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Posttransplantation Bone Marrow Assessment by Quantifying Hematopoietic Cell–Derived mRNAs in Plasma Exosomes/Microvesicles

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BACKGROUND: Bone marrow (BM) aspiration often can be a painful medical procedure. It is unavoidable, however, because hematopoietic precursor cells (HPC) exist only in BM and few escape to peripheral blood (PB). We hypothesized that HPCs might release exosomes and microvesicles (EMV) in BM, and the resulting EMV would penetrate into PB. Such BM-derived EMV might be identified in PB by measuring specific mRNAs produced by HPC.

METHODS: Human plasma was applied to an EMV-capture filter plate. After centrifugation, captured EMV were lysed on the filter plate. Resulting lysates were transferred to an oligo(dT)-immobilized microplate for mRNA isolation followed by reverse transcription PCR (RT-PCR). Using this system, myeloid-, erythroid-, and megakaryocyte-lineage–specific poly(A)$^+$ mRNAs were quantified in plasma obtained from 18 patients who had undergone hematopoietic stem cell transplantation (HSCT).

RESULTS: When fluorescent liposomes were applied to the filter plate, more than 95% of applied liposomes were absorbed. When human plasma was applied, a scanning electron microscope showed EMV-like particles on the membrane of the filter plate. After RT-PCR, various HPC-specific mRNAs were detected, and the results were equivalent to those derived from the standard ultracentrifugation method. The levels of these mRNAs were undetectable after HSCT and became detectable 1–2 weeks after HSCT, a substantially earlier time point than with traditional hematological analysis. The recovery of EMV mRNA at day 15 corresponded to the final clinical outcome at day 180.

CONCLUSIONS: HPC-derived mRNAs in plasma EMV may represent new biomarkers for the assessment of BM condition and could reduce the necessity for frequent BM aspiration.

Bone marrow aspiration (BMA)[3] is used routinely for the diagnosis of various hematologic diseases, such as leukemia, anemia, thrombocytopenia, and myelodysplastic syndrome. BMA is also used for the assessment of treatment efficacy during chemotherapy, radiation, and hematopoietic stem cell transplantation (HSCT), for which patients undergo multiple cycles of BMA during a treatment protocol. Although BMA often is a painful medical procedure, and most patients suffer some discomfort, BMA is unavoidable because hematopoietic precursor cells (HPC) exist only in BM and few escape to peripheral blood (PB). Currently, there is no option to analyze BM status without BMA.

Recent studies have demonstrated that a variety of cells release exosomes and microvesicles (EMV) into nearby biological fluids, such as blood, saliva, and breast milk *(1)*. During the exocytotic process, various proteins *(2)*, mRNA *(3, 4)*, and micro RNA (miRNA) *(5)* are included in the EMV. We hypothesized that HPC might release EMV in BM, and such EMV might be small and flexible enough to penetrate into PB. If this were to occur, BM-derived EMV might be identified in PB by measuring HPC-derived mRNAs. To validate this possibility, we chose allogeneic HSCT as a model system, because BM content varies widely from the short period of time between myeloablation and full recovery.

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[3] Nonstandard abbreviations: BMA, Bone marrow aspiration; HSCT, hematopoietic stem cell transplantation; HPC, hematopoietic precursor cells; PB, peripheral blood; EMV, exosomes and microvesicles; mRNA, messenger RNA; miRNA, micro RNA; RT-PCR, reverse transcription PCR; SEM, scanning electron microscopy; Ct, cycle threshold; CBC, complete blood count; WBC, white blood cell; CR, complete remission; NH, normal hematopoiesis; RBC, red blood cells.

The traditional method of EMV isolation is ultracentrifugation with 100 000*g* for 2 h *(6)*. This is a time-consuming procedure, and the assay throughput is quite limited. After centrifugation, the removal of the supernatant without disturbing the EMV pellet is difficult and impedes accurate and reproducible quantification. Subsequent RNA preparation, cDNA synthesis, and PCR introduce additional impediments. In each additional step, technical variation is introduced, and such variation is augmented after exponential amplification by PCR.

Previously we introduced a unique system of leukocyte mRNA analysis from whole blood, in which leukocytes were trapped on a 96-well filter plate and leukocyte lysate was transferred to a 96-well oligo(dT)-immobilized microplate for direct poly(A)$^+$ mRNA purification followed by cDNA synthesis and PCR *(7–9)*. In this study, the leukocyte-capture filter plate was replaced with the newly developed EMV-capture filter plate, and HPC-derived mRNAs were quantified in plasma. Here, we report that HPC-derived mRNAs in plasma EMV may represent new biomarkers for the assessment of BM condition after HSCT.

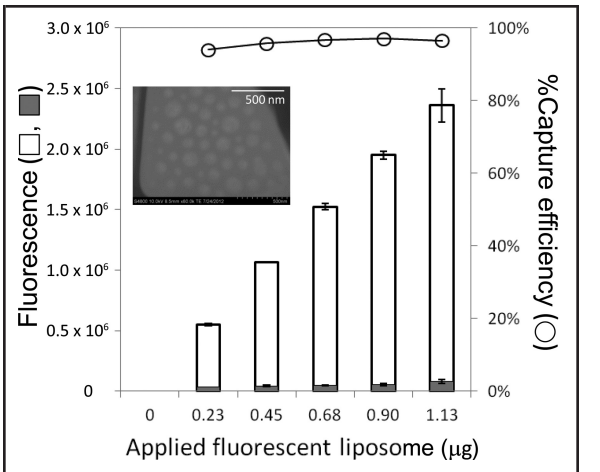

Fig. 1. Liposome capture by the filter plate.
Fluorescent liposomes (0–1.13 μg) were suspended in 100 μL PBS, pH 7.4, and applied to the filter plate and centrifuged at 2000*g* for 5 min. Fluorescent intensities of the liposome were measured before (□) and after (■) filtration, and capture efficiencies were calculated (○). Inset, SEM images of liposomes.

Materials and Methods

FLUORESCENT LIPOSOME CAPTURE BY THE FILTE RPLATE

To calculate the capture efficiency of the EMV-capture filter plate (Hitachi Chemical Research Center), triplicate aliquots of 0.23–1.13 μg fluorescent liposomes [87.4 (0.6)-nm diameter; FormuMax Scientific] were suspended in 100 μL PBS, pH 7.4, and applied to the filter plate and centrifuged at 2000*g* for 5 min. Fluorescent intensities of the liposomes were measured before and after filtration, and capture efficiencies were calculated.

SCANNING ELECTRON MICROSCOPY

To visualize the captured EMV or EMV-like materials, pooled human plasma obtained from a commercial source (Nova Biologics) was applied to the filter plate and centrifuged at 2000*g* for 5 min. Without any washing, the filter membrane was removed, dried, sputter coated, and analyzed by scanning electron microscopy (SEM) (S-4800, Hitachi High-Technologies).

EMV mRNA ANALYSIS WITH THE FILTER PLATE

Human plasma samples (0.3 mL) were applied to the filter plate and centrifuged at 2000*g* for 5 min. In each well, 60 μL of lysis buffer containing a cocktail of antisense primers was added and incubated at 55 °C for 10 min. The filter plate was then placed on a oligo(dT)-immobilized microplate (Hitachi Chemical Research Center) and centrifuged at 2000*g* for 5 min. The cDNA was directly synthesized in the same microplate by adding dNTPs (final concentration, 5 mmol/L), Moloney murine leukemia virus reverse transcriptase (final concentration, 2.7 U/μL), and RNasin (0.13 U/μL) (Invitrogen) and incubated at 37 °C for 2 h. The cDNA solution was used for subsequent real-time SYBR green PCR (iTaq, Bio-Rad) in the final volume of 5 μL in a 384-well plate. Each gene was amplified individually. The PCR conditions were 1 cycle of 95 °C for 10 min, followed by 50 cycles of 1 min 95 °C denaturing and 1 min 65 °C annealing/extension in PRISM7900 (Applied Biosystems). Melting curve analysis was performed every time to confirm that the amplification was derived from a single peak. The cycle threshold (Ct) was determined by the analytical software (SDS, Applied Biosystems). A Ct of 32 was used as a baseline. By use of the known concentrations of PCR templates, Ct values were converted to the copy number per milliliter of plasma. Primer sequences used in this study are summarized in Table 1 in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol60/issue4.

ULTRACENTRIFUGATION

Human plasma samples (0.3 mL) were added into small containers (Beckman), and centrifuged at 100 000*g* for 2 h at 4 °C (Optima XL-90 ultracentrifuge; Beckman) using the type 90Ti rotor (Beckman). After centrifugation, supernatants were decanted, and the pellets were suspended in 1× lysis buffer. Lysates were incubated at 55 °C for 10 min, then applied to the oligo(dT)-immobilized microplate for mRNA analysis.

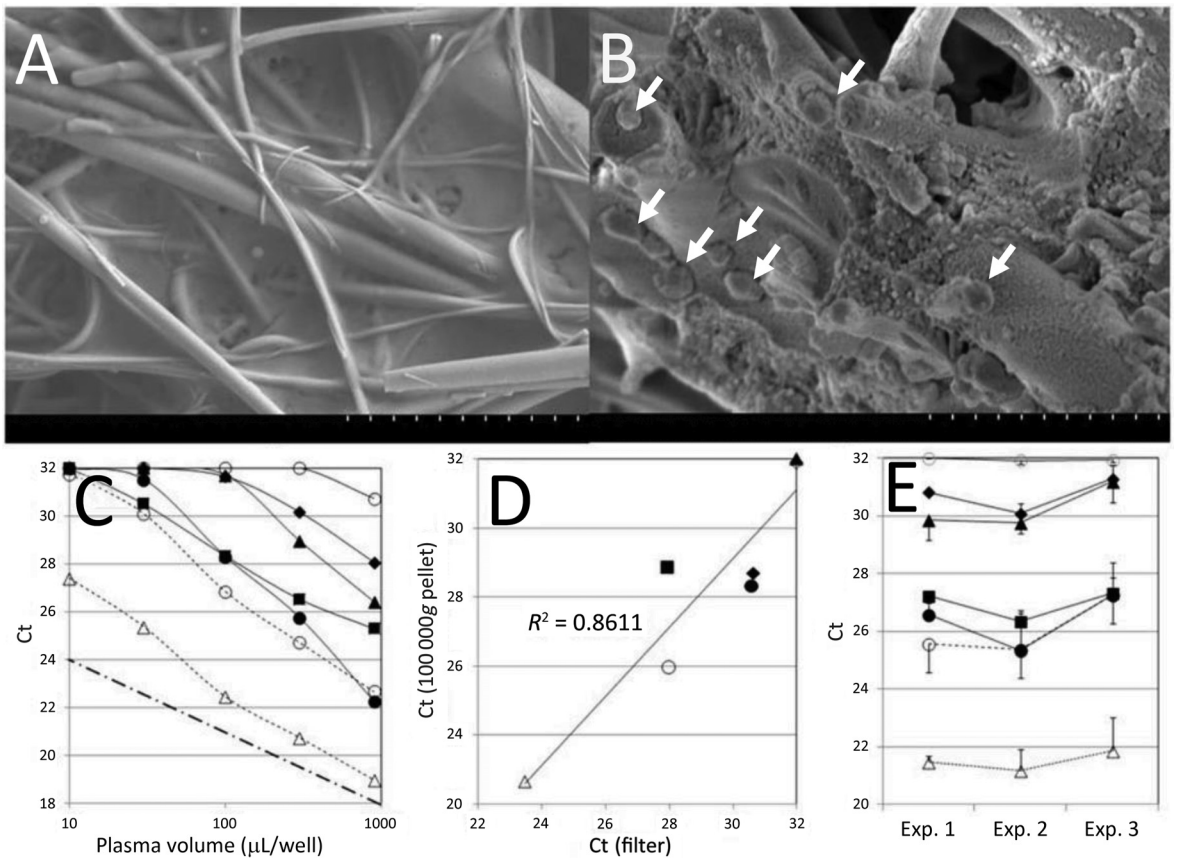

Fig. 2. Method validation.

(A, B), SEM analysis. After pooled human plasma was applied to the filter plate, filter membranes were removed, air dried, sputter coated with gold, and imaged at an accelerating voltage of 5 kV (A) and 3 kV (B) using the lower and upper secondary electron detectors, respectively [top surface (A), cross section (B)]. Arrows indicate EMV-like particles with a diameter of approximately 100 nm. (C), Dose responses. Plasma (10–1000 μL) was applied to the filter plate, and various mRNAs were measured by our method. Each symbol was the mean of the Ct values from triplicate plasma samples. ○, *ACTB*; Δ, B2M; ■, *ITGA2B*; ◇, *UROD*; ●, *HBB*; ▲, *SRGN*; ◆, *DEFA3*; – · –, theoretical slope. (D), Comparison between ultracentrifugation and our method. Plasma (300 μL) was ultracentrifuged at 100 000*g* for 2 h, then the pellets were resuspended in our lysis buffer, followed by mRNA analysis by our method. The results (*y* axis) were compared with the data of our filter plate method (*x* axis) using the identical plasma samples. Graphical symbols are the same as in Fig. 2C. (E), Reproducibility. Using the same plasma samples (300 μL, triplicate), our method was repeated 3 times. Graphical symbols are the same as in Fig. 2C. The bars indicate SD. Exp., experiment.

CLINICAL PROTOCOL

Plasma samples obtained from 18 patients who underwent allogeneic HSCT were analyzed retrospectively. Approval from the institutional review board was obtained for this study. Plasma samples were collected before conditioning and on days 7, 14, and 28 and stored frozen at −80 °C. All patients received routine screening blood tests, including complete blood count (CBC) with reticulocyte count at least 3 times per week after transplantation. BMA was performed on days 14 and 28. Clinical outcome was determined at day 180. The PB criteria for BM engraftment were as follows. Myeloid recovery was defined as an absolute neutrophil count of more than 500/μL, and erythroid and megakaryocyte lineage reconstitution were defined as reticulocyte counts of more than 3×10^4/μL and platelet counts of more than 20 000/μL without the transfusion of platelet concentrates, respectively.

Results

As shown in Fig. 1, fluorescent liposomes were absorbed by the filter plate, and the mean (SD) capture efficiency

Table 1. Patient characteristics.

Case no.	Age, years	Sex	Disease	Initial disease status	Donor	Conditioning	CMV reactivation, yes/no	Acute GvHD, grade
1	33	M	Ph$^+$ALL[a]	CR2	RPB	M	No	No
2	38	M	MDS	RCMD	BM	M	Yes	No
3	16	M	SAA	TD	BM	M	Yes	2
4	71	F	AML	CR3	BM	Non-M	Yes	1
5	60	M	MDS	RCMD	BM	M	Yes	2
6	25	M	ALL	CR1	BM	M	Yes	2
7	39	F	AML	CR1	RPB	M	No	1
8	39	F	Ph$^+$ALL	CR1	CB	M	Yes	1
9	28	M	SAA	TD	BM	M	Yes	2
10	33	F	AML	CR2 post BMT	BM	M	No	No
11	37	M	AML	RL1 post BMT	BM	M	Yes	1
12	60	M	AML	CR1	BM	M	Yes	1
13	28	M	Ph$^+$ALL	CR1	CB	M	No	1
14	38	M	ALL	RL1	BM	M	No	1
15	60	M	MDS	RAEB 1	RBM	M	Yes	2
16	33	F	AML	RL2	BM	M	No	2
17	62	M	ALL	CR1	BM	M	Yes	No
18	46	F	AML	RL1	CB	M	No	1

[a] Ph$^+$ALL, Philadelphia chromosome-positive acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; SAA, severe aplastic anemia; AML, acute myeloid leukemia; CR2, second CR; RCMD, refractory cytopenia with multilineage dysplasia; TD, transfusion dependent; RL1, first relapse; BMT, BM transplantation; RAEB, refractory anemia with excessive blast; RPB, related PB; CB, cord blood; M, myeloablative; CMV, cytomegalovirus; GvHD, graft vs host disease.

was 96.0% (1.2%). The filter plate was not saturated at the range of 0.23–1.13 μg of liposome (Fig. 1).

As shown in Fig. 2A, SEM demonstrated a thick membrane-like layer on the surface of post–plasma-applied membrane. EMV-like particles with sizes of approximately 100 nm were also observed on the cross section of the membrane (Fig. 2B, arrows), although the exact identification of EMV is quite difficult by SEM alone. After application of our lysis buffer, these trapped materials disappeared (data not shown). In separate experiments, plasma-derived EMV were lysed on the filter plate and mRNA was analyzed as described in the Materials and Methods. As shown in Fig. 2C, various mRNA were quantified in a dose-dependent manner from 30 μL to 1 mL of plasma, with the slope similar to the theoretical values (dotted line). The mRNAs included control housekeeping genes [actin, beta (*ACTB*)[4] and beta-2-microglobulin (*B2M*)] and myeloid [defensin, alpha 3, neutrophil-specific (*DEFA3*) and serglycin (*SRGN*)], erythroid [hemoglobin, beta (*HBB*) and uroporphyrinogen decarboxylase (*UROD*)], and megakaryocyte [integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41) (*ITGA2B*) and integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61) (*ITGB3*)] lineage-specific genes. The results from out method were also equivalent to the values derived from standard ultracentrifugation, with $r^2 = 0.8611$ (Fig. 2D). Most importantly, the Ct values of the target mRNA were reproducible among multiple independent experiments (Fig. 2E). We also measured mRNA genes derived from stem cells [CD34 molecule (*CD34*)], T cells [CD3e molecule, epsilon (CD3-TCR complex) (*CD3E*)], CD4 molecule (*CD4*), CD8a molecule (*CD8A*), and B cells [CD19 molecule (*CD19*)], but these genes were not amplified (data not shown).

The characteristics of the 18 patients who underwent allogeneic HSCT are listed in Table 1. The median age of the 12 male and 6 female patients was 38 years (range, 16–71 years). For these 18 patients, the donor samples consisted of 12 unrelated BM, 3 cord bloods, 2 related PB, and 1 related BM. A typical case is shown in Fig. 3A–C, in which the baseline expression of plasma EMV mRNA became undetectable (Ct, >32) just be-

[4] Human genes: *ACTB,* actin, beta; *B2M,* beta-2-microglubulin; *DEFA3,* defensin, alpha 3, neutrophil-specific; *SRGN,* serglycin; *HBB,* hemoglobin, beta; *UROD,* uroporphyrinogen decarboxylase; *ITGA2B,* integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41); *ITGB3,* integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61); *CD34,* CD34 molecule; *CD3E,* CD3e molecule, epsilon (CD3-TCR complex); *CD4,* CD4 molecule; *CD8A,* CD8a molecule; *CD19,* CD19 molecule.

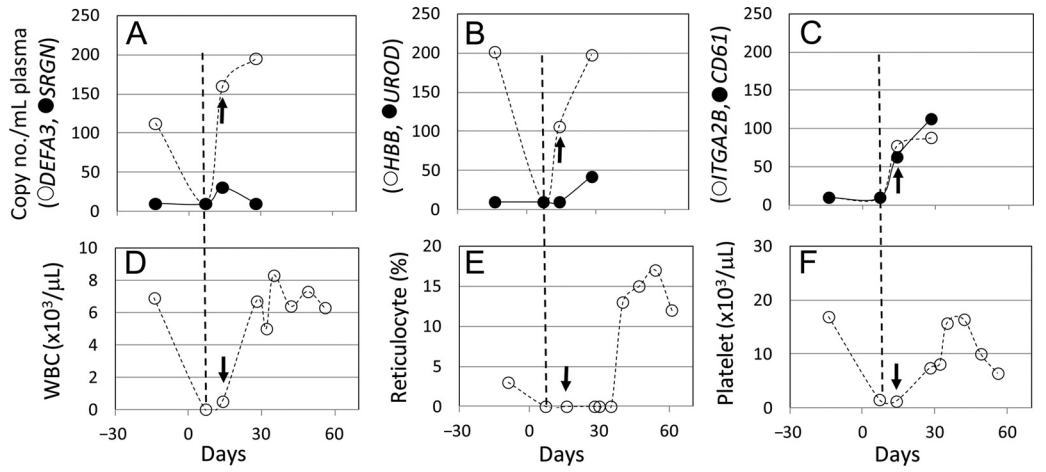

Fig. 3. Comparison between EMV mRNA analysis and conventional CBC, typical case.

The upper panels (A–C) plot the levels of EMV mRNAs [(A), *DEFA3* (○) and *SRGN* (●); (B), *HBB* (○) and *UROD* (●); (C), *ITGA2B* (○) and *ITGB3* (●)]. The bottom panels (D–F) plot the results of CBC analysis from a single typical case [(D), WBC; (E), reticulocytes; (F), platelets]. In (A–C), the results of PCR (Ct) were converted to the copy number per milliliter plasma as described in the Materials and Methods. Vertical dashed lines indicate day 7, when neither mRNA or CBC results could detect BM recovery, whereas the arrows indicate the points where mRNA was first recovered.

fore HSCT and increased after HSCT. Moreover, the recovery of *DEFA3*, *HBB*, *ITGA2B*, and *ITGB3* mRNAs in EMV preceded the recovery of white blood cell (WBC), reticulocyte, and platelet counts in the CBC (Fig. 3 arrows). The performances of *DEFA3* and *HBB* were better than those of *SRGN* and *UROD*, respectively, and *ITGB3* was similar to *ITGA2B* (Fig. 3, A–C).

As shown in Table 2, patients with *DEFA3*, *HBB*, and *ITGA2B* mRNA recovery by day 15 all showed CBC recovery (WBC >500/μL, reticulocytes >3×10⁴/μL, platelets >20 000/μL) by day 30, respectively, whereas patients with CBC recovery after day 30 showed slower EMV mRNA recovery occurring after day 15. Fig. 4, A–C, shows a graphical representation of all 18 cases, with the first recovery day of CBC on the *y* axis and the same data of EMV mRNA on the *x* axis. Any data points above the 45° dotted line indicated that the recovery of EMV mRNA gave an earlier indication of engraftment than the CBC. As shown in Fig. 4, A–C, individuals with EMV mRNA recovery by day 15 all showed data points above this 45° dotted line, indicating that EMV mRNA recovery was earlier than that for CBC for all 3 lineages, myeloid, erythroid, and megakaryocyte. By contrast, the EMV mRNA data were not conclusive if mRNA recovery occurred after day 15. Interestingly, EMV mRNA recovery was noted as early as day 7 in 2 individuals with *DEFA3* and in 1 individual each with *HBB* and *ITGA2B* (Fig. 4). Moreover, as shown in Fig. 4D–F, EMV mRNA recoveries of *DEFA3*, *HBB*, and *ITGA2B* were correlated with the number of nucleated cells in BMA samples at day 15 (n = 11).

The next question was whether early recovery of EMV mRNA would predict the success or failure of HSCT. Thus, the results of EMV mRNA recovery by day 15 were compared with the clinical outcome on day 180 (Table 2). Eight of 11 patients with myeloid lineage mRNA recovery (73%) by day 15 achieved complete re-

Table 2. Correlations among EMV mRNA, CBC, and clinical outcomes.

	RNA elevation, days[a]	CBC recovery, n		Status on day 180, n	
		≤30 days[b]	>30 days	CR/NH	RL/TD[c]
WBC	≤15	11	0	8	3
	>15	6	1	4	3
RBC	≤15	6	0	6	0
	>15	2	10	6	6
Platelet	≤15	6	0	6	0
	>15	1	11	6	6

[a] RNA elevation, *DEFA3* for WBC, *HBB* for RBC, and *ITGA2B* for platelets, respectively.
[b] CBC recovery was WBC >500/μL, reticulocyte >3×10⁴/μL, and platelets >20,000/μL.
[c] RL, relapse; TD, transfusion dependent.

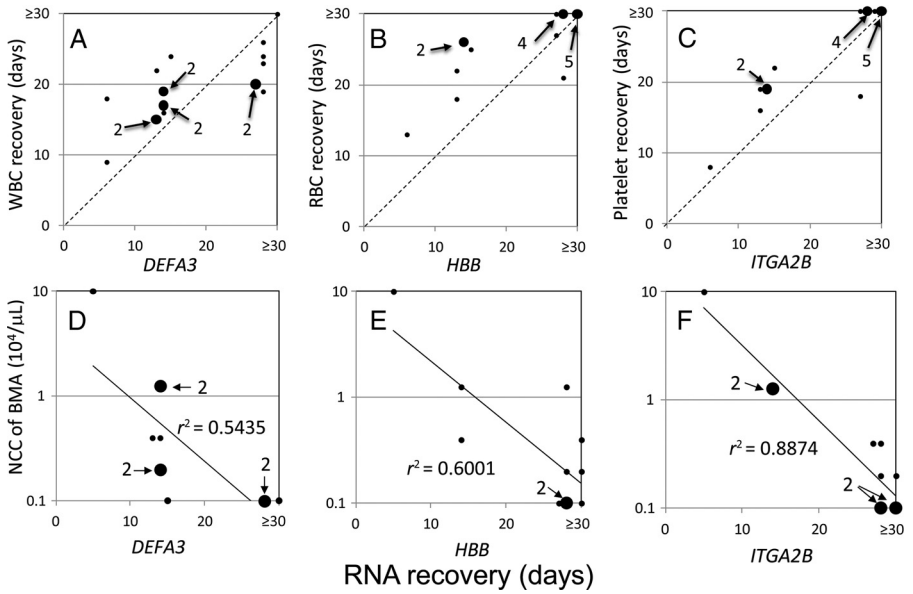

Fig. 4. Comparison between EMV mRNA analysis and conventional assays.

(A–C), EMV mRNA vs CBC for all 18 cases combined. The recovery days of EMV mRNA (first detectable mRNA) are shown on the *x* axis, and CBC recovery days (WBC, >500/μL; reticulocytes, >4/μL; platelets, >20 000 without blood transfusion) are shown on the *y* axis, up to day 30. (A), WBC vs *DEFA3*; (B), RBC vs *HBB*; (C), platelets vs *ITGA2B*. Dotted lines are plotted at an angle of 45°. The data points above this line represent the patients who demonstrated earlier mRNA evidence of recovery than CBC. Small dots represent a single patient, and large dots represent multiple patients at the same location. The number of patients for each large dot is shown by arrows. (D–F), EMV mRNA analysis vs BM data at day 14–15. The recovery days of EMV mRNA (*x* axis) were the same as those shown in (A–C). The *y* axis represents nucleated cell counts (NCC) in BM aspirations at day 14–15 ($n = 11$). (A), NCC vs *DEFA3*; (B), NCC vs *HBB*; (C), NCC vs *ITGA2B*. Solid lines indicate regression lines with r^2 values. Small and large dots are the same as (A–C).

mission (CR) or normal hematopoiesis (NH). Furthermore, all patients with the recovery of erythroid or megakaryocyte lineage–specific mRNAs achieved CR or NH (Table 2), although poor mRNA recovery at day 15 or thereafter did not provide an accurate prediction of outcome (Table 2).

Discussion

Standard total RNA preparation contains rRNA, tRNA, noncoding RNA, and miRNA, as well as full-length and fragmented mRNAs. Thus, the PCR amplification indicates only the presence of primer lesions of RNA. However, owing to the use of the oligo(dT)-immobilized microplate and PCR primers designed in the coding region of each target gene, the PCR results in this study indicated the presence of long mRNA from the poly(A)$^+$ tail to the coding region. Although plasma is a rich source of RNase, EMV mRNA was clearly protected by the encapsulation of EMV membranes. Recent studies also revealed a new biological role of EMV to promote cell-to-cell communication by transferring genetic information such as mRNA and miRNA to remote sites *(10, 11)*. Thus, the identification of long poly(A)$^+$ mRNA in EMV will be a model platform for the analysis of EMV biology in future.

BM-derived EMV has been reported previously. However, the BM cells in these reports were stromal cells *(12)*, stem cells *(13)*, dendritic cells *(14)*, and mesenchymal stem cells *(15)*, not HPC. *DEFA3* is a gene of microbicidal and cytotoxic peptides found in the granules of neutrophils and precursor myeloid cells. These granules are produced in myeloid precursor cells in BM, indicating that *DEFA3* mRNA is expressed in myeloid precursor cells more prominently than mature PB neutrophils. In fact, according to the EST (expressed sequence tag) database (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.654448), the expression frequency of *DEFA3* mRNA is 10 times more in BM than PB. *HBB* is the gene for the β subunit of hemoglobin. Hemoglobin protein is abundantly expressed in red blood cells (RBC), but owing to the lack of a nucleus in RBC, *HBB* mRNA cannot be

produced in RBC. Thus the origin of *HBB* mRNA is the erythroblasts in BM and reticulocytes *(16)*. *ITGA2B* and *ITGB3* are platelet-specific integrins. Because platelets do not have a nucleus, the origin of these 2 integrins is the megakaryocytes in BM.

The levels of these mRNAs became undetectable at day 7 in many cases (Fig. 3). This means that BM-derived EMV is cleared through the myeloablative procedure. This also indicates that EMV mRNA may be applicable to monitor the efficacy of the myeloablative procedure. The EMV mRNAs were recovered earlier than traditional CBC (Figs. 3 and 4). This is quite reasonable, because hematopoietic stem cells first grow in BM to produce various HPC, then after a series of maturation processes, WBC, RBC, and platelets are released into PB. Our results (Figs. 3 and 4) indicate that myeloid and erythroid precursor cells and megakaryocytes produce EMV, and such EMV are released into PB earlier than mature WBC, RBC, and platelets. Not only are *DEFA3*, *HBB*, and integrin mRNAs present in BM-derived EMV, but also WBC, reticulocytes, and platelets. However, because BM recovery was not detectable using the CBC at the time when EMV mRNAs first appeared in plasma after HSCT, these EMV mRNAs of BM origin can be considered as early indicators of BM recovery.

Although BMA is not very quantitative owing to contamination with PB during aspiration and location-specific variation of the bone, EMV mRNA analysis provides accurate quantitative results. The volume of plasma required was as small as 0.3 mL, and the plasma could be acquired by simple phlebotomy, a procedure far less invasive than BMA. Although BMA cannot be replaced entirely, plasma EMV mRNA analysis may provide an opportunity to reduce the frequency of BMA. Although the number of patients in this study was limited, we believe that the results of this single institution exploratory study are sufficient to encourage the initiation of much larger clinical studies as well as clinical applications in other BM diseases. Although we have demonstrated only a plasma application in this study, this filter plate appears to be applicable to urine, saliva, cell culture supernatant, and other fluids. Data from experiments examining these other sample sources will be made available separately.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

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