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ORIGINAL ARTICLE

Elevated levels of platelet- and red cell-derived extracellular vesicles in transfusion-dependent β-thalassemia/HbE patients with pulmonary arterial hypertension

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Abstract

Pulmonary arterial hypertension (PAH) is a serious complication in β-thalassemia. The mechanism of PAH development is believed to be through chronic platelet activation and red cell (RBC) membrane abnormality contributing to a hypercoagulable state and thrombosis, which consequently leads to the development of PAH. Extracellular vesicles (EVs) shed from the plasma membrane of platelets and RBCs are found to be associated with thrombotic risk. This study aimed to investigate the involvement of phosphatidylserine (PS)-bearing cells and EVs in accelerating the progression of the hypercoagulable state in transfusion-dependent thalassemia (TDT) patients. Fresh whole blood samples from splenectomized TDT-β-thalassemia/HbE patients (11 with PAH and 14 without PAH) and 15 normal subjects were analyzed for platelet activation by measuring P-selectin expression using flow cytometry and the number of dense granular using an electron microscope. The amounts of PS-bearing RBCs, large RBC-EVs, platelets, and medium EVs were determined by flow cytometry. Platelet activation in PAH patients was not significantly different from other groups; however, the amounts of PS-bearing large RBC-EVs, platelets, and medium platelet-derived EVs were significantly increased in PAH patients as compared to normal subjects, but they were not different from patients without PAH. This could be affected by antiplatelet therapy that reduced the levels of platelet activation and the amount of PS-bearing cells, including EVs, in PAH patients as well as in patients without PAH.

Keywords Extracellular vesicles · Beta-thalassemia · Pulmonary arterial hypertension · Hypercoagulable state · Antiplatelet

Abbreviations

EV	Extracellular vesicle
PAH	Pulmonary arterial hypertension
P-EV	Platelet-derived EV
TDT	Transfusion-dependent thalassemia

Introduction

Pulmonary arterial hypertension (PAH) is the major cause of heart failure and pulmonary deterioration resulting in morbidity and mortality in β-thalassemia [1, 2], a common genetic

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disorder characterized by the absence or reduction of β-globin chain production. Studies of β-thalassemia major (βTM), β-thalassemia intermedia (βTI), and β-thalassemia/hemoglobin (Hb) E diseases demonstrated that the progression of PAH could be mild to severe. The etiology of PAH in β-thalassemia is multifactorial including aging, iron overload, splenectomy, damaged red blood cells (RBCs), chronic platelet activation, and a hypercoagulable state causing thrombotic lesions in the lungs [1, 3]. The hypercoagulable state in β-thalassemia is a consequence of RBC abnormality that leads to procoagulant phosphatidylserine (PS) exposure on the outer membrane leaflet of RBCs, PS-bearing RBC extracellular vesicle (EV) release, and RBC adhesion and aggregation on endothelial cells. PS is a negatively charged phospholipid that normally binds to coagulation factors to form the tenase complex and the prothrombinase complex that rapidly converts prothrombin into thrombin [4]. Thrombin is a strong agonist that can activate platelets, white blood cells (WBCs), and endothelial cells, leading to a blood clot [3, 5–8].

Extracellular vesicles (EVs) shed from the plasma membrane during apoptosis or cell activation are divided into three types according to the International Society for Extracellular Vesicles (ISEV): large EVs or apoptotic bodies (1–5 μm in diameter), medium EVs or microvesicles/microparticles (0.1–1 μm in diameter), and small EVs or exosomes (30–100 nm in diameter) [9]. Large RBC-EVs, also termed RBC vesicles, are defined as apoptotic bodies as described in our previous study [10]. Both large RBC-EVs and medium EVs are characterized by enriched PS exposure and can carry proteins, mRNA, miRNA, and other machinery from their parent cells [11]. Increased levels of PS-bearing large RBC-EVs and medium EVs, especially from platelets and RBCs, were found in splenectomized β-thalassemia/HbE patients as compared to non-splenectomized patients and normal subjects [7]. Increased platelet factor 3-like activity, a platelet activation marker, was correlated with increased amounts of PS-bearing MPs in β-thalassemia/HbE patients [7]. Moreover, PS-bearing medium EVs from splenectomized β-thalassemia/HbE patients were clearly shown to have procoagulant activity in inducing prothrombinase complex activity [12], platelet activation, platelet aggregation, and platelet-neutrophil aggregation [13]. Accordingly, quantitative analysis of the cellular origins of PS-bearing medium EVs has been considered as a biomarker of cellular abnormality, vascular injury, and pro-thrombotic risk in thalassemia [7, 14] and in other diseases [15].

Therefore, this study aimed to analyze the amount of PS-bearing RBCs, large RBC-EVs, and platelets in transfusion-dependent (TDT)-β-thalassemia/HbE patients who had PAH or did not have PAH in comparison to normal subjects. Moreover, the amounts of PS-bearing medium EVs and their cellular origin were also examined, in an attempt to develop markers for the hypercoagulable state in β-thalassemia/HbE patients with PAH.

Materials and methods

Subjects

The study protocol was approved by the Mahidol University Central Institutional Review Board (approval numbers COA.No.MU-CIRB 2014/013.0502 and COA.No.MU-CIRB 2015/076.1906) and the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University (approval number MURA2014/326). Written informed consent was obtained from all individual participants included in the study. Fourteen TDT-β-thalassemia/HbE patients without PAH, 11 TDT-β-thalassemia/HbE patients with PAH, and 15 normal subjects at ages ranging from 24 to 61 years were recruited. Patients with PAH who were diagnosed by hemodynamic assessment using cardiac catheterization had received aspirin for treatment. All patients had been splenectomized for at least 5 years and received deferiprone (GPO-L-ONE®), folic acid, calcium, and vitamin D. None of the patients had been hospitalized or blood transfused within the 4 weeks prior to their blood donation. Clinical and laboratory characteristics are summarized in Table 1. Three of the 11 patients with PAH and five of the 14 patients without PAH had serum ferritin > 1000 ng/mL. The first 2 mL of all blood samples was discarded to avoid platelet activation, and then samples were collected into 3.2% sodium citrate anticoagulant at room temperature and immediately processed.

Dense granular analysis

Fresh platelet-rich plasma was diluted and dropped on formvar-coated copper mesh grids. Grid samples were fixed with 0.5% glutaraldehyde solution in 0.1 M phosphate-buffered saline to examine the dense granules in platelets by transmission electron microscopy (TEM) (Hitachi TEM System, HT7700, Hitachi High-Technologies Corporation). Images of platelets were randomized and the dense granules per platelet counted under the counting criteria described by Westmoreland D et al. [16].

P-selectin expression

Diluted whole blood samples were stained with fluorochrome-conjugated monoclonal antibodies (mAb) specific to CD41a (glycoprotein (GP) IIb/IIIa) as a platelet marker and CD62P (P-selectin) as a platelet activation marker. The percentage of P-selectin-positive platelets and the mean fluorescent intensity (MFI) of P-selectin expression on platelets (CD41a+) were determined using CellQuest software, FACScan flow cytometer (Becton Dickinson Biosciences, BDB) as described in a previous study [13]. All mAbs and reagents were purchased from BDB.

Table 1 Hematological parameters

Description[a]	Normal subjects	Transfusion-dependent β-thalassemia/HbE patients with splenectomy	
		No PAH	PAH
Number	15	14	11
Age (years)	28 ± 5 (23–36)	37 ± 12 (24–61)	39 ± 11 (24–52)
Hemoglobin typing			
HbA (%)	88.3 ± 3.7	35.9 ± 22.1[b]	48.4 ± 19.9[b]
HbA_2 (%)	2.9 ± 0.3		
HbE (%)		37.5 ± 19.8	28.1 ± 14.1
HbF (%)	0.3 ± 0.2	18.5 ± 11.4[b]	14.5 ± 10.3[b]
RBC count ($\times 10^6/\mu L$)	4.8 ± 0.4	2.9 ± 0.5[b]	3.0 ± 0.4[b]
Hemoglobin (g/dL)	13.7 ± 1.2	6.5 ± 1.2[b]	7.0 ± 0.7[b]
Hematocrit (%)	40.9 ± 3.1	21.7 ± 3.0[b]	22.6 ± 2.7[b]
MCV (fL)	87.0 ± 5.1	76.4 ± 5.2[b]	74.7 ± 4.4[b]
MCH (pg)	29.2 ± 2.4	23.0 ± 2.4[b]	23.2 ± 2.0[b]
MCHC (g/dL)	33.5 ± 1.1	30.1 ± 2.3[b]	31.1 ± 1.6[b]
RDW (%)	13.1 ± 1.1	26.9 ± 4.5[b]	26.9 ± 4.0[b]
NRBCs (cells/100WBC)	0 ± 0	459 ± 400[b]	448 ± 447[b]
corWBC count ($\times 10^3/\mu L$)	5.4 ± 0.9	13.7 ± 5.6[b]	11.4 ± 4.2[b]
Differential WBC count			
Neutrophils (%)	55 ± 4	52 ± 13	50 ± 11
Lymphocytes (%)	34 ± 3	36 ± 14	38 ± 10
Monocytes (%)	6 ± 2	7 ± 4	7 ± 3
Eosinophils (%)	3 ± 2	3 ± 3	3 ± 2
Basophils (%)	1 ± 1	1 ± 1	2 ± 1
Platelet count ($\times 10^3/\mu L$)	256 ± 39	612 ± 186[b]	553 ± 140[b]
Serum ferritin (ng/mL)	62 ± 50	1072 ± 981[b]	769 ± 791[b]

corWBC count, corrected white blood cell count; *Hb*, hemoglobin; *MCH*, mean corpuscular hemoglobin; *MCHC*, mean corpuscular hemoglobin concentration; *MCV*, mean corpuscular volume; *NRBC*, nucleated red blood cells; *PAH*, pulmonary arterial hypertension; *RBC*, red blood cells; *RDW*, red cell distribution width. [a] Statistical analysis using mean ± standard deviation (S.D.). [b] Significant difference when compared to normal subjects at $P < 0.05$

Phosphatidylserine-bearing cell and extracellular vesicle analysis

Diluted whole blood samples were stained with fluorochrome-conjugated annexin V (PS marker) and a mAb specific to CD41a (a platelet marker), CD45 (leukocyte common antigen, a leukocyte marker), CD105 (endoglin, an endothelial cell marker), and CD235a (glycophorin A, a RBC marker) in annexin V binding buffer containing TruCount™ beads (BDB) for absolute number calculation. The EV population was determined by comparison with beads with a 1-μm diameter (Spherotech, Lake Forest, IL, USA). PS-bearing cells and EVs were acquired and analyzed using CellQuest software, FACScan flow cytometer (BDB) as described in a previous study [7, 13]. All mAbs and reagents were purchased from BDB.

Statistical analysis

Data were analyzed using SPSS Version 18.0 (IBM Collaboration) and GraphPad PRISM 6.0 (GraphPad Software, Inc.). Comparisons between parameters were evaluated with a non-parametric Mann-Whitney *U* test. The correlation coefficient was calculated with Spearman's rho (r_s). The threshold for statistical significance for all comparisons was $P < 0.05$.

Results

Platelet activation in transfusion-dependent β-thalassemia/HbE patients with PAH

Thrombocytosis is found in patients as a post-splenectomy consequence. However, the percentage of P-selectin positive

platelets (Fig. 1a) and P-selectin expression (mean fluorescent intensity) of the individual platelets (Fig. 1b) as measured by flow cytometry was not different among groups in this cohort study. Dense granules were observed by TEM (Fig. 1c), as it is a more sensitive technique, to confirm that there was no significant difference of platelet activation among these groups (Fig. 1d).

Phosphatidylserine-bearing cells and extracellular vesicles in transfusion-dependent β-thalassemia/HbE patients with PAH

Platelet activation is not the only factor that leads to PAH, as plasma membrane abnormalities and EV release also play roles. Increased amounts of PS-bearing platelets and PS-bearing large RBC-EVs in patients with PAH (mean ± S.D. $8.0 \pm 4.3 \times 10^3$ cells/μL and $4.7 \pm 2.9 \times 10^3$ cells/μL, respectively) and in patients without PAH ($8.1 \pm 4.8 \times 10^3$ cells/μL and $4.5 \pm 2.2 \times 10^3$ cells/μL, respectively) were found when compared to normal subjects ($3.3 \pm 1.3 \times 10^3$ cells/μL and $2.5 \pm 1.7 \times 10^3$ cells/μL, respectively) ($P < 0.05$) (Fig. 2a). However, the amounts of PS-bearing RBCs and EVs were not significantly different between patients and normal subjects in this cohort study. The cellular origin of PS-bearing medium EVs from individual samples was also analyzed. The amount of PS-bearing medium platelet-derived EVs (P-EVs) in the blood circulation from patients (with PAH $19.6 \pm 7.1 \times 10^3$ particles/μL and without PAH $18.5 \pm 9.3 \times 10^3$ particles/μL) was significantly higher than that from normal subjects ($5.1 \pm 2.7 \times 10^3$ particles/μL) ($P < 0.05$) (Fig. 2b). However, there were no significant differences of PS-bearing RBCs, PS-bearing platelets, PS-bearing large RBC-EVs, and PS-bearing medium EVs or different cellular origins of PS-bearing medium EVs (P-EVs, RBC-EVs, EC-EVs, and L-EVs) between patients with PAH and without PAH (Fig. 2).

Medium P-EVs and large RBC-EVs as biomarkers of chronic platelet activation and RBC membrane damage

Medium P-EVs derived from platelets during cell activation or under stress lead to PS exposure on the outer membrane leaflet, and Spearman's rho coefficient analysis showed that medium P-EVs were significantly correlated with platelet activation ($r_s = 0.471$, $P = 0.007$) and PS-bearing platelets ($r_s = 0.795$, $P < 0.001$) (Table 2). Therefore, PS-bearing medium P-EVs could be a marker to monitor the status of chronic platelet activation in splenectomized TDT-β-thalassemia/HbE patients who have had antiplatelet therapy.

Moreover, PS-bearing large RBC-EV analysis could be a biomarker to determine the pathology of RBCs in TDT-BES patients in addition to MCV, MCH, and MCHC. It is possible

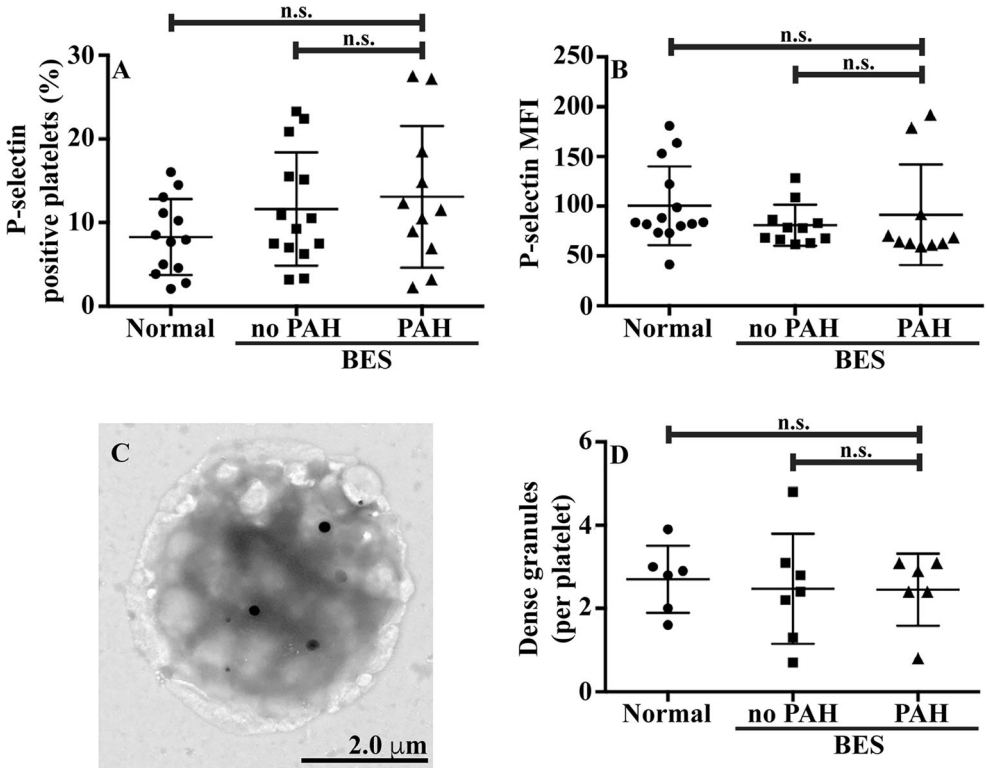

Fig. 1 Analysis of platelet activation in splenectomized transfusion-dependent β-thalassemia/HbE patients (BES) with pulmonary arterial hypertension (PAH) or without PAH and normal subjects. **a** Percentages and **b** mean fluorescent intensity (MFI) of P-selectin on platelets were determined using flow cytometry. **c** Image of dense granules in platelet and **d** mean of the amounts of dense granules per platelet were examined using a transmission electron microscope. The lines show mean ± S.D. n.s., not significant difference

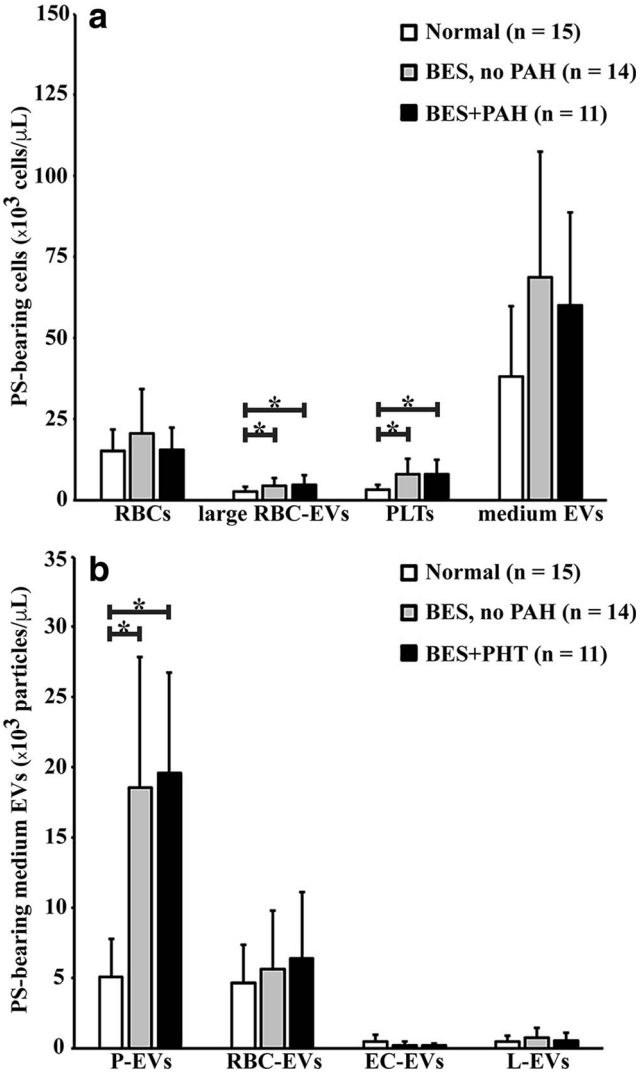

Fig. 2 Absolute number of **a** phosphatidylserine-bearing cells/extracellular vesicles and **b** cellular origin of medium extracellular vesicles in peripheral blood from splenectomized transfusion-dependent β-thalassemia/HbE patients (BES) with pulmonary arterial hypertension (PAH) or without PAH. Data are presented as mean ± S.D. EC-EVs, endothelial cell-derived EVs; EVs, extracellular vesicles; L-EVs, leukocyte-derived EVs; PLTs, platelets; P-EVs, platelet-derived EVs; PS, phosphatidylserine; RBCs, red blood cells; RBC-EVs, red blood cell-derived EVs. *Significant *P* values are indicated as determined by the Mann-Whitney *U* test

that donor RBCs remained in the blood circulation of patients who have received multiple blood transfusions. As seen in this study, although patients had no blood transfusion for at least 4 weeks before blood sample collection, however, HbA levels were still detectable (Table 1). Although, a correlation between CBC index and PS-bearing RBC vesicles was not found, the amount of PS-bearing large RBC-EVs was significantly correlated with PS-bearing RBCs ($r_s = 0.491$, $P < 0.001$) and PS-bearing medium RBC-EVs ($r_s = 0.650$, $P < 0.001$) (Table 3).

Discussion

PAH is a significant risk factor for vaso-occlusive crisis (VOC) leading to early sudden death in β-thalassemia patients, and careful management of blood transfusions and iron chelation therapy is required for the general management of β-thalassemia patients to prevent PAH development [17] and other complications. However, despite this, 56% of βTM [5] and 37% of β-thalassemia/HbE patients [18] who have received regular blood transfusions and iron chelation therapy show evidence of PAH development, suggesting that chronic transfusion coupled with iron chelation therapy may not be enough to prevent PAH development in β-thalassemia, and therefore specific therapies for PAH in β-thalassemia are required. However, the etiology of PAH in β-thalassemia is complex and multifactorial and so understanding the mechanism of PAH development in β-thalassemia could be useful to improve clinical guidelines as well as to develop novel therapeutic approaches.

Chronic platelet activation was hypothesized to be one of the major factors that play an important role in PAH development in patients with hemoglobinopathies such as β-thalassemia [3, 5] and sickle cell disease (SCD) [19]. P-selectin expression on platelets from β-thalassemia patients [5, 20] and SCD patients with PAH [19] is significantly higher than P-selectin expression in patients without PAH. Significantly decreased platelet activation in SCD patients with PAH at VOC who had an oral dose of sildenafil, a phosphodiesterase-5 inhibitor, was observed [19], and as such antiplatelet therapy to inhibit platelet activation could provide additional benefits for β-thalassemia patients with PAH. In this cohort study, splenectomized TDT-β-thalassemia/HbE patients with PAH who had received aspirin showed inhibition of platelet activation, which could reduce the risk of a hypercoagulable state and consequently progressive pulmonary vascular disease.

However, splenectomized TDT-β-thalassemia/HbE patients with PAH had increased amounts of PS-bearing platelets, large RBC-EVs, and medium P-EVs, which could be a high risk factor for thromboembolism. A good correlation between the amount of PS-bearing medium P-EVs and other platelet markers including the amount of platelets, percentage of P-selectin+ platelet activation, and the amount of PS-bearing platelets was shown with statistical significance in this study (Table 2). It is emphasized that chronic platelet activation led to platelets releasing PS-bearing medium P-EVs into the blood circulation. Moreover, increased PS-bearing platelets and EVs could activate other cells such as RBCs and WBCs to release their own PS-bearing medium EVs (Table 2) [7]. It is possible that PS-bearing medium EVs, especially P-EVs, could bind to coagulation factors to form the prothrombinase complex, followed by cleavage of prothrombin into thrombin [12]. Thrombin activates platelets

Table 2 Spearman's correlation coefficient (r_s) of medium platelet-derived extracellular vesicles and platelet properties

Parameters	Medium P-EVs	PS-bearing medium EVs	PS-bearing PLTs	P-selectin+ PLTs (%)	PLT count
Medium P-EVs	–	n.s.	$r_s = 0.795$ $P < 0.001$	$r_s = 0.471$ $P = 0.007$	$r_s = 0.811$ $P < 0.001$
PS-bearing medium EVs	n.s.	–	n.s.	$r_s = 0.595$ $P < 0.001$	n.s.
PS-bearing PLTs	$r_s = 0.795$ $P < 0.001$	n.s.	–	n.s.	$r_s = 0.762$ $P < 0.001$
P-selectin+ PLTs (%)	$r_s = 0.471$ $P = 0.007$	$r_s = 0.595$ $P < 0.001$	n.s.	–	n.s.
PLT count	$r_s = 0.811$ $P < 0.001$	n.s.	$r_s = 0.762$ $P < 0.001$	n.s.	–

EVs, extracellular vesicles; *n.s.*, not significant difference; *PLTs*, platelets; *P-EVs*, platelet-derived EVs; *PS*, phosphatidylserine

and other cells such as endothelial cells and leukocytes, causing loss of plasma membrane asymmetry and leading to translocation of PS onto the outer membrane bilayer and the generation of PS-bearing EVs [21]. Moreover, our previous studies clearly demonstrated that high amounts of PS-bearing EVs affected the induction of platelet-platelet aggregation, and MP-platelet-neutrophil aggregation in β-thalassemia/HbE patients and normal subjects in a dose-dependent manner [13].

PS-bearing platelets also correlated with the amount of PS-bearing large RBC-EVs (Table 3). Damaged RBCs as characterized by PS-bearing RBCs could have defective cytoskeleton membranes and shed PS-bearing large RBC-EVs into the blood circulation as statistical analysis showed a good correlation between the amount of PS-bearing RBCs and the amount of PS-bearing large RBC-EVs. PS-bearing large RBC-EVs might further shed their membranes to generate PS-bearing medium RBC-EVs. Both PS-bearing large RBC-EVs and PS-bearing medium RBC-EVs could contain cell-free Hb that could affect the scavenging activity of the vasodilator nitric oxide [22, 23].

PS-bearing EVs might be applied for use as a biomarker associated with thrombotic risk in β-thalassemia/HbE patients. Platelets are also a major source of EVs in β-thalassemia/HbE patients [7] and βTM [24], while endothelial cells are a major source of EVs in βTI, suggesting endothelial activation or injury [14]. Platelet activation, PS-bearing cells, and their EVs in β-thalassemia patients could be involved in vasculopathic development. Therefore, therapeutic management for vasculopathy in β-thalassemia could be concerned with the degree or the main factors of disease progression and take into account the phenotype spectrum of patients with βTM, βTI, or β-thalassemia/HbE, as βTM patients have had severe anemia that require initiation of blood transfusion since infancy, while βTI patients have a less severe phenotype that only requires occasional blood transfusion. β-Thalassemia/HbE patients encompass a broad phenotype spectrum from mild cases who do not need regular blood transfusion to severe cases who receive regular blood transfusion as Hb levels range between 3 and 13 g/dL [25, 26]. Therefore, it is necessary to maintain Hb levels greater than 10 g/dL. All patients who receive regular blood transfusion should be offered iron chelation therapy. Splenectomized β-thalassemia patients, even those with βTI or β-thalassemia/HbE disease, who have moderate to severe anemia should receive life-long antiplatelet therapy to prevent PAH development. Chronic blood transfusion, iron chelation, long-term antiplatelet therapy, and other supplements such as vasodilators to prevent PAH should be considered as a conventional therapeutic approach for β-thalassemia [1, 2, 27].

Table 3 Spearman's correlation coefficient (r_s) of large red cell extracellular vesicles and their related parameters

Parameters	Medium RBC-EVs	Large RBC-EVs	PS-bearing RBCs	PS-bearing PLTs
Medium RBC-EVs	–	$r_s = 0.650$ $P < 0.001$	n.s.	n.s.
Large RBC-EVs	$r_s = 0.650$ $P < 0.001$	–	$r_s = 0.491$ $P < 0.001$	$r_s = 0.653$ $P < 0.001$
PS-bearing RBCs	n.s.	$r_s = 0.491$ $P < 0.001$	–	n.s.
PS-bearing PLTs	n.s.	$r_s = 0.653$ $P < 0.001$	n.s.	–

EVs, extracellular vesicles; *n.s.*, not significant difference; *PLTs*, platelets; *PS*, phosphatidylserine; *RBCs*, red blood cells; *RBC-EVs*, red blood cell-derived EVs

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Authors' contributions KM, PP, and KP performed the experiments, analyzed the data, and drafted the manuscript. SC contributed to the concept of the study and specimen collection. DT, SW, and SS contributed to the concept of the study, design the experiments, the analysis of the data, and drafting the manuscript. PC was the principal investigator and takes primary responsibility for the concept and design of the project, the analysis of the data, and drafting the manuscript. All authors reviewed and approved the final version to be published.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Redundant publication No substantial overlap with previous papers

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Mahidol University Central Institutional Review Board (approval numbers COA.No.MU-CIRB 2014/013.0502 and COA.No.MU-CIRB 2015/076.1906) and the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University (approval number MURA2014/326) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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