may not be the same for all clinicians. Furthermore, the definition of BM blast cells, especially in MDS, is sometimes difficult because of the variety of dysplasia and the regional heterogeneity of genetic abnormalities in MDS clones.<sup>(15,21)</sup> However, PB collection is usually much easier than BM aspiration, and PB is likely more homogeneous regardless of the collection site. Our data and speculation about sample collection suggest that PBcfDNA may be much more accurate for detection of genetic abnormalities that exist in a patient's entire hematopoietic cell population than BM-DNA if a high enough DNA concentration for sequencing can be obtained from PBcfDNA.

Analyses using serially harvested PBcfDNA samples from a patient who was treated with 5-Aza followed by CBT indicated that the DNA concentration was markedly elevated (63.1–688.6 ng/mL) at the time of disease progression as defined by the clinical findings of documented tumor fever and LDH elevation. The ratio of the *U2AF1* mutated allele in plasma-PBcfDNA was also increased (Fig. 4a,b), probably due to significant expansion of MDS clones in his entire body. These data suggest that the PBcfDNA concentration and the ratio of the mutated allele can be utilized as biomarkers to predict the disease status and confirm minimal residual disease. We tried to perform pyro-sequencing analysis to quantitate the *U2AF1* mutation, but, unfortunately, the assay was not successful. Inappropriate assay conditions probably related to fragmentation of the template DNA may explain this lack of success. Further improvement of the assay system and/or introducing allele-specific quantitative PCR are required.

Because PBcfDNA showed a fragmented pattern that likely reflects nucleosomal structures, we optimized the PCR conditions for Sanger sequencing with primer sets that amplified approximately 160-bp DNA products (Fig. 3). Most of the genetic mutations were confirmed by Sanger sequencing after obtaining specific PCR products (Table 1). However, to detect FLT3-ITD, PCR amplification using previously reported primer sets did not work well (data not shown, Table 1), probably because the size of the PCR products was >200 bp.<sup>(38)</sup> We tried to design PCR primers on the flanking position of the

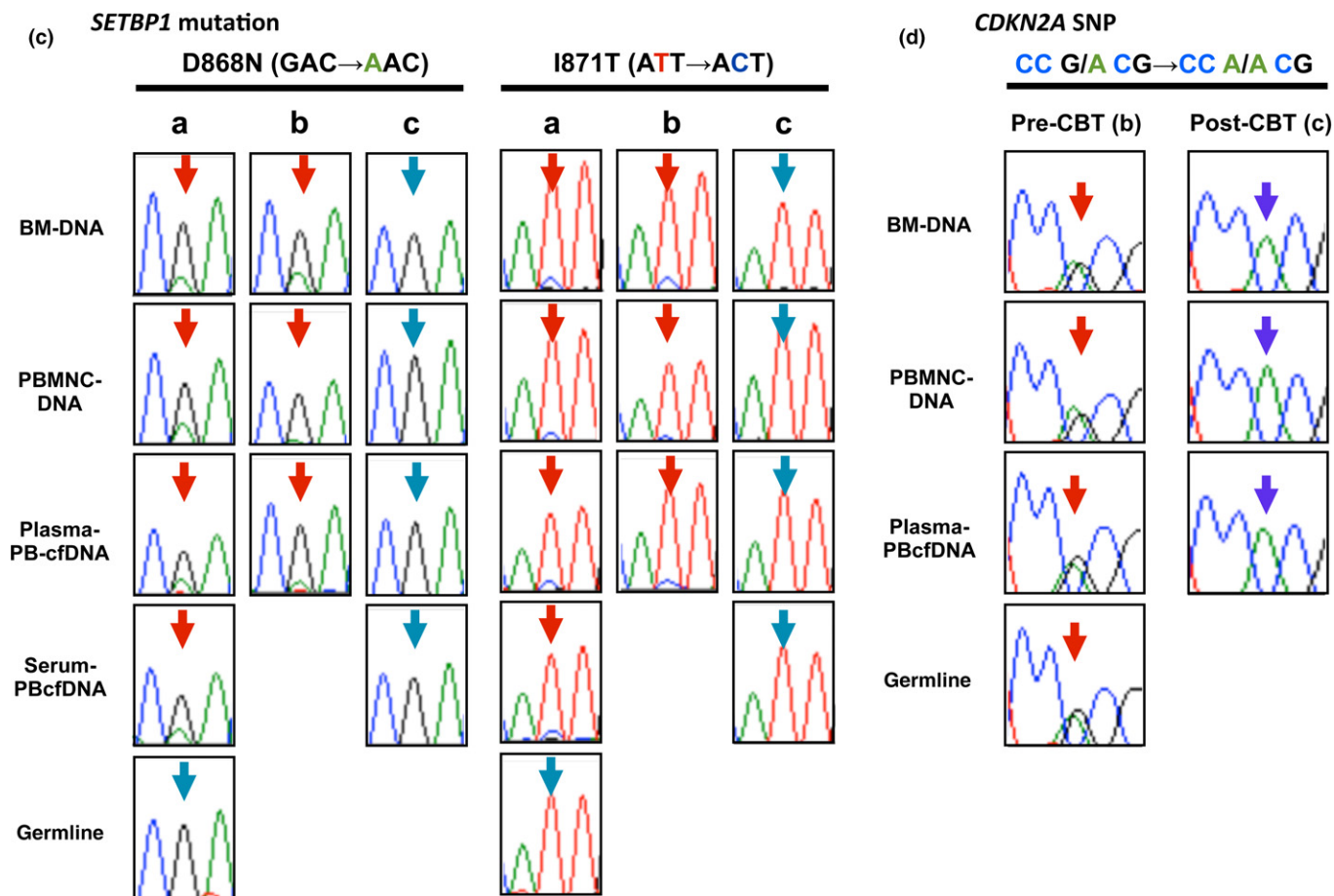


Fig. 4. Continued

duplicated sequences, but this was quite difficult because the duplicated sequences are sometimes longer than 200 bp, and also because the duplicated sequences vary from patient to patient. Further improvement of the detection strategy for FLT3-ITD using PBcfDNA is required.

PBcfDNA can be obtained from both plasma and serum,<sup>(31,39–41)</sup> but which sample is more suitable for detecting genetic abnormalities in malignant situations, especially MDS, was unclear. In healthy individuals, the concentration of serum-PBcfDNA tended to be higher than that of plasma-PBcfDNA.<sup>(31,39)</sup> In our experiment, the concentration of plasma-PBcfDNA in MDS tended to be higher than that of serum-PBcfDNA, but the difference was not significant (Fig. 1a). Our previous limited data for genetic analyses using PBcfDNA indicate that both plasma-PBcfDNA and serum-PBcfDNA can be utilized for Sanger sequencing, and the mutant allele frequencies in plasma-PBcfDNA and serum-PBcfDNA are almost the same and relatively higher compared with the frequency in DNA from BM CD34(+)/38(–) blast fraction cells.<sup>(31)</sup> Mohamedali *et al.*<sup>(32)</sup> report that serum-PBcfDNA can be utilized for Sanger sequencing in some cases. However, the concordance with BM-DNA was only 42%, and Sanger sequencing could be performed in only 9 (75%) of 12 cases. In our experiment, a genetic mutation in *IDH2* that was confirmed in BM-DNA and plasma-PBcfDNA was not detected in serum-PBcfDNA in one case (Table 1 and Fig. S4). Furthermore, Board *et al.*<sup>(39)</sup> report that the serum-PBcfDNA concentration increases in a time-dependent manner after blood coagulation, suggesting release of genomic DNA

from white blood cells containing normal clones. This phenomenon may be a reasonable explanation for the increasing contamination with the wild-type genome in serum-PBcfDNA. Thus, plasma-PBcfDNA may be more suitable for genetic analysis in MDS than serum-PBcfDNA.

Our data strongly suggest that PBcfDNA is a useful alternative source of tumor DNA in MDS, not only for conventional sequencing strategies but also for newer, comprehensive genetic analysis. Collection of PB is much safer, easier and relatively less painful compared to BM aspiration, and serial sample collection is also feasible. Furthermore, storing plasma/serum samples in the clinical setting is much easier and faster than storing PBMNC and BMMNC. PBcfDNA may also become a useful biomarker that closely reflects the patient's clinical situation. Further optimization and improvement of the sensitivity of sequencing analyses is still required for this strategy to be widely utilized in the clinical setting, especially for patients with a low PBcfDNA concentration.

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**Supporting Information**

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1.** Confirmation of the fragmentation pattern of plasma-/serum-PBcfDNA in agarose gel electrophoresis.

**Figure S2.** The difference of PBcfDNA concentration in peripheral blood samples harvested at the different time points.

**Figure S3.** Detection of genetic mutations in PBcfDNA.

**Figure S4.** Detection of *IDH2* mutation using plasma-/serum-PBcfDNA in UPN #7.

**Table S1.** Patients' characteristics.

**Table S2.** PCR primers.