In vivo tracking of transfused platelets to measure platelet survival by NGS method using mitochondrial DNA

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In vivo tracking of transfused platelets to measure platelet survival by NGS method using mitochondrial DNA

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<TABLE OF CONTENTS>

ABSTRACT ····· ··vi

I. INTRODUCTION ····· 1
II. MATERIALS AND METHODS ····· 3
1. Subjects and study design ····· 3
2. In vitro platelet mixing study ····· 3
3. Platelet mtDNA isolation ····· 4
4. Extraction of mtDNA ····· 4
5. Library preparation, target enrichment, and sequencing ····· 4
6. Bioinformatic analysis ····· 5
7. Patient data analysis ····· 5
8. Statistical analysis ····· 6
III. RESULTS ····· 6
1. Linear regression analysis of expected and observed dilution ratios in platelet mixtures ····· 6
2. Application of mtDNA NGS technique to whole blood samples ····· ··13
IV. DISCUSSION ····· ··35
V. CONCLUSION ····· ··40

REFERENCES ····· ··41
ABSTRACT(IN KOREAN) ····· ··46

LIST OF FIGURES

Figure 1. Simple linearity regression testing using diluted (1:1, 1:5, 1:10, 1:20, 1:50 and 1:100) mixtures from two unrelated platelets. ……13

Figure 2. Time course of selected SNP frequencies after the administration of apheresis platelets in patients 3 (A), 4 (B), and 5 (C). ……21

Figure 3. Time course of counts for total, endogenous and transfused platelets after administration of a single apheresis platelet in patient 7. ……24

Figure 4. Time course of counts for total, endogenous and transfused platelets after administration of a total of four apheresis platelets in patient 8. ……30

Figure 5. Time course of counts for total, endogenous and transfused platelets after administration of a total of five apheresis platelets in patient 9. ……35

LIST OF TABLES

Table 1. Selected single nucleotide polymorphism markers in 7 platelet mixtures ····· 6
Table 2. Read depth of single nucleotide polymorphisms in each of ratio points of 7 platelet mixtures ····· 8
Table 3. Quantitative results of 7 mixtures with single nucleotide polymorphism markers ····· ··10
Table 4. Time course of the blood collections and platelet transfusions in 9 patients ····· ··14
Table 5. Selected single nucleotide polymorphism markers in patient 1 ····· ··15
Table 6. Quantitative results of whole blood samples from patient 1 with single nucleotide polymorphism markers ····· ··15
Table 7. Selected single nucleotide polymorphism markers in patient 2 ····· ··16
Table 8. Quantitative results of whole blood samples from patient 2 with single nucleotide polymorphism markers ····· ··16
Table 9. Selected single nucleotide polymorphism markers in patient 3 ····· ··17
Table 10. Quantitative results of whole blood samples from patient 3 with single nucleotide polymorphism markers ····· ··17

Table 11. Selected single nucleotide polymorphism markers in patient 4 ……18
Table 12. Quantitative results of whole blood samples from patient 4 with single nucleotide polymorphism markers ……19
Table 13. Selected single nucleotide polymorphism markers in patient 5 ……20
Table 14. Quantitative results of whole blood samples from patient 5 with single nucleotide polymorphism markers ……20
Table 15. Selected single nucleotide polymorphism markers in patient 6 ……22
Table 16. Quantitative results of whole blood samples from patient 6 with single nucleotide polymorphism markers ……22
Table 17. Selected single nucleotide polymorphism markers in patient 7 ……23
Table 18. Quantitative results of whole blood samples from patient 7 with single nucleotide polymorphism markers ……24
Table 19. Selected single nucleotide polymorphism markers in patient 8 ……25
Table 20. Quantitative results of whole blood samples from patient 8 with single nucleotide polymorphism markers ……27
Table 21. The calculated platelet counts of the transfused and endogeneous platelets in the whole blood samples from patient 8 ……29
Table 22. Selected single nucleotide polymorphism markers in patient 9 ……31

Table 23. Quantitative results of whole blood samples from patient 9 with single nucleotide polymorphism markers ……32
Table 24. The calculated platelet counts of the transfused and endogeneous platelets in the whole blood samples from patient 9 ……34

ABSTRACT

In vivo tracking of transfused platelets to measure platelet survival by NGS method using mitochondrial DNA

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Introduction: The measurement of recovery and survival of platelets is an important decisive factor when 'new' platelet products have been developed. Mitochondrial DNA (mtDNA) markers have been identified as potential targets for the quantitication of endogeneous and allogeneic platelets in the blood of individuals who received platelet transfusions. This study endeavors to achieve whole mitochondrial genome sequencing via next-generation sequencing (NGS) to detect polymorphisms capable of effectively distinguishing between endogenous platelets and transfused counterparts. By leveraging NGS-based techniques, we aim to evaluate a comprehensive and practical approach for assessing platelet transfusion outcomes.

Materials and methods: A total of 30 whole blood samples were collected from nine patients who had recently undergone single donor platelet apheresis, with no recent history of platelet transfusion. A series of in vitro platelet mixing experiments were conducted using ABO-identical platelet concentrates, with varying ratios (1:1, 1:5, 1:10, 1:20, 1:50 and 1:100) of mixed platelet suspensions. Mt DNA was extracted from platelet components and peripheral blood, followed by library construction and sequencing using the Novaseq platform. Bioinformatic analysis involved sequence alignment, variant calling, and selection of single nucleotide polymorphisms (SNPs) for analysis. SNPs specific to transfused platelets were identified by comparing pre- and post-transfusion sequence data, enabling platelet quantification.

Results and discussions: Fourteen platelet suspensions were used to create seven pairs. Each pair combined two suspensions in dilution ratios from 1:1 to 1:100. About 33 SNPs on average were identified as markers for each mixture. Analysis showed strong linear correlation (R^2-value $\geq$ 0.970) between expected and observed ratios for dilutions 1:1 to 1:100, with slopes near 1.0 and intercepts close to 0.0. In patient 1 to 7, one unit of apheresis platelet product was transfused in each patient. A total of 33, 32, 15, 46, 39, 7 and 23 SNP markers were selected to diffrentiate between the patient's and transfused platelets, respectively. Based on the average frequency of selected SNP markers and the total platelet count in 14 blood samples, it was possible to estimate the patient's platelet count and the transfused platelet count in each specimen. In patient 8 and 9, 4 and 5 units of apheresis platelet products were transfused, respectively. A total of 50 (14 for first, 9 for second, 16 for third and 11 for fourth transfusion) and 49 (11 for first, 5 for second, 18 for third, 3 for fourth and 12 for fifth transfusion) SNP markers were selected. Based on the average frequency of selected SNP markers and the total platelet count in 16 blood samples, it was possible to calculate the patient's platelet count and the transfused platelet count from all apheresis platelet products in each specimen.

Conclusion: In this study, we have evaluated an NGS method for sequencing the whole mitochondrial genome. This method has been employed to detect polymorphisms that enable the differentiation between endogenous platelets and transfused platelets. Our findings demonstrate the utility of this method as a valuable tool for assessing platelet survival from apheresis platelet products within patients. In cases where the platelet count does not increase as anticipated following platelet transfusion in patients with thrombocytopenia, this method is expected to helpful that discerning whether the cause originates from the patient or from the transfused platelets would be beneficial.

Key words : mitochondrial DNA, next-generation sequencing, single nucleotide polymorphism, platelet transfusion, in vivo tracking

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I. INTRODUCTION

The assessment of platelet transfusion efficacy for patients with thrombocytopenia and the evaluation of novel platelet products (such as extended storage, enhanced production automation, application of platelet additive solutions, pathogen-reduction technologies, and other modifications) significantly relies on in vivo measurements of transfused platelet recovery and survival rates. Conventionally, quantification of radioactively labeled platelets prior to transfusion has been the standard technique employed for monitoring these parameters[1-9]. The Biomedical Excellence for Safer Transfusion Collaborative has proposed a standardized procedure involving the use of indium-111 and chromium-51 to radiolabel platelets[10]. However, the administration of radioactive substances to healthy volunteers is now restricted by institutional review boards in numerous countries due to concerns regarding radiation exposure[9,11]. Moreover, the radiolabeling method is beset by technical limitations[9] and a scarcity of radionuclide supplies, necessitating the exploration of alternative measurement techniques[12]. Another approach of discriminating transfused from autologous platelets is by detecting their difference in human leukocyte antigen (HLA) phenotypes on the platelet membrane[13,14]. Clear advantages of the HLA-tracking method are that there is no need for special handling or manipulation of the platelet product prior to transfusion and platelets are not

altered in any way, in contrast to methods using radioactive labeling. Neither is there any radioactive or immunological risk for the volunteer or patient. However, this method also has evident drawbacks. Firstly, it requires the HLA typing results of the patient. Furthermore, obtaining genomic DNA for donor HLA typing analysis is challenging since all apheresis platelet products available in Korea have undergone leukocyte reduction, making it difficult to access the genomic DNA of the donors. Additionally, this method cannot be applied to patients with platelet refractoriness who have received HLA-matched platelet products.

An emerging alternative entails the identification of mitochondrial DNA (mtDNA) polymorphisms in platelets using quantitative real-time polymerase chain reaction (RT-PCR)[11] or droplet digital PCR (ddPCR)[15], enabling the monitoring of transfused platelet recovery and survival rates without the need for platelet modification. Mitochondria have a double membrane structure and use aerobic respiration to generate adenosine triphosphate, which is used throughout the cell as a source of chemical energy. An individual's mitochondrial genes are inherited only from the mother, with rare exceptions[16]. Mitochondria contain their own genome. The relative small (16,569bp), circular double-stranded mtDNA is completely sequenced in 1981[17]. An average of 4 mitochondria per platelet indicating that each mitochondrion contains a single mtDNA molecule[18]. For many years, mtDNA has been widely used for personal identification in forensic science[19]. The viability of the mtDNA markers for the purpose of monitoring platelet survival in patients has been previously discussed by Garritsen et al[20,21]. These researchers leveraged the genetic variability within the noncoding regions of the mtDNA genome to distinguish platelets from various individuals within blood samples. Through sequencing the noncoding regions of 100 platelet donors, a total of 516 base pair substitutions and 151 insertions were identified (ranging from 2 to 17 polymorphisms per donor)[22,23]. Subsequent studies developed both RT-PCR and ddPCR assays to quantitatively monitor transfused platelets using five or seven SNPs[11,15].

In a previous study[24], mtDNA sequencing by next-generation sequencing (NGS) provides

concordant results to Sanger-type sequencing. The NGS can be an attractive alternative for mtDNA sequencing and has the potential of being an efficient and sensitive method for forensic analyses. Additionally, Kim et al. demonstrated the feasibility of utilizing SNP-based NGS chimerism assays to monitor engraftment status following allogeneic hematopoietic stem cell transplantation[25]. In their investigation, the NGS assay effectively detected occult relapse by distinguishing original malignant cells from donor-derived cells through short tandem repeat analysis. Based on the studies mentioned above, it is considered that using NGS to examine mtDNA polymorphism is a promising approach for distinguishing between a patient's platelets and transfused platelets.

This study aims to sequence the whole mitochondrial genome through NGS to detect polymorphisms that can effectively distinguish between endogenous platelets and transfused platelets.

II. MATERIALS AND METHODS

1. Subjects and study design

To evaluate the platelet quantification method, we recruited patients who received single donor platelet apheresis recently, without recent history of platelet transfusion for at least the past 2 weeks. Total 9 patients were enrolled prospectively. For enrolled patients, pre- and post-transfusion peripheral blood samples were collected daily. A total of 30 whole blood samples were collected from those 9 patients. This study was reviewed and approved by the Institutional Review Board of Yonsei University Health System (IRB No. 4-2021-1670). Written informed consents were obtained from each participant enrolled in this study.

2. In vitro platelet mixing study

From two ABO-identical platelet concentrates, small amounts of platelets were

11collected and diluted with phosphate-buffered saline (PBS) to obtain platelet suspension of 100 x 10^9/L platelet. Two diluted platelet concentrates were mixed with the ratio of 1:1, 1:5, 1:10, 1:20, 1:50 and 1:100. Subsequent sequencing was performed for each platelet concentrates and mixed platelet concentrate.

3. Platelet mtDNA isolation

   Whole blood samples were collected from patients into 1 to 3 mL ethylenediaminetetraacetic acid (EDTA)-coated Vacutainer® tubes (Becton-Dickenson, NJ, USA). The collected whole blood was initially filtered through Acrodisc® WBC 25 mm PSF (Pall Corporation, NY, USA). Platelet isolation was performed using a centrifugation protocol adapted from a previous study[26]. To purify platelets, the filtered whole blood was first subjected to centrifugation at 300 x g for 5 minutes at room temperature, with the brake disengaged. The supernatant, along with the buffy coat, was then transferred to an empty sterile tube. A second centrifugation stem was carried out at 700 x g for 17 minutes at room temperature, also with the brake disengaged. After this, the upper two-thirds of the supernatant was carefully removed.

4. Extraction of mtDNA

   Extraction of mtDNA was performed using the AccuPrep® Plasmid Mini Extraction Kit (Bioneer, Republic of Korea). The extraction was carried out according to the manufacturer's guidelines, with a protocol adapted from the other previous study[27].

5. Library preparation, target enrichment, and sequencing

   Libraries compatible with the NovaSeq 6000 sequencing system were prepared from platelet mtDNA samples using the Twist Library Preparation EF 2.0 with Enzymatic Fragmentation and Twist Universal Adapter system kit, each equipped

with unique dual indices (Twist Bioscience, CA, USA). Upon successful library preparation, eight uniquely barcoded libraries were pooled in equimolar concentrations, amounting to 187.5 ng per library, to form an 8-plex hybridization reaction. Target enrichment was subsequently carried out using the Twist Target Enrichment kit along with the mtDNA panel (Twist Bioscience). The purified libraries were quantified using KAPA Library Quantification kits (Roche Diagnostics, Basel, Switzerland) according to the qPCR Quantification Protocol Guide and qualified using the Agilent Technologies 2200 TapeStation (Agilent Technologies, CA, USA). And then the paired-end (2×150 bp) sequencing was performed using the Novaseq platform (Illumina, San Diego, USA)

6. Bioinformatic analysis

Sequenced data were converted and demultiplexed with bcl2fastq. Sequencing reads were subsequently trimmed by Trimmomatic. Reads were aligned with Burrow-Wheel Aligner using mem algorithm, to human mitochondria reference sequence of Revised Cambridge Reference Sequence (NC_129920). Aligned sequences were further processed using Samtools and duplicate removal were performed using Picard. After the further process for alignments according to the GATK version 4 best practices, Mutect2 (mitochondria mode) was used to call variants of mtDNA. Only single nucleotide polymorphisms (SNPs) were selected for further analysis. Variants with heteroplasmy level of $\geq$1% were excluded from the analysis.

7. Patient data analysis

By the comparison of pre- and post-transfusion sequence data, SNP of transfused platelet which are different from those of the patient were selected for platelet quantification. For patients who received multiple platelet transfusion, SNP specific for each donor were selected. From sequencing data of daily collected

patient samples, the number of transfused platelet remains in the patient were calculated by averaging the allele frequency of unique SNP.

8. Statistical analysis

The simple linear regressions for analyzing the relations between the expected and the observed dilution in platelet mixtures diluted to ratio from 1:1 to 1:100 were performed using Analyze-it for Microsoft Excel 6.15.4. (Analyze-it Software, Leeds, United Kingdom).

III. RESULTS

1. Linear regression analysis of expected and observed dilution ratios in platelet mixtures

A total of 7 pairs were established using 14 platelet suspensions. Within each pair, two platelet suspensions were combined at dilution ratios of 1:1, 1:5, 1:10, 1:20, 1:50, and 1:100. To differentiate between the two types of platelets, an average of 33 SNPs (with a range of 26 to 37) were identified as markers in each of the 7 mixtures through pre-mixing sequencing (Table 1).

Table 1. Selected single nucleotide polymorphism markers in 7 platelet mixtures

Mixture	Number of total SNP markers	Selected SNP markers
A	31	548T, 1005T, 1824T,3645C, 4086T, 6445C, 6899A, 6962A, 7828A, 8426C, 8572G, 9053A, 9548A, 10211T, 10535T, 10586G, 10609C, 12338T, 12406A, 12645C, 12720A, 12882T, 13708G, 13759A, 16093T, 16129A, 16162G, 16172C, 16203A, 16239C, 16291C

B	35	338C, 372T, 482T, 489C, 709A, 5417G, 5601T, 6941T, 7600A, 7859G, 8701G, 9377G, 9540C, 9575A, 10400T, 10873C, 13563G, 14178T, 14200C, 14562C, 14569A, 14693A, 14783C, 14914A, 15043A, 15244A, 15301A, 16051G, 16126T, 16150T, 16223T, 16231T, 16278T, 16311T, 16362C
C	37	150C, 186T, 489C, 4386T, 4715G, 5231G, 5417G, 6179A, 6719C, 7196A, 8453G, 8584A, 8684T, 8701G, 9540C, 10398G, 10400T, 10873C, 12007G, 12358A, 12372G, 14470C, 14783C, 15043A, 15047A, 15148A, 15301A, 15355A, 15487T, 15697C, 16111C, 16129G, 16257C, 16261C, 16298C, 16311C, 16319A
D	36	151C, 199T, 334T, 489T, 3834A, 4071C, 4850C, 5417A, 5442T, 6455C, 8701A, 9278T, 9540T, 9824T, 10097G, 10400C, 10873T, 11167A, 11665C, 12091T, 12561G, 14053A, 14178C, 14693G, 14783T, 15043G, 15221A, 15301G, 15460T, 15891C, 16126C, 16223T, 16231C, 16266T, 16274G, 16295C
E	26	71G, 150C, 709G, 1118G, 3010A, 4793A, 4833A, 4883T, 5108T, 5178A, 7867C, 8200T, 8414T, 11215T, 11914G, 12414T, 14569G, 14668T, 15323G, 15448C, 15497G, 15860A, 15874G, 16209T, 16325T, 16519T
F	34	146T, 152T, 4140C, 4833G, 5108C, 5601T, 7250A, 7600A, 9377G, 9575A, 10646G, 12549C, 13135G, 13152A, 13563G, 13968A, 14200C, 14502T,

G	33	14569A, 15040C, 15071T, 15218A, 15850C, 16129G, 16148C, 16193C, 16227G, 16278T, 16302G, 16311T, 16357T, 16362C, 16497A, 16519C 146T, 152T, 204C, 207A, 235G, 663G, 709G, 1736G, 4248C, 4655A, 4824G, 5465T, 7052A, .7271A, 8563G, 8686T, 8794T, 9123G, 10238T, 11536T, 11647T, 12705T, 12909G, 13020T, 15661C, 16092T, 16217T, 16223T, 16261C, 16290T, 16317A, 16319A, 16519T

SNP: single nucleotide polymorphism

For mixture A, sequencing results for the 1:5 ratio data point were excluded from analysis due to technical dilution errors. The mean read depth of the selected SNP markers ranged from 7,064x to 25,543x, except for the four 1:100 ratio data points across four distinct mixtures (B, C, D, and E). In these instances, the mean read depth ranged from 396x to 410x (Table 2).

Table 2. Read depth of single nucleotide polymorphisms in each of ratio points of 7 platelet mixtures

Mixture and ratio	Read depth		
	Mean	Minimum	Maximum
A			
1:1	10,951x	9,315x	12,699x
1:5		NA[1]	
1:10	11,668x	10,132x	13,502x
1:20	10,413x	9,429x	12,200x
1:50	12,001x	10,703x	13,607x
1:100	12,925x	11,829x	14,798x

B			
1:1	18,886x	13,435x	21,002x
1:5	19,898x	12,765x	23,630x
1:10	16,457x	10,779x	18,802x
1:20	17,927x	11,470x	20,389x
1:50	18,525x	10,276x	21,337x
1:100	405x	352x	487x
C			
1:1	12,215x	9,811x	13,014x
1:5	9,557x	7,242x	10,420x
1:10	9,435x	7,332x	10,600x
1:20	10,419x	8,250x	11,536x
1:50	11,318x	8,924x	12,368x
1:100	410x	364x	532x
D			
1:1	12,059x	8,151x	13,324x
1:5	13,803x	9,719x	15,558x
1:10	15,599x	12,177x	17,139x
1:20	14,754x	10,820x	16,360x
1:50	15,948x	12,677x	17,070x
1:100	396x	350x	443x
E			
1:1	14,236x	11,132x	16,392x
1:5	18,839x	14,541x	21,456x
1:10	15,067x	11,728x	17,648x
1:20	16,459x	12,758x	19,018x
1:50	18,734x	14,529x	21,512x
1:100	410x	329x	583x

F			
1:1	13,765x	9,974x	15,899x
1:5	25,543x	16,944x	30,582x
1:10	17,490x	10,014x	21,125x
1:20	21,132x	13,122x	24,550x
1:50	17,675x	9,815x	21,231x
1:100	22,067x	13,298x	26,572x
G			
1:1	7,064x	4,503x	9,015x
1:5	15,574x	7,645x	18,694x
1:10	13,801x	8,503x	17,758x
1:20	23,993x	11,952x	28,400x
1:50	14,138x	8,562x	17,840x
1:100	25,009x	13,057x	30,133x

[1]Sequencing results of 1:5 ratio point were excluded and not analyzed due to technical error of dilution.

NA: not available

Quantitative analysis outcomes for each mixture (1:1, 1:5, 1:10, 1:20, 1:50, and 1:100) are presented in Table 3.

Table 3. Quantitative results of 7 mixtures with single nucleotide polymorphism markers

Mixture and ratio	Variant allele frequency[1]
A	
1:1	0.390 ± 0.036
1:5	NA[2]
1:10	0.101 ± 0.012
1:20	0.034 ± 0.006

1:50	0.016 ± 0.004
1:100	0.008 ± 0.003
B	
1:1	0.607 ± 0.015
1:5	0.284 ± 0.014
1:10	0.151 ± 0.011
1:20	0.075 ± 0.006
1:50	0.033 ± 0.002
1:100	0.023 ± 0.019
C	
1:1	0.432 ± 0.008
1:5	0.161 ± 0.005
1:10	0.077 ± 0.003
1:20	0.039 ± 0.015
1:50	0.015 ± 0.001
1:100	0.008 ± 0.007
D	
1:1	0.497 ± 0.017
1:5	0.190 ± 0.010
1:10	0.094 ± 0.005
1:20	0.048 ± 0.003
1:50	0.020 ± 0.001
1:100	0.011 ± 0.010
E	
1:1	0.497 ± 0.010
1:5	0.196 ± 0.006
1:10	0.100 ± 0.005
1:20	0.048 ± 0.002

1:50	0.020 ± 0.001
1:100	0.022 ± 0.038
F	
1:1	0.523 ± 0.025
1:5	0.184 ± 0.016
1:10	0.096 ± 0.011
1:20	0.050 ± 0.006
1:50	0.021 ± 0.003
1:100	0.013 ± 0.003
G	
1:1	0.397 ± 0.052
1:5	0.138 ± 0.019
1:10	0.066 ± 0.013
1:20	0.034 ± 0.006
1:50	0.014 ± 0.003
1:100	0.007 ± 0.002

[1]All results were presented as mean ± SD.

[2]Sequencing results of 1:5 ratio point were excluded and not analyzed due to technical error of dilution.

NA: not available, SD: standard deviation

Platelet mixtures diluted from 1:1 to 1:100 exhibited a linear correlation between the exoected and observed dilution ratios, featuring R^2-values surpassing 0.970, slopes approximating 1.0 across distinct pairs (with a range of 0.7765 to 1.196), and intercepts approximately close to 0.0 (ranging from -0.007738 to 0.02012) (Figure 1).

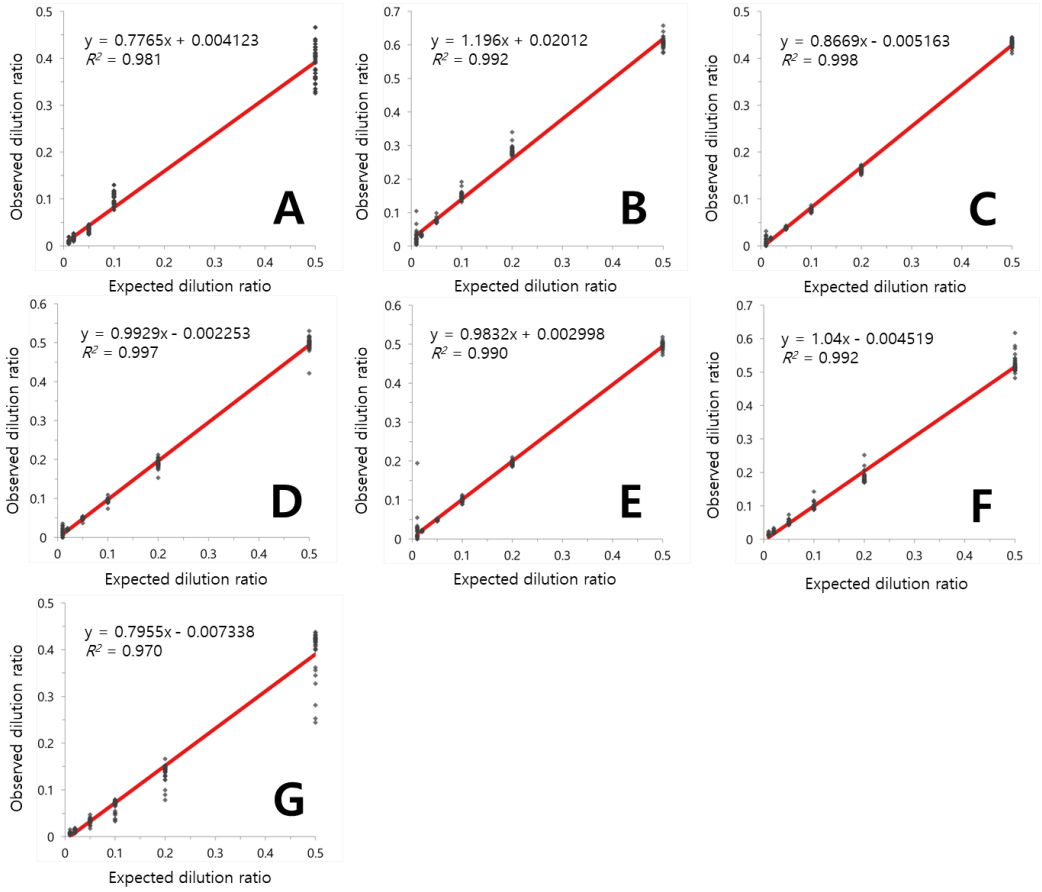

Figure 1. Simple linearity regression testing using diluted (1:1, 1:5, 1:10, 1:20, 1:50 and 1:100) mixtures from two unrelated platelets. Total of 7 platelet mixtures diluted to ratio from 1:1 to 1:100 showed linear relations between the expected and the observed dilution ratio with R^2-values above 0.97, slopes close to 1.0 for the different pairs (range, 0.7765 to 1.196) and intercepts of approximately 0.0 (range, -0.007738 to 0.02012).

2. Application of mtDNA NGS technique to whole blood samples

A total of 30 whole blood samples were collected from 9 patients who had not received platelet transfusions for at least the past 2 weeks. The time course of blood collections and platelet transfusions is summarized in Table 4.

Table 4. Time course of the blood collections and platelet transfusions in 9 patients

Patient	Time course of the blood collections and platelet transfusions
1	TF 1 > sample 1
2	TF 1 > sample 1
3	TF 1 > sample 1 > sample 2
4	TF 1 > sample 1 > sample 2
5	TF 1 > sample 1 > sample 2
6	sample 1 > TF 1 > sample 2
7	sample 1 > TF 1 > sample 2 > sample 3 > sample 4
8	sample 1 > TF 1 > sample 2 > sample 3 > TF 2 > sample 4 > sample 5 > sample 6 > TF 3 > sample 7 > TF 4 > sample 8
9	sample 1 > TF 1 > sample 2 > TF 2 > sample 3 > sample 4 > TF 3 > sample 5 > TF 4 > sample 6 > sample 7 > TF 5 > sample 8

TF: transfusion of an apheresis platelet

In the case of patient 1, an apheresis platelet was transfused on day 0, and a whole blood sample was collected on day 5. A total of 33 SNP markers were chosen to differentiate between endogenous and transfused platelets (Table 5). The mean frequency of the selected SNPs was 16.06% (ranging from 13.71% to 18.80%). Given the 5-day interval since the transfusion, it was estimated that 16.06% of the platelets in the sample originated from the platelet product. With the platelet count of 65 x 10^9/L in the sample, it is estimated that the patient's platelet count was 54.6 x 10^9/L, and the transfused platelet count was 10.4 x 10^9/L (Table 6).

Table 5. Selected single nucleotide polymorphism markers in patient 1

Number of total SNP markers	Selected SNP markers
33	58A, 143G, 152T, 827G, 4820A, 4833A, 5108T, 6086T, 6211T, 7521A, 7621T, 8701A, 9101G, 9540T, 10398A, 10400C, 10873T, 11239G, 12705C, 13590A, 14007A, 14569G, 14587G, 14766C, 14783T, 15301G, 15434T, 15497A, 15535T, 15746A, 16136C, 16175G, 16519C

SNP: single nucleotide polymorphism

Table 6. Quantitative results of whole blood samples from patient 1 with single nucleotide polymorphism markers

Selected SNP frequency (%)		Platelet count (x 10^9/L)		
Mean	Range	Total	Patient[1]	Transfused[1]
16.06	13.71 to 18.80	65	54.6	10.4

[1]Thoes platelet counts were estimated based on the total platelet count and the mean frequency of the selected SNP markers.

SNP: single nucleotide polymorphism

For patient 2, an apheresis platelet was transfused on day 0, and a whole blood sample was gathered on day 1. A total of 32 SNP markers were selected (Table 7). The mean frequency of the selected SNPs was 76.10% (ranging from 71.38% to 78.39%). Considering the patient's platelet count before and after transfusion (15 x 10^9/L and 38 x 10^9/L, respectively), it was presumed that 76.10% of the platelets in the sample originated from the transfused platelets. With the platelet count of 38 x 10^9/L in the sample collected after transfusion, it is estimated that the patient's platelet count was 9.1 x 10^9/L, and the transfused platelet count was 28.9 x 10^9/L (Table 8).

Table 7. Selected single nucleotide polymorphism markers in patient 2

Number of total SNP markers	Selected SNP markers
32	152T, 235A, 489C, 663A, 709A, 1736A, 4248T, 4824A, 5108C, 5601T, 6267A, 7600A, 8563A, 8701G, 8794C, 9377G, 9540C, 9575A, 10398G, 10400T, 10873C, 11536C, 12816C, 13563G, 13780G, 14200C, 14569A, 14783C, 15237T, 15301A, 16362C, 16519C

SNP: single nucleotide polymorphism

Table 8. Quantitative results of whole blood samples from patient 2 with single nucleotide polymorphism markers

Selected SNP frequency (%)		Platelet count (x 10^9/L)		
Mean	Range	Total	Patient[1]	Transfused[1]
76.10	71.38 to 78.39	38	9.1	28.9

[1]Thoes platelet counts were estimated based on the total platelet count and the mean frequency of the selected SNP markers.

SNP: single nucleotide polymorphism

In the case of patient 3, an apheresis platelet was transfused on day 0, and whole blood samples were taken on days 1 and 2. A total of 15 SNP markers were selected (Table 9). The mean frequencies of selected SNPs were 84.23% (range: 81.10% to 89.79%) and 81.46% (range: 76.85% to 88.33%) for blood samples collected on day 1 and 2, respectively (Table 10). Given the decreasing pattern of selected SNP frequencies over time, it was hypothesized that these SNPs were indicative of the transfused platelets (Figure 2). With the platelet count of 68 x 10^9/L in the sample collected on day 1, it is estimated that the patient's platelet count was 9.5 x 10^9/L, and the transfused platelet count was 58.5 x 10^9/L (Table

10). With the platelet count of 54 x 10^9/L in the sample collected on day 2, it is estimated that the patient's platelet count was 10.0 x 10^9/L, and the transfused platelet count was 44.0 x 10^9/L (Table 10).

Table 9. Selected single nucleotide polymorphism markers in patient 3

Number of total SNP markers	Selected SNP markers
15	3357A, 5231A, 5417A, 7217G, 7304G, 11368C, 12358G, 12372A, 12705T, 15090C, 16162G, 16223T, 16257A, 16261T, 16264T

SNP: single nucleotide polymorphism

Table 10. Quantitative results of whole blood samples from patient 3 with single nucleotide polymorphism markers

Day[1]	Selected SNP frequency (%)		Platelet count (x 10^9/L)		
	Mean	Range	Total	Patient[2]	Transfused[2]
1	84.23	81.10 to 89.79	68	9.5	58.5
2	81.46	76.85 to 88.33	54	10.0	44.0

[1]Day after transfusion

[2]Thoes platelet counts were estimated based on the total platelet count and the mean frequency of the selected SNP markers.

SNP: single nucleotide polymorphism

In the instance of patient 4, an apheresis platelet was transfused on day 0, and whole blood samples were collected on days 3 and 4. A total of 46 SNP markers were selected (Table 11).

Table 11. Selected single nucleotide polymorphism markers in patient 4

Number of total SNP markers	Selected SNP markers
46	152T, 489C, 2706A, 3010A, 3487C, 3788A, 3970C, 4538T, 4883T, 5178A, 5302T, 5585G, 6392T, 6962G, 7270C, 8158A, 8414T, 8548T, 8701G, 8764A, 9053G, 9540C, 10310G, 10398G, 10400T, 10454T, 10609T, 11255C, 11279T, 11479C, 11776T, 12406G, 12705T, 12882C, 13759G, 13928G, 13980G, 14668T, 14783C, 15043A, 15301A, 15616C, 16129G, 16223T, 16304T, 16380C

SNP: single nucleotide polymorphism

The mean frequencies of selected SNPs were 61.24% (range: 58.36% to 64.91%) and 35.30% (range: 31.67% to 39.84%) for blood samples obtained on days 3 and 4, respectively (Table 12). As the selected SNP frequency demonstrated a decreasing trend over time, it was deduced that these selected SNPs reflected the transfused platelets (Figure 2). With the platelet count of 26 x 10^9/L in the sample collected on day 3, it is estimated that the patient's platelet count was 10.1 x 10^9/L, and the transfused platelet count was 15.9 x 10^9/L (Table 12). With the platelet count of 26 x 10^9/L in the sample collected on day 4, it is estimated that the patient's platelet count was 16.8 x 10^9/L, and the transfused platelet count was 9.2 x 10^9/L (Table 12).

Table 12. Quantitative results of whole blood samples from patient 4 with single nucleotide polymorphism markers

Day[1]	Selected SNP frequency (%)		Platelet count (x 10^9/L)		
	Mean	Range	Total	Patient[2]	Transfused[2]
3	61.24	58.36 to 64.91	26	10.1	15.9
4	35.30	31.67 to 39.84	26	16.8	9.2

[1]Day after transfusion

[2]Thoes platelet counts were estimated based on the total platelet count and the mean frequency of the selected SNP markers.

SNP: single nucleotide polymorphism

In Patient 5, an apheresis platelet transfusion took place on day 0, and whole blood samples were procured on both day 0 after transfusion and day 1. A total of 39 SNP markers were selected (Table 13). The mean frequency of the selected SNPs in the blood samples obtained on day 0 and day 1 were 22.68% (range: 19.27% to 26.19%) and 13.19% (range: 11.24% to 15.46%), respectively (Table 14). Given the observable trend of decreasing frequency of the selected SNPs over time, it was inferred that these particular SNPs were representative of the transfused platelets (Figure 2). With the platelet count of 98 x 10^9/L in the sample collected on day 0, it is estimated that the patient's platelet count was 75.8 x 10^9/L, and the transfused platelet count was 22.2 x 10^9/L (Table 14). With the platelet count of 91 x 10^9/L in the sample collected on day 1, it is estimated that the patient's platelet count was 79.0 x 10^9/L, and the transfused platelet count was 12.0 x 10^9/L (Table 14).

Table 13. Selected single nucleotide polymorphism markers in patient 5

Number of total SNP markers	Selected SNP markers
39	489T, 3010G, 3206C, 3970T, 4732G, 4883C, 5147A, 5178C, 5466A, 6392C, 6962A, 7912G, 8292A, 8414C, 8473T, 8701A, 9540T, 10310A, 10398A, 10400C, 10601T, 10609C, 10873T, 10976T, 12406A, 12633T, 12705C, 12882T, 13928C, 14455T, 14476A, 14668C, 14783T, 14979T, 15043G, 15301G, 15440T, 16344T, 16362T

SNP: single nucleotide polymorphism

Table 14. Quantitative results of whole blood samples from patient 5 with single nucleotide polymorphism markers

Day[1]	Selected SNP frequency (%)		Platelet count (x 10^9/L)		
	Mean	Range	Total	Patient[2]	Transfused[2]
0	22.68	19.27 to 26.19	98	75.8	22.2
1	13.19	11.24 to 15.46	91	79.0	12.0

[1]Day after transfusion

[2]Thoes platelet counts were estimated based on the total platelet count and the mean frequency of the selected SNP markers.

SNP: single nucleotide polymorphism

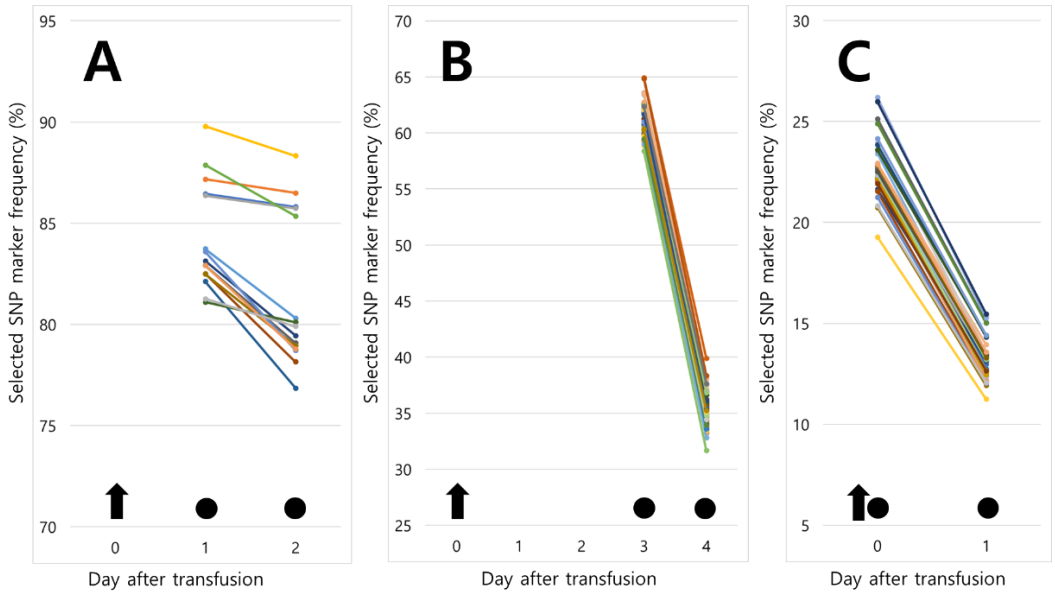

Figure 2. Time course of selected SNP frequencies after the administration of apheresis platelets in patients 3 (A), 4 (B), and 5 (C). A total of 15, 46, and 39 SNP markers were selected for these patients, respectively. The decreasing pattern observed in the mean frequencies of selected SNPs over time led to the presumption that these selected SNPs are indicative of the transfused platelets. Black arrows indicated the time of apheresis platelet transfusion, while black dots indicated the time of whole blood sample collection.
SNP: single nucleotide polymorphism

In the case of patient 6, a single unit of apheresis platelets was administered on day 0, and whole blood samples were procured both prior to the transfusion on day 0 and on day 1. Unlike the approach adopted for other patients, only 7 specific SNPs were designated as markers (Table 15). Despite the limited quantity of markers, differentiation between the transfused platelets and the patient's endogenous platelets was achievable through analysis of the sequencing data from the pre-transfusion sample. The mean frequency of SNPs exclusively identified in the blood sample obtained on day 1 was computed as 71.49% (with a range of

70.42% to 72.39%) (Table 16). The platelet count for the blood specimens taken on day 1 was measured at 35 x 10^9/L. The estimated platelet counts for the patient's platelets and the transfused platelets, as deduced from SNP marker frequencies, were 10.0 x 10^9/L and 25.0 x 10^9/L, respectively (Table 16).

Table 15. Selected single nucleotide polymorphism markers in patient 6

Number of total SNP markers	Selected SNP markers
7	2766T, 3391A, 8383T, 8419T, 9431C, 9755A, 16223C

SNP: single nucleotide polymorphism

Table 16. Quantitative results of whole blood samples from patient 6 with single nucleotide polymorphism markers

Day[1]	Selected SNP frequency (%)		Platelet count (x 10^9/L)		
	Mean	Range	Total	Patient[2]	Transfused[2]
1	71.49	70.42 to 72.39	35	10.0	25.0

[1]Day after transfusion

[2]Thoes platelet counts were estimated based on the total platelet count and the mean frequency of the selected SNP markers.

SNP: single nucleotide polymorphism

In the case of patient 7, an apheresis platelet was transfused on day 0, and whole blood samples were collected on day 0 before transfusion, day 1, 2 and 3. A total of 23 SNP markers were chosen to differentiate between endogenous and transfused platelets (Table 17).

Table 17. Selected single nucleotide polymorphism markers in patient 7

Number of total SNP markers	Selected SNP markers
23	709G, 1189T, 3010A, 3753C, 4833A, 4883T, 5108T, 5178A, 5601C, 7600G, 8414T, 9377A, 9575G, 11696A, 12372G, 13563A, 14569G, 14668T, 16093T, 16157C, 16184T, 16278C, 16311C

SNP: single nucleotide polymorphism

The mean frequency of the selected SNPs in the blood samples obtained on day 1, 2 and 3 were 46.21% (range: 44.29% to 48.91%), 41.53% (range: 37.15% to 45.39%) and 42.06% (range: 37.07% to 47.42%), respectively. The platelet count was 33 x 10^9/L in the sample collected on day 0 before transfusion. With the platelet count of 72 x 10^9/L in the sample collected on day 1, it is calculated that the patient's platelet count was 38.7 x 10^9/L, and the transfused platelet count was 33.3 x 10^9/L. With the platelet count of 58 x 10^9/L in the sample collected on day 2, it is calculated that the patient's platelet count was 33.9 x 10^9/L, and the transfused platelet count was 24.1 x 10^9/L. With the platelet count of 44 x 10^9/L in the sample collected on day 3, it is calculated that the patient's platelet count was 25.5 x 10^9/L, and the transfused platelet count was 18.5 x 10^9/L (Figure 3 and Table 18).

Table 18. Quantitative results of whole blood samples from patient 7 with single nucleotide polymorphism markers

Day[1]	Selected SNP frequency (%)		Platelet count (x 10^9/L)		
	Mean	Range	Total	Patient[2]	Transfused[2]
1	46.21	44.29 to 48.91	77	38.7	33.3
2	41.53	37.15 to 45.39	58	33.9	24.1
3	42.06	37.07 to 47.42	44	25.5	18.5

[1]Day after transfusion

[2]Thoes platelet counts were estimated based on the total platelet count and the mean frequency of the selected SNP markers.

SNP: single nucleotide polymorphism

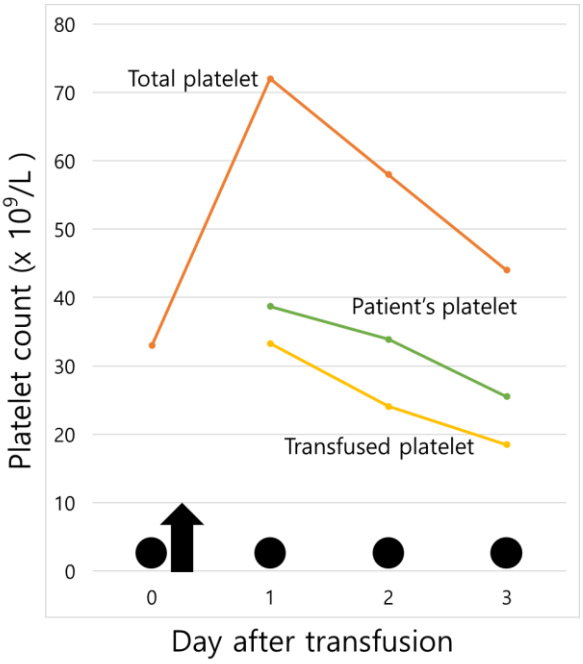

Figure 3. Time course of counts for total, endogenous and transfused platelets after administration of a single apheresis platelet in patient 7. Black arrows indicated apheresis platelet transfusion time, and black dots indicated whole blood sample collection time.

In the instance of patient 8, a total of 4 apheresis platelets were transfused, with 1 unit each on day 0, 2, 5 and 6. The whole blood samples were collected on day 0 before the first transfusion, day 1, day 2 before the second transfusion, day 3, day 4, day 5 before the third transfusion, day 6 before the fourth transfusion and day 7. A total of 50 SNP markers were selected to differentiate between endogenous and transfused platelets. In those 50 SNP markers, 14 SNP markers were selected for the first transfusion, 9 SNP markers were selected for the second transfusion, 16 SNP markers were selected for the third transfusion, and 11 SNP markers were selected for the fourth transfusion (Table 19).

Table 19. Selected single nucleotide polymorphism markers in patient 8

Platelet transfusion	Number of SNP markers	Selected SNP markers
First	14	150T, 195C, 214G, 1119C, 3497T, 5268G, 5441G, 9425C, 11329G, 12705C, 13629G, 14203C, 15346A, 15941C
Second	9	143A, 152C, 5108C, 6086C, 6221C, 7621C, 14569A, 15001C, 15746G
Third	16	7250G, 8793C, 8856A, 9932A, 10245C, 10646A, 10921C, 12549T, 13135A, 13152G, 15040T, 15071C, 15218G, 16129A, 16265C, 16274A
Fourth	11	508G, 3010A, 4883T, 5178A, 8020A, 8414T, 8648A, 13743C, 14668T, 15072G, 16249C

SNP: single nucleotide polymorphism

For the first transfusion, the mean frequencies of the selected 14 SNPs in the blood samples obtained on day 1, 2, 3, 4, 5, 6 and 7 were 43.73% (range: 38.20% to 51.42%), 39.71% (range: 33.74% to 48.18%), 16.69% (range: 13.43% to 23.19%), 12.36% (range: 5.45% to 22.22%), 11.35% (range: 8.90% to 14.84%), 6.63%

(range: 5.17% to 8.67%), and 1.91% (range: 0.90% to 2.38%), respectively. For the second transfusion, the mean frequencies of the selected 9 SNPs in the blood samples collected on day 3, 4, 5, 6 and 7 were 45.78% (range: 39.66% to 51.15%), 40.64% (range: 34.57% to 52.63%), 43.05% (range: 39.22% to 46.15%), 29.03% (range: 26.18% to 31.17%), and 12.02% (range: 9.98% to 13.90%), respectively. For the third transfusion, the mean frequencies of the selected 16 SNPs in the blood samples collected on day 6 and 7 were 12.80% (range: 6.11% to 16.57%), and 1.48% (range: 0.84% to 3.95%), respectively. For the fourth transfusion, the mean frequency of the selected 11 SNPs in the blood samples obtained on day 7 was 46.44% (range: 42.24 to 54.12) (Table 20).

Table 20. Quantitative results of whole blood samples from patient 8 with single nucleotide polymorphism markers

Day[1]	SNP frequency for 1st TF (%)	SNP frequency for 2nd TF (%)	SNP frequency for 3rd TF (%)	SNP frequency for 4th TF (%)
	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)
1	43.73 (38.20 to 51.42)			
2	39.71 (33.74 to 48.18)			
3	16.69 (13.43 to 23.19)	45.78 (39.66 to 51.15)		
4	12.36 (5.45 to 22.22)	40.64 (34.57 to 52.63)		
5	11.35 (8.90 to 14.84)	43.05 (39.22 to 46.15)		
6	6.63 (5.17 to 8.67)	29.03 (26.18 to 31.17)	12.80 (6.11 to 16.57)	
7	1.91 (0.90 to 2.38)	12.02 (9.98 to 13.90)	1.48 (0.84 to 3.95)	46.44 (42.24 to 54.12)

[1]Day after transfusion

SNP: single nucleotide polymorphism, TF: transfusion

The platelet count was 29 x 10^9/L in the sample collected on day 0 before transfusion. With the platelet count of 62 x 10^9/L in the sample collected on day 1, it is calculated that the patient's platelet count was 34.9 x 10^9/L, and the firstly transfused platelet count was 27.1 x 10^9/L. With the platelet count of 49 x 10^9/L in the sample collected on day 2, it is calculated that the patient's platelet count was 29.5 x 10^9/L, and the firstly transfused platelet count was 19.5 x 10^9/L. With the platelet count of 82 x 10^9/L in the sample collected on day 3, it is calculated that the patient's platelet count was 30.8 x 10^9/L, the firstly transfused platelet count was 13.7 x 10^9/L, and the secondly transfused platelet count was 37.5 x 10^9/L. With the platelet count of 64 x 10^9/L in the sample collected on day 4, it is calculated that the patient's platelet count was 30.1 x 10^9/L, the firstly transfused platelet count was 7.9 x 10^9/L, and the secondly transfused platelet count was 26.0 x 10^9/L. With the platelet count of 42 x 10^9/L in the sample collected on day 5, it is calculated that the patient's platelet count was 19.1 x 10^9/L, the firstly transfused platelet count was 4.8 x 10^9/L, and the secondly transfused platelet count was 18.1 x 10^9/L. With the platelet count of 35 x 10^9/L in the sample collected on day 6, it is calculated that the patient's platelet count was 18.0 x 10^9/L, the firstly transfused platelet count was 2.3 x 10^9/L, the secondly transfused platelet count was 10.2 x 10^9/L, and the thirdly transfused platelet count was 4.5 x 10^9/L. With the platelet count of 56 x 10^9/L in the sample collected on day 7, it is calculated that the patient's platelet count was 21.4 x 10^9/L, the firstly transfused platelet count was 1.1 x 10^9/L, the secondly transfused platelet count was 6.7 x 10^9/L, the thirdly transfused platelet count was 0.8 x 10^9/L, and the fourthly transfused platelet count was 26.0 x 10^9/L (Figure 4 and Table 21).

Table 21. The calculated platelet counts of the transfused and endogeneous platelets in the whole blood samples from patient 8

Day[1]	Platelet count (x 10^9/L)					
	Total	Patient[2]	1st TF[2]	2nd TF[2]	3rd TF[2]	4th TF[2]
1	62	34.9	27.1			
2	49	29.5	19.5			
3	82	30.8	13.7	37.5		
4	64	30.1	7.9	26.0		
5	42	19.1	4.8	18.1		
6	35	18.0	2.3	10.2	4.5	
7	56	21.4	1.1	6.7	0.8	26.0

[1]Day after transfusion

[2]Thoes platelet counts were estimated based on the total platelet count and the mean frequencies of the selected SNP markers.

SNP: single nucleotide polymorphism, TF: transfusion

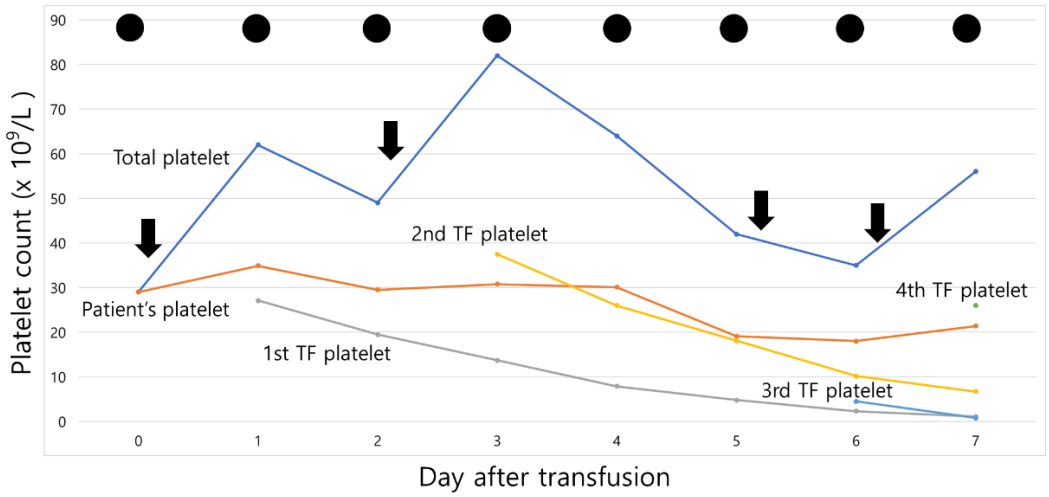

Figure 4. Time course of counts for total, endogenous and transfused platelets after administration of a total of four apheresis platelets in patient 8. Black arrows indicated apheresis platelet transfusion time, and black dots indicated whole blood sample collection time.

In the instance of patient 9, a total of 5 apheresis platelets were transfused, with 1 unit each on day 0, 1, 3, 4 and 7. The whole blood samples were collected on day 0 before the first transfusion, day 1 before the second transfusion, day 2, day 3 before the third transfusion, day 4 before the fourth transfusion, day 5, day 7 before the fifth transfusion and day 8. A total of 49 SNP markers were selected to differentiate between endogenous and transfused platelets. In those 49 SNP markers, 11 SNP markers were selected for the first transfusion, 5 SNP markers were selected for the second transfusion, 18 SNP markers were selected for the third transfusion, 3 SNP markers were selected for the fourth transfusion, and 12 SNP markers were selected for the fourth transfusion (Table 22).

Table 22. Selected single nucleotide polymorphism markers in patient 9

Platelet transfusion	Number of SNP markers	Selected SNP markers
First	11	4833G, 5108C, 5601T, 7600A, 9377G, 9575A, 12978G, 13563G, 14200T, 14569A, 16362C
Second	5	3970T, 4732G, 10398A, 10976T, 12406A
Third	18	146C. 199C. 3882A. 4071T, 4850T, 5442C, 6053T, 6455T, 7521A, 7961C, 8251A, 9824C, 11665T, 12091C, 12804C, 13269G, 14281G, 14755T
Fourth	3	93G, 16187T, 16266G
Fifth	12	153G, 1041G, 3394C, 4491A, 7861C, 9242G, 14308C, 14417G, 16234T, 16291T, 16316G, 16519T

SNP: single nucleotide polymorphism

For the first transfusion, the mean frequencies of the selected 11 SNPs in the blood samples obtained on day 1, 2, 3, 4, 5, 7 and 8 were 74.94% (range: 71.34% to 79.49%), 25.27% (range: 23.63% to 30.55%), 25.52% (range: 24.33% to 28.02%), 5.49% (range: 4.24% to 6.41%), 1.16% (range: 0.87% to 1.50%), less than 1%, and less than 1%, respectively. For the second transfusion, the mean frequencies of the selected 5 SNPs in the blood samples collected on day 2, 3, 4, 5, 7 and 8 were 68.62% (range: 66.00% to 70.94%), 67.18% (range: 64.64% to 69.83%), 15.54% (range: 14.02% to 17.67%), 3.24% (range: 2.97% to 3.48%), less than 1%, and less than 1%, respectively. For the third transfusion, the mean frequencies of the selected 18 SNPs in the blood samples collected on day 4, 5, 7 and 8 were 74.89% (range: 73.16% to 76.49%), 21.87% (range: 20.56% to 23.62%), 3.99% (range: 1.24% to 5.90%), and 1.56% (range: 0.81% to 2.01%), respectively. For the fourth transfusion, the mean frequencies of the selected 3 SNPs in the blood

samples obtained on day 5, 7 and 8 were 47.92% (range: 40.08 to 53.23), 7.86% (range: 2.73% to 10.59%), and 2.98% (range: 1.52% to 3.85%), respectively. For the fifth transfusion, the mean frequencies of the selected 12 SNPs in the blood samples obtained on day 7 and 8 were 54.28% (range: 51.81 to 62.43), and 30.23% (range: 21.02% to 54.65%), respectively (Table 23).

Table 23. Quantitative results of whole blood samples from patient 9 with single nucleotide polymorphism markers

Day[1]	SNP frequency (%)				
	1st TF Mean (Range)	2nd TF Mean (Range)	3rd TF Mean (Range)	4th TF Mean (Range)	5th TF Mean (Range)
1	74.94 71.34 to 79.49)				
2	25.57 (23.63 to 30.55)	68.62 (66.00 to 70.94)			
3	25.52 (24.33 to 28.02)	67.18 (64.64 to 69.83)			
4	5.49 (4.24 to 6.41)	15.54 (14.02 to 17.67)	74.89 (73.16 to 76.49)		
5	1.16 (0.87 to 1.50)	3.24 (2.97 to 3.48)	21.87 (20.56 to 23.62)	47.92 (40.08 to 53.23)	
7	< 1[2]	<1[2]	3.99 (1.24 to 5.90)	7.86 (2.73 to 10.59)	54.28 (51.81 to 62.43)
8	< 1[2]	<1[2]	1.56 (0.81 to 2.01)	2.98 (1.52 to 3.85)	30.23 (21.02 to 54.65)

[1]Day after transfusion

[2]Variants with heteroplasmy level of ≥1% were excluded from the analysis.

SNP: single nucleotide polymorphism, TF: transfusion

The platelet count was 12 x 10^9/L in the sample collected on day 0 before transfusion. With the platelet count of 17 x 10^9/L in the sample collected on day 1, it is calculated that the patient's platelet count was 4.3 x 10^9/L, and the firstly transfused platelet count was 12.7 x 10^9/L. With the platelet count of 24 x 10^9/L in the sample collected on day 2, it is calculated that the patient's platelet count was 1.4 x 10^9/L, the firstly transfused platelet count was 6.1 x 10^9/L and the secondly transfused platelet count was 16.5 x 10^9/L. With the platelet count of 11 x 10^9/L in the sample collected on day 3, it is calculated that the patient's platelet count was 0.8 x 10^9/L, the firstly transfused platelet count was 2.8 x 10^9/L, and the secondly transfused platelet count was 7.4 x 10^9/L. With the platelet count of 16 x 10^9/L in the sample collected on day 4, it is calculated that the patient's platelet count was 0.6 x 10^9/L, the firstly transfused platelet count was 0.9 x 10^9/L, the secondly transfused platelet count was 2.5 x 10^9/L and the thirdly transfused platelet count was 12.0 x 10^9/L. With the platelet count of 23 x 10^9/L in the sample collected on day 5, it is calculated that the patient's platelet count was 6.0 x 10^9/L, the firstly transfused platelet count was 0.3 x 10^9/L, and the secondly transfused platelet count was 0.7 x 10^9/L the thirdly transfused platelet count was 5.0 x 10^9/L and the fourthly transfused platelet count was 11.0 x 10^9/L. With the platelet count of 36 x 10^9/L in the sample collected on day 7, it is calculated that the patient's platelet count was 12.3 x 10^9/L, the thirdly transfused platelet count was 1.4 x 10^9/L, the fourthly transfused platelet count was 2.8 x 10^9/L, and the fifthly transfused platelet count was 19.5 x 10^9/L. With the platelet count of 43 x 10^9/L in the sample collected on day 8, it is calculated that the patient's platelet count was 28.0 x 10^9/L, the thirdly transfused platelet count was 0.7 x 10^9/L, the fourthly transfused platelet count was 1.3 x 10^9/L, the fifthly transfused platelet count was 13.0 x 10^9/L (Figure 5 and Table 24).

Table 24. The calculated platelet counts of the transfused and endogeneous platelets in the whole blood samples from patient 9

Day[1]	Platelet count (x 10^9/L)						
	Total	Patient[2]	1st TF[2]	2nd TF[2]	3rd TF[2]	4th TF[2]	5th TF[2]
1	17	4.3	12.7				
2	24	1.4	6.1	16.5			
3	11	0.8	2.8	7.4			
4	16	0.6	0.9	2.5	12.0		
5	23	6.0	0.3	0.7	5.0	11.0	
7	36	12.3			1.4	2.8	19.5
8	43	28.0			0.7	1.3	13.0

[1]Day after transfusion

[2]Thoes platelet counts were estimated based on the total platelet count and the mean frequencies of the selected SNP markers.

SNP: single nucleotide polymorphism, TF: transfusion

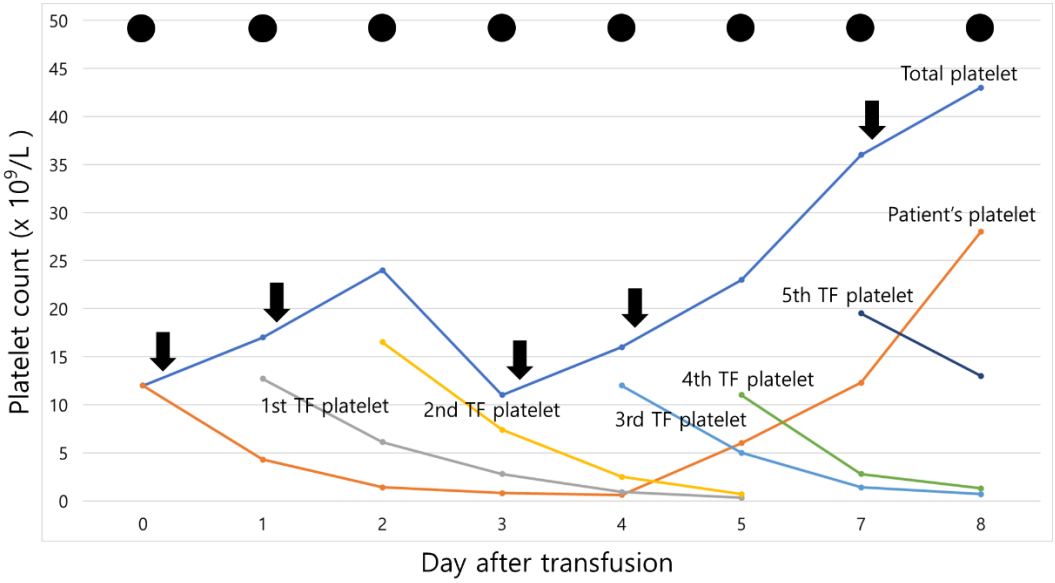

Figure 5. Time course of counts for total, endogenous and transfused platelets after administration of a total of five apheresis platelets in patient 9. Black arrows indicated apheresis platelet transfusion time, and black dots indicated whole blood sample collection time.

IV. DISCUSSION

Our findings illustrate the potential of utilizing mtDNA markers obtained through NGS for the purpose of monitoring platelet survival in patients. The viability of these mtDNA markers for such an application has been previously discussed by Garritsen et al[20,21]. These researchers leveraged the genetic variability within the noncoding regions of the mtDNA genome to distinguish platelets from various individuals within blood samples. Through sequencing the noncoding regions of 100 platelet donors, a total of 516 base pair substitutions and 151 insertions were identified (ranging from 2 to 17 polymorphisms per donor)[22,23]. Subsequent studies developed both RT-PCR and ddPCR assays to quantitatively monitor transfused platelets using five or seven SNPs[11,15]. Additionally, Kim et al. demonstrated the feasibility of utilizing SNP-based NGS chimerism assays to

monitor engraftment status following allogeneic hematopoietic stem cell transplantation[25]. In their investigation, the NGS assay effectively detected occult relapse by distinguishing original malignant cells from donor-derived cells through short tandem repeat analysis. Our current study focused on the refinement of an NGS methodology for the selection of SNP markers capable of discriminating between patients' platelets and transfused platelets. This NGS method offers distinct advantages, particularly for patients who receive multiple platelet products or undergo repeated platelet transfusions. This is due to the potential for tailored SNP marker selection based on the specific patient and transfusion circumstances. Notably, the SNP markers chosen through this NGS approach surpassed those employed in prior studies in terms of scope[11,15]. Despite the application of our NGS methodology, we identified a total of only 7 SNP markers for patient 6. Nonetheless, these markers adequately facilitated tracking and monitoring of transfused platelets. In contrast, the conventional methods utilized by previous reports were unable to achieve the same for patient 6, potentially due to the inadequacy of previously selected markers. In this study, patients 8 and 9 received multiple units of apheresis platelets for their medical treatment. However, when applying the NGS method employed in this research, it was possible to detect a sufficient number of SNP markers in each transfused platelet product, allowing for comprehensive tracking transfused platelets. Consequently, this NGS-based method demonstrated its superiority over conventional PCR-based methods, particularly in cases involving multiple units of platelet transfusions or recurrent platelet transfusions, making it a more valuable method for monitoring the effectiveness of transfused platelets in such patient populations.

In our in vitro platelet mixing study, we conducted dilutions ranging from 1:1 to 1:100 for 7 platelet mixtures. These mixtures consistently exhibited a linear relationship between expected and observed dilution ratios, characterized by R^2-values exceeding 0.97. The slopes of the regression lines were close to 1.0 for various pairs, with intercepts approximating 0.0. While the slopes displayed proximity to 1.0, they exhibited a somewhat broad range (0.7765 to 1.196). This variability could be attributed to differing

quantities of mtDNA among individuals, as the amount of mtDNA was linked to total nuclear DNA content and varied across individuals[28,29]. Nevertheless, due to the nearly 1.0 slope and robust linear relationship, our NGS method demonstrates sufficient analytical capability for the quantitative monitoring of changes in transfused platelets over time. For precise monitoring of transfused platelets, an improved level of accuracy might be achieved by analyzing a combination of pre-transfusion patient platelets and transfused platelets in a 1:1 ratio.

Among the 9 patients, blood samples collected prior to transfusion were not obtained in 5 patients. Nonetheless, our NGS method effectively distinguished between transfused platelets and endogenous platelets. For example, in patient 1, the mean frequency of selected SNPs was 16.06%, which, as the sample was obtained 5 days after platelet transfusion, likely represented the transfused platelet population. In addition, in patient 2, with a mean SNP frequency of 76.10%, the selected SNPs likely corresponded to the transfused platelets given that the sample was collected a mere day after transfusion. In patients 3, 4, and 5, who underwent single platelet transfusion with subsequent daily sample collections, demonstrated consistent SNP marker frequencies and decreasing patterns between consecutive samples. These selected markers reliably represented the transfused platelets. Notably, the assessment of recovery and survival of transfused platelets could be easily facilitated by obtaining sequencing data from samples collected prior to transfusion.

Patients 6, 7, 8, and 9 were able to obtain pre-transfusion samples prior to their first transfusion. This enabled the differentiation between the patient's own platelets and the transfused platelets within post-transfusion samples, facilitating the tracking of the transfused platelets. Particularly noteworthy, patients 8 and 9 received multiple units of platelets, yet it was possible to detect a sufficient number of SNP markers for each platelet, allowing for comprehensive tracking. While both patients exhibited a pattern of increasing platelet counts following repeated platelet transfusions, patient 8 showed minimal changes in autologous platelet count, whereas patient 9 displayed an increase in

endogenous platelet count after the day 5. Platelet transfusions are employed to supplement deficient platelet components, inducing hemostasis to treat bleeding-related disorders or prevent complications arising from hemorrhage[30]. The efficacy of platelet transfusions has traditionally been assessed using parameters such as corrected count increment (CCI), percent platelet recovery (PPR), and percent platelet increment (PPI), with CCI being the most commonly employed metric. In patients receiving recurrent platelet transfusions, the phenomenon of platelet refractoriness, characterized by an insufficient increase in platelet count following platelet transfusion, can occur[31]. Therefore, to discern the causes of platelet refractoriness and establish appropriate platelet transfusion strategies, CCI has been utilized to evaluate the effectiveness of platelet transfusions. To assess the extent of immune-mediated platelet destruction, it is common practice to analyze the CCI within 10 to 60 minutes after platelet transfusion. Even in the presence of non-immunologic factors such as splenomegaly or coagulation disorders, platelet destruction can occur, leading to a decrease in CCI. This non-immunologic reduction in platelet lifespan can be evaluated using CCI assessed within 1 to 24 hours post-transfusion. When CCI is greater than 7,500 one hour after platelet transfusion but falls below 4,500 after 24 hours, suspicion may arise regarding non-immunologic factors contributing to the reduction in platelet lifespan[32-34]. In contrast, when ABO-matched platelet transfusions result in CCI values below 5,000 to 7,500 for two consecutive assessments within 1-hour post-transfusion, suspicion arises for alloimmune platelet refractoriness[33-35]. As described above, CCI offers the advantage of rapid and clinically feasible application. However, it has certain limitations, including the inability to precisely determine the exact number of transfused platelets required for the CCI calculation and its susceptibility to fluctuations influenced by the platelet production within the patients[11]. Consequently, these limitations can affect the accurate discernment of the underlying causes of platelet refractoriness. In both patients 8 and 9, a pattern of increasing platelet counts has been observed following repeated platelet transfusions. While CCI is expected to increase in both patients, it is anticipated that the level of

interference exerted by the patients' own platelets on CCI may differ between the two individuals. By employing the NGS method utilized in this study to differentiate between the patient's own platelets and the transfused platelets and to monitor the survival of transfused platelets, it is expected that the establishment of tailored platelet transfusion strategies for each patient will lead to more effective treatments. Additionally, in cases where immune-mediated platelet refractoriness is suspected, antibodies against HLA and HPA have been identified as contributing factors[30]. In such instances, it is recommended to conduct antibody testing for HLA or HPA and subsequently utilize HLA or HPA-compatible platelet products. In Korea, only HLA-compatible platelet products are available through the Korean Red Cross[36]. However, to supply HLA-compatible platelet products, additional testing, including the patient's HLA typing, is required, incurring additional costs and time for matching the patient's HLA type with compatible platelet products. Nonetheless, it's important to note that not all cases of immune-mediated platelet refractoriness are attributed to HLA antibodies. Therefore, if the NGS method employed in this study can be used to monitor the effectiveness of platelet transfusions in patients receiving HLA-compatible platelet products, it is expected to aid in the efficient allocation of limited resources, ensuring timely and effective platelet product supply to patients in need. While not yet introduced in Korea, “new” platelet products, including extension of storage time from 5 to 7 days[2,37], storage in the cold[38] or cryopreserved platelets[1], use of platelet additive solution[39,40], and induced pluripotent stem cell (iPSC)-derived platelet products[41-44], are already in development or have been developed. The assessment of platelet recovery and survival is a critical factor when evaluating the transfusion of these 'new' platelet products. By utilizing the NGS method evaluated in this study, it is anticipated that monitoring the post-transfusion effects of these new platelet products will aid in establishing personalized platelet transfusion strategies for patients.

This study has several limitations. Firstly, it may not be feasible to routinely apply the evaluated testing method in all clinical situations. The test is more expensive and has a

longer turnaround time compared to the conventional CCI calculation method. For these reasons, it is more practical to selectively apply the test for specific clinical scenarios rather than incorporating it into routine clinical practice. Particularly in patients with platelet refractoriness, this test may prove helpful in distinguishing whether the lack of expected platelet transfusion efficacy is due to patient-derived factors or the transfused platelet products. Secondly, since this study focused exclusively on patients receiving apheresis platelet products, it remains uncertain whether the test can differentiate between patient's platelets and transfused platelets in those who receive platelet concentrates. Typically, when adults receive one platelet transfusion, they are administered six units of platelet concentrates, implying the presence of platelets derived from six different donors within the patient. While the test evaluated in this study successfully differentiated all apheresis product-derived platelets in patients receiving a total of five units of apheresis platelet products, it should be investigated whether the test can perform similarly in patients receiving six units of platelet concentrates. Furthermore, the application of this test was not examined in patients who received HLA-matched platelet products for platelet refractoriness. Despite attempts to include such patients in the study, suitable candidates could not be identified. Additional research is necessary to evaluate the test's performance in patients who have received HLA-matched platelet products. Lastly, it is also important to note that this method is not suited to determine autologous platelet survival and recovery.

V. CONCLUSION

In this study, we have evaluated an NGS method for sequencing the whole mitochondrial genome. This method has been employed to detect polymorphisms that enable the differentiation between endogenous platelets and transfused platelets. Our findings demonstrate the utility of this method as a valuable tool for assessing platelet survival from apheresis platelet products within patients. In cases where the platelet count does not increase as anticipated following platelet transfusion in patients with

thrombocytopenia, this method is expected to helpful that discerning whether the cause originates from the patient or from the transfused platelets would be beneficial.

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ABSTRACT(IN KOREAN)

미토콘드리아 DNA 차세대 염기서열 분석법을 이용한 수혈된 혈소판의 생체내 추적을 통한 혈소판 생존 측정

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서론: 혈소판 회복 및 생존율 측정은 '새로운' 혈소판 제제가 개발될 때 중요한 결정적인 요소다. 수혈을 받은 환자의 혈액 내에서 환자의 혈소판 및 수혈된 혈소판을 정량화하기 위해 미토콘드리아 DNA 표지자들의 가능성이 알려져 왔다. 본 연구는 차세대 염기서열 분석법을 통해 전체 미토콘드리아 유전체의 염기서열분석을 시도하여 환자의 혈소판과 수혈된 혈소판을 효과적으로 구별할 수 있는 단일염기다형성을 검출하고자 한다. 차세대 염기서열 분석법을 기반으로 한 검사법을 활용하여 혈소판 수혈 결과를 평가하기 위한 포괄적이고 실용적인 접근 방법을 평가하고자 하였다.
재료 및 방법: 혈소판 수혈 이력이 없는 9명의 환자로부터 총 30개의 전체 혈액 검체를 수집하였다. 먼저, 두 개의 ABO 동형 혈소판 농축액을 1:1, 1:5, 1:10, 1:20, 1:50 및 1:100의 다양한 비율로 혼합하여 시험관 내 혼합실험을 수행하였다. 혈소판 성분과 말초 혈액으로부터 미토콘드리아 DNA를 추출한 후 Novaseq 플랫폼을 사용하여 염기서열 라이브러리를 구축하고 염기서열을 분석하였다. 생물정보학적 분석은 서열 정렬, 변이 호출 및 분석을 위한 단일 염기다형성 선택을 포함하여 수행되었다. 수혈 전과 후의 염기서열 분석 결과를 비교하여 수혈된 혈소판을 특정할 수 있는 단일염기다형성을 확인함으로써 검체 내 수혈된 혈소판을 정량화하였다.
결과 및 고찰: 14개의 혈소판 농축액을 통해, 총 7쌍의 혈소판 혼합물을 실험에 이용하였다. 각 혈소판 혼합물은 1:1부터 1:100의 희석 비율로 희석되었다. 평균적으로 33개의 단일염기다형성들이 각 혼합물에 대한

표지자로 선택되었다. 분석 결과, 1:1에서 1:100의 희석 비율에 대한 예상 및 관측된 비율 사이에 강력한 선형 상관관계 (R^2-값 ≥ 0.970), 기울기가 1.0 근처에 있고 절편이 0.0 근처에 있는 것을 확인하였다. 환자 1에서 7까지, 각 환자에게 성분채혈혈소판제제 1 단위가 수혈되었다. 환자의 혈소판과 수혈된 혈소판을 구별하기 위해 총 33, 32, 15, 46, 39, 7 및 23개의 단일염기다형성 표지자들이 선택되었다. 14개의 혈액 검체에서 선택된 단일염기다형성 표지자들의 평균 빈도와 총 혈소판 수를 기반으로 각 검체에서 환자의 혈소판 수와 수혈된 혈소판 수를 계산할 수 있었다. 환자 8과 9에서 각각 성분채혈혈소판제제 4단위와 5단위가 수혈되었다. 총 50개 (첫 번째, 두 번째, 세 번째 및 네 번째 수혈에 각각 14, 9, 16, 11개) 및 49개 (첫 번째, 두 번째, 세 번째, 네 번째 및 다섯 번째 수혈에 각각 11, 5, 18, 3, 12개)의 단일염기다형성 표지자들이 선택되었다. 16개의 혈액 검체에서 선택된 단일염기다형성 표지자들의 평균 빈도와 총 혈소판 수를 기반으로 모든 성분채혈혈소판제제에서 유래된 수혈된 혈소판 수와 환자의 혈소판 수를 계산할 수 있었다.

결론: 본 연구에서는 전체 미토콘드리아 유전체를 염기서열 분석하기 위한 차세대 염기서열 분석법을 평가하였다. 이 검사법은 환자의 혈소판과 수혈된 혈소판을 구별할 수 있었고, 환자 내 혈소판 제제에서 유래된 혈소판의 생존을 평가하는 데 유용한 도구로서의 가치를 확인하였다. 이 검사법은 혈소판감소증 환자에서 혈소판 수혈 후에 기대한 대로 혈소판 수가 증가하지 않는 경우, 환자 자체에서 비롯된 원인인지 수혈된 혈소판에서 비롯된 원인인지를 구별하는 데 도움이 될 것으로 기대된다.

핵심되는 말 : 미토콘드리아 DNA, 차세대 염기서열 분석법, 단일염기다형성, 혈소판 수혈, 생체내 추적