Association of Tissue-Specific DNA Methylation Alterations with α-Thalassemia Southeast Asian Deletion

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ABSTRACT: In the wild-type allele, DNA methylation levels of 10 consecutive CpG sites adjacent to the upstream 5′-breakpoint of α-thalassemia Southeast Asian (SEA) deletion are not different between placenta and leukocytes. However, no previous study has reported the map of DNA methylation in the SEA allele. This report aims to show that the SEA mutation is associated with DNA methylation changes, resulting in differential methylation between placenta and leukocytes. Methylation-sensitive high-resolution analysis was used to compare DNA methylation among placenta, leukocytes, and unmethylated control DNA. The result indicates that the DNA methylation between placenta and leukocyte DNA is different and shows that the CpG status of both is not fully unmethylated. Mapping of individual CpG sites was performed by targeted bisulfite sequencing. The DNA methylation level of the 10 consecutive CpG sites was different between placenta and leukocyte DNA. When the 10th CpG of the mutation allele was considered as a hallmark for comparing DNA methylation level, it was totally different from the unmethylated 10th CpG of the wild-type allele. Finally, the distinct DNA methylation patterns between both DNA were extracted. In total, 24 patterns were found in leukocyte samples and 9 patterns were found in placenta samples. This report shows that the large deletion is associated with DNA methylation change. In further studies for clinical application, the distinct DNA methylation pattern might be a potential marker for detecting cell-free fetal DNA.

KEYWORDS: A-thalassemia Southeast Asian (SEA) deletion, DNA methylation, epimutation

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Introduction

The α-thalassemia Southeast Asian (SEA) deletion is a type of mutation on the α-globin gene cluster which is prevalent in Southeast Asia. The mutation deletes 4 genes and 1 pseudogene: μ-globin gene (*HBM*), pseudo α-globin gene (*HBAP1*), α-globin 2 gene (*HBA2*), α-globin 1 gene (*HBA1*), and θ-globin gene (*HBQ1*). The alternative form of this gene cluster is a recessive allele, and the homozygous form of the allele is the cause of common hydrops fetalis, which is also called hemoglobin Bart's hydrops fetalis.[1]

Apart from fetal death, which almost always occurs in utero or shortly after birth,[1–5] other critical clinical concerns are serious maternal complications during pregnancy.[6] The maternal mortality rate can approach 50% in women with a hydropic fetus who have not received adequate medical care. At present, South Asia at-risk countries such as Hong Kong, Southern China, Thailand, Taiwan, Malaysia, Singapore, Maldives, and Sri Lanka have set up prospective screening in comprehensive national prevention programs to detect couples at risk for hemoglobin Bart's hydrops fetalis.[7] When both parents carry the SEA mutation, prenatal diagnosis is offered for pregnancy management and for parents to make an informed decision.[1,7]

Standard prenatal diagnosis is an invasive technique, including chorionic villus sampling and amniocentesis, which risks miscarriage of 1.0% and 0.5%, respectively.[8] There is a 3 in 4 chance that the fetus will not have the homozygous pathogenic allele, but it is susceptible to the risk of miscarriage.

Several attempts have been made to conduct noninvasive prenatal testing (NIPT) for hemoglobin Bart's hydrops fetalis using cell-free fetal DNA (cffDNA) in the maternal circulation. The allelic dosage[9–11] and paternal marker[12,13] are 2 major lines; however, these studies were not able discriminate fetal DNA from the maternal background.[14]

This research team initially attempted to develop a new approach for hemoglobin Bart's hydrops fetalis NIPT based on 2 premises. First, cffDNA is mainly released from placental tissue,[15,16] and cell-free maternal DNA (cfmDNA) mostly originates from hematopoietic cells.[17] Second, the trophectoderm which appears in much of the placenta has relative global hypomethylation when compared with other tissues.[18–20] These 2 premises led to the suggestion that the methylation profile of the CpG sites around the SEA breakpoint junction in cffDNA

may, possibly, be different from cfmDNA, whereas previous studies indicate that the CpG sites in the wild-type allele show that there is no difference in the methylation levels between placenta and leukocyte samples (Supplementary Figure B).[21–24]

Here, the study aims to compare the methylation modification on DNA to identify a potential target for NIPT. Chorionic villus samples (CVS) were used to represent cffDNA and buffy coat to represent cfmDNA. They were compared to screen the distinction of the methylation profile of the SEA breakpoint junction region using methylation-sensitive high-resolution melting (HRM) analysis. Finally, DNA methylation patterns were extracted from the heterogeneous sample using targeted deep bisulfite sequencing. Distinct DNA methylation patterns were found which could be used as a potential target for discriminating the fetal pathogenic allele in maternal blood.

Previous studies have, so far, not indicated that the large deletion mutation can influence the alteration of DNA methylation modification around the breakpoint junction and bring distinct DNA methylation patterns between the placenta and leukocytes. This report shows that not only environmental factors[25] but also genetic mutation could play a role in epimutation.

Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki, the Belmont Report, Council for International Organizations of Medical Sciences (CIOMS) guidelines, and the International Conference on Harmonization in Good Clinical Practice. Appropriate institutional review board approval for this study was obtained from the Ethics Committee at Naresuan University (COA no. 075/2015, IRB no. 502/57). All patients provided written informed consent for the collection of the samples and subsequent analysis.

Sample collection

A total of 53 buffy coat samples from SEA carriers and 10 CVS from therapeutically aborted hemoglobin Bart's hydrops fetuses (16-26 weeks of gestation, mean and standard deviation of 21 ± 4 weeks) were obtained, which were diagnosed by the Thalassemia Unit, Human Genetics, University of Phayao. Real-time gap-polymerase chain reaction (PCR) was used to confirm the SEA mutation as previously described.[26] Only unambiguous samples were used for the study.

DNA preparation for analysis

DNA extraction. Buffy coat DNA and chorionic villus DNA were extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Bisulfite conversion. Genomic DNA (300 ng) derived from buffy coat samples and CVS was used for the bisulfite conversion reaction using an EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions and eluted in a final volume of 10 µL.

Synthesis of nonmethylated DNA control. A 203-bp mimic nonmethylated SEA breakpoint junction was commercially synthesized by the gBlocks Gene Fragments service. All cytosines in this region were substituted with thymine.

Methylation-sensitive HRM analysis

Methylation-sensitive HRM analysis was performed on a CFX96 Touch Real-Time platform (Bio-Rad, Hercules, CA, USA). Each sample was run in duplicate. Primers for analysis of the region surrounding the breakpoint junction of the SEA deletion were designed by MethPrimer software[27]; the forward primer was 5′-TTTGATTTTAATAAATGGATGAGGA-3′ and the reverse primer was 5′-ACTTAAATAATCCTC CTACCCCAAC-3′. The amplified region corresponds to nucleotides 165 227 to 184 733 on NCBI (National Center for Biotechnology Information) Reference Sequence NC 000016.10, of which a DNA segment of about 19 kb was deleted by SEA mutation from nucleotides 165 397 to 184 700 (the 2 underlined letters of the following sequence represent the breakpoint site of the deletion: 5′-GGGAGGTTCT AGCCCCTGAG-TCCCAGTTACTTGG-3′; Figure 1).

Polymerase chain reaction was performed in Bio-Rad Hard-Shell 96-well skirted PCR plates with a final reaction volume of 25 µL all containing 200 nmol/L of each primer, 200 µmol/L of each deoxynucleotide triphosphate, 2 µmol/L of SYTO9, 1.5 mmol/L of $MgCl_2$, 1 unit of Platinum DNA Polymerase in its supplied buffer (1×), and 10 ng bisulfite-modified DNA. Polymerase chain reaction was performed as follows: 1 cycle at 94°C for 2 minutes, 40 cycles at 94°C for 15 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. This was immediately followed by a hold at 95°C for 30 seconds, 50°C for 15 seconds, and an HRM step from 50°C to 95°C rising at 0.5°C per second and holding for 1 second after each stepwise increment. The data analysis was performed on Precision Melt Analysis software.

Targeted deep bisulfite sequencing

Amplicon sequencing. After the HRM process, the PCR products were purified using a QIAquick PCR Purification Kit (Qiagen). A fragment analyzer was used to confirm the band size of eluate DNA and to determine the concentration. To reduce sequencing costs, bisulfite-treated DNA from all 10 CVS and 10 randomly selected buffy coat samples were barcoded and combined into a single sequencing run. The single-end library of 200 bp (base pairs) was barcoded and constructed according to requisite instructions. The library was sequenced on an Ion Torrent Personal Genome Machine platform using chip 314 as previously described.[28]

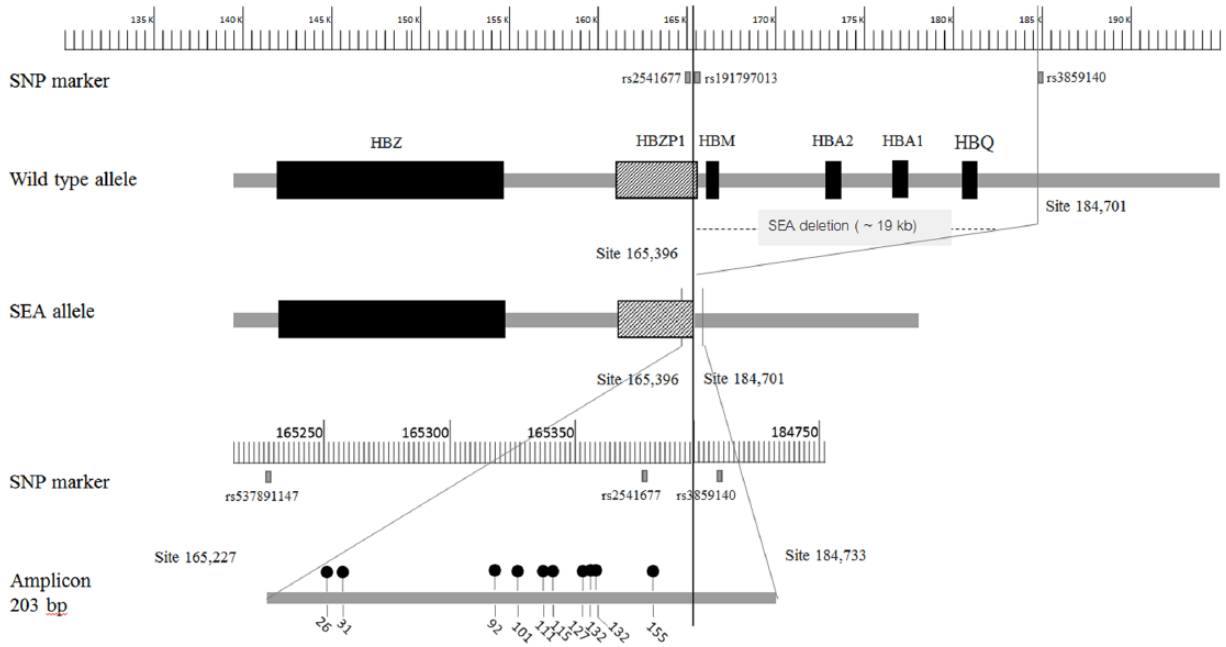

Figure 1. The designed amplicon. The region across the SEA breakpoint junction was used to design a 203-bp amplicon covering 10 CpG sites using MethPrimer Software. The figure shows the position of the CpG site on the amplicon and on chromosome 16—NC_000016. SEA indicates Southeast Asian; SNP, single-nucleotide polymorphism.

Data analysis

BAM files, the output data from amplicon sequencing, were converted to FASTA format by BAM tools (https://usegalaxy.org/). The converted data were loaded into BiQ Analyzer HT (http://biq-analyzer-ht.bioinf.mpi-inf.mpg.de/) and minimal conversion rates set at 1.0. From the results, the mean methylation levels of each sample, methylation profile of individual CpG sites, and whole DNA methylation patterns in each sample were obtained. For in-depth analysis, the DNA methylation patterns in each sample were exported in TSV format and the frequency counted on a Microsoft Excel spreadsheet. The heat map for comparing DNA methylation patterns in CVS and buffy coat was generated by RStudio.

Results

There were different DNA methylation profiles between leukocytes and CVS from the 10 CpG sites at the SEA breakpoint junction.

The length of the SEA deletion type is 19 304 bp. The deletion was mapped onto assembly genome sequence GRCh38.p7 (GenBank assembly accession: GCA_000001405.22); it showed the position of the deletion from 165 397 to 184 700, including *HBAP1, HBM, HBA2, HBA1*, and *HBQ* genes, a result of single-nucleotide polymorphism marker rs2541677 close to rs3859140. The designed primers were situated on both sides of the breakpoint and covered 10 CpG sites located between rs537891147 and rs191797013. The amplicon size was 203 bp, which is appropriate for methylation-sensitive HRM analysis and targeted deep bisulfite sequencing in downstream analysis (Figure 1).

First, we used methylation-sensitive HRM analysis to screen whether the DNA methylation status of the 10 CpG sites between leukocytes and placenta was not nonmethylated and showed a sufficiently different profile between the groups. The result showed a different melt profile curve between bisulfite-treated amplicons of the 2 sample types and similar profiles in the same sample type (Figure 2). The result implies that the 10 CpG sites have differential DNA methylation patterns. The profile curve of the nonmethylated DNA standard control showed fewer differences to the placenta group than the leukocyte group. This could imply that the DNA methylation profile of the placenta is more hypomethylated than the leukocyte group; however, the profiles of the 2 sample types were not nonmethylated.

According to confirmation results by targeted deep bisulfite sequencing, the mean DNA methylation level of the placenta was significantly lower than the leukocytes (*t* test 1-tailed $P < .0001$, Figure 2); the levels for placenta and leukocytes were 17.37% ± 3.75% and 43.85% ± 5.95%, respectively. The levels were in the range of 0% to 100%, indicating that the 10 CpG sites have distinct status and were not homogeneously marked with methylated or unmethylated status. The results, however, demonstrate that the 10 CpG sites are sufficient to distinguish between placenta samples and the leukocyte samples.

Profile of individual CpG sites

After the DNA methylation profiles of this region were compared, it could be seen that the samples were different. They were shown as not fully methylated or as nonmethylated. Mapping DNA methylation is necessary for discriminating

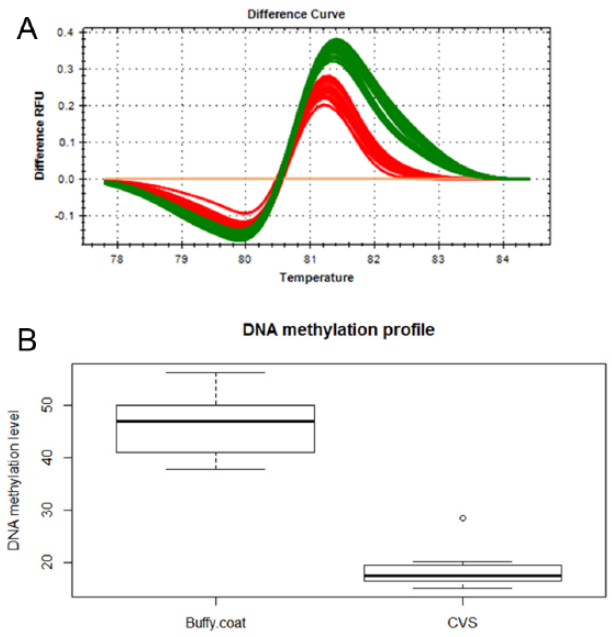

Figure 2. Comparison of DNA methylation profiles of the SEA breakpoint junction. (A) A total of 53 DNA samples from buffy coat and 10 CVS were amplified across the breakpoint region, and then high-resolution melting analysis was performed; the green and red lines show the HRM pattern of buffy coat and CVS, respectively. The yellow line served as a nonmethylated DNA control. (B) The box plot shows the different DNA methylation levels between the 2 types of sample. Statistical analysis comparing sample groups was conducted using a 1-tailed Student *t* test with unequal variance. The mean was significantly different ($P < .0001$). CVS indicates chorionic villus samples; HRM, high-resolution melting; SEA, Southeast Asian.

between the 2 samples. Targeted bisulfite deep sequencing and BiQ_Analyzer HT software were used to measure the DNA methylation level of individual CpG sites to find DNA methylation patterns.

Every sample in the same group showed a similar methylation level profile (Figure 3). When the profiles were used for data clustering by dendrogram (Figure 3A), they were clustered into 2 groups which depended on sample type. The subordinate node of each clade indicates that the profile patterns are independent of sex and age. The DNA methylation levels of individual CpG sites were, however, still in the range of 0% to 100% (Figure 3B), which implies that the methylation levels of individual CpG sites have heterogeneous appearance of methyl groups. In other words, each sample exhibits diverse DNA methylation patterns.

Comparative analysis of DNA methylation levels in individual CpG sites indicates that the 10th CpG has, significantly, the highest methylation level, in both leukocytes and placenta (Tukey test $P < .0001$, Figure 3B); the mean was 84.80% ± 3.01% and 44.20% ± 9.10%, respectively. The lowest methylation level was seen in CpG2, with a mean of 20.90% ± 4.67% and 5.70% ± 1.48%, respectively. CpG2 only showed significant methylation in leukocytes (Tukey test $P < .0001$, Figure 3B).

When the DNA methylation levels of individual CpG sites were compared across sample types, the indication was that every CpG site is significantly different, giving $P < .001$ at CpG3, whereas other CpG sites showed $P < .0001$ by *t* test (Figure 3B).

Differential DNA methylation patterns

BiQ_Analyzer HT software was used to discern the DNA methylation patterns in each sample. Quality control was performed by setting the conversion rate at 1.0 for filtering false-positive data. The exported data were compared with the same sample type to find a consensus pattern. Leukocytes had 35 consensus patterns. If fewer than 5 methylated CpG sites in the region were given as hypomethylated patterns, then 71.45% (25 patterns in 35 patterns) of patterns were hypomethylated in leukocytes. In placenta samples, all 20 consensus patterns had the addition of a methyl group on not more than 3 CpG sites (Figure 4A).

When the consensus patterns between leukocytes and placenta were compared, they were clustered into 3 groups: Group A, the 24 consensus patterns which were found only in leukocytes; Group B, the 11 consensus patterns which were found in both sample types; and Group C, the 9 consensus patterns which were found only in the placenta (Figure 4B).

The consensus pattern which had the highest signal in group A was m1111111111, significantly different from the signal of the placenta (*t* test, $P = .000142$, Figure 4B); however, the pattern with the most significant difference was m0001111111 (*t* test, $P = .000053$, Figure 4A), whereas the consensus pattern which had the highest signal in group C was m0000010000. The signal was significantly different from the leukocyte signal (*t* test, $P = .00360$, Figure 4A); however, the pattern which had the most significant difference was m0001000000 (*t* test, $P = .00213$, Figure 4A). The consensus patterns which gave the strongest signal in group B were m0000000000 and m0000000001.

Discussion

Recently, whole-genome bisulfite sequencing studies have identified the DNA methylation profile of many tissues.[22–24,29] In these tissues, the DNA methylation profiles of the α-globin gene cluster show a trend toward low methylation levels. No evidence has indicated that a large deletion mutation in the locus can change the DNA methylation profile of the rest of the CpG sites and result in distinction of the DNA methylation profile among tissues.

This study found different DNA methylation profiles of CpG sites around the SEA breakpoint junction between leukocyte and placenta samples. This could be applied as a biomarker for detecting the fetal pathogenic allele in the maternal circulation system. It may also be seen as a breakthrough in the limitations of current techniques in distinguishing the pathogenic allele of the fetus from an identical allele of the mother.[9–13,30]

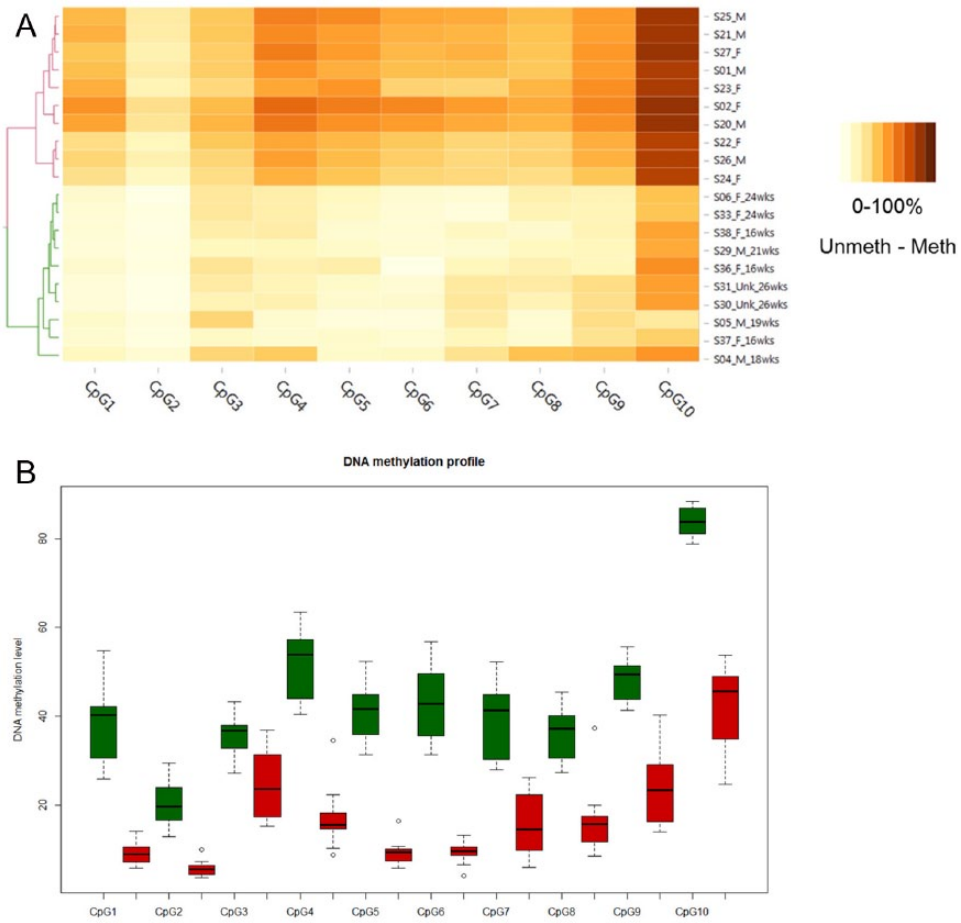

Figure 3. DNA methylation profile of individual CpG sites. (A) Heat map for DNA methylation levels on individual CpG sites. The dendrogram shows clustering data by similarity of profile into 2 groups: buffy coats (red line) and the CVS group (green line). The labels of the dendrogram branches give sample details: S and number are sample codes, F or M are sexes, and wks refers to the gestation age in weeks. (B) A box plot of the methylation levels of individual CpG sites. The green boxes are buffy coat samples, and the red boxes are CVS. CVS indicates chorionic villus samples.

In this study, due to the limitations of targeted deep bisulfite sequencing (average read length on Ion Torrent PGM platforms equals 200 bp),[28] it was necessary to design a pair of primers across the breakpoint junction. Ten CpG sites, adjacent to the 5′-breakpoint junction and located between rs537891147 and rs191797013, were used to distinguish between leukocyte and placenta DNA.

The screening step revealed that the 10 CpG sites are sufficient to discriminate between these 2 samples (Figure 2). When analyzing the DNA methylation level of individual CpG sites, to map the DNA methylation pattern on the locus, it was found that the DNA methylation profile from the 10 CpG sites is specific to the sample type. However, the DNA methylation levels were not absolutely 0% or 100% in individual CpG sites (Figure 3), indicating that the DNA methylation profile of each tissue is formed by the merged heterogeneous mixture of DNA methylation patterns. The diversity of the DNA methylation patterns might be from several cell types which are in biologically complex tissue,[31,32] such as leukocytes and placenta. Thus, all the consensus DNA methylation patterns were found, as shown in Figure 4.

To uncover the candidate target for use in NIPT, the consensus patterns of the placenta were subtracted from the consensus patterns of the leukocytes, based on the idea that the placenta is a source of cffDNA[15,33,34] and the background for the plasma DNA from leukocytes.[16,17,35] Nine candidate targets were identified, as shown in Figure 4.

Although comparing plasma DNA between a carrier mother who has a fetus with or without the α^0-thalassemia allele is a direct way to discover biomarkers, cffDNA is only 9% of the total cell-free DNA in maternal plasma during the first and second trimesters,[36,37] and it can be destroyed by the bisulfite conversion reaction and therefore give a false-negative result. This study compared the sources of cffDNA and cfmDNA, which have a high concentration of DNA. However, whether methylation analysis can distinguish cffDNA from the maternal DNA background is still unclear. This study only shows evidence of different DNA methylation between 2 sources of plasma DNA.

Another application is the consensus DNA methylation pattern, which is found only in leukocytes, and might be used to determine the contamination of CVS from maternal blood.

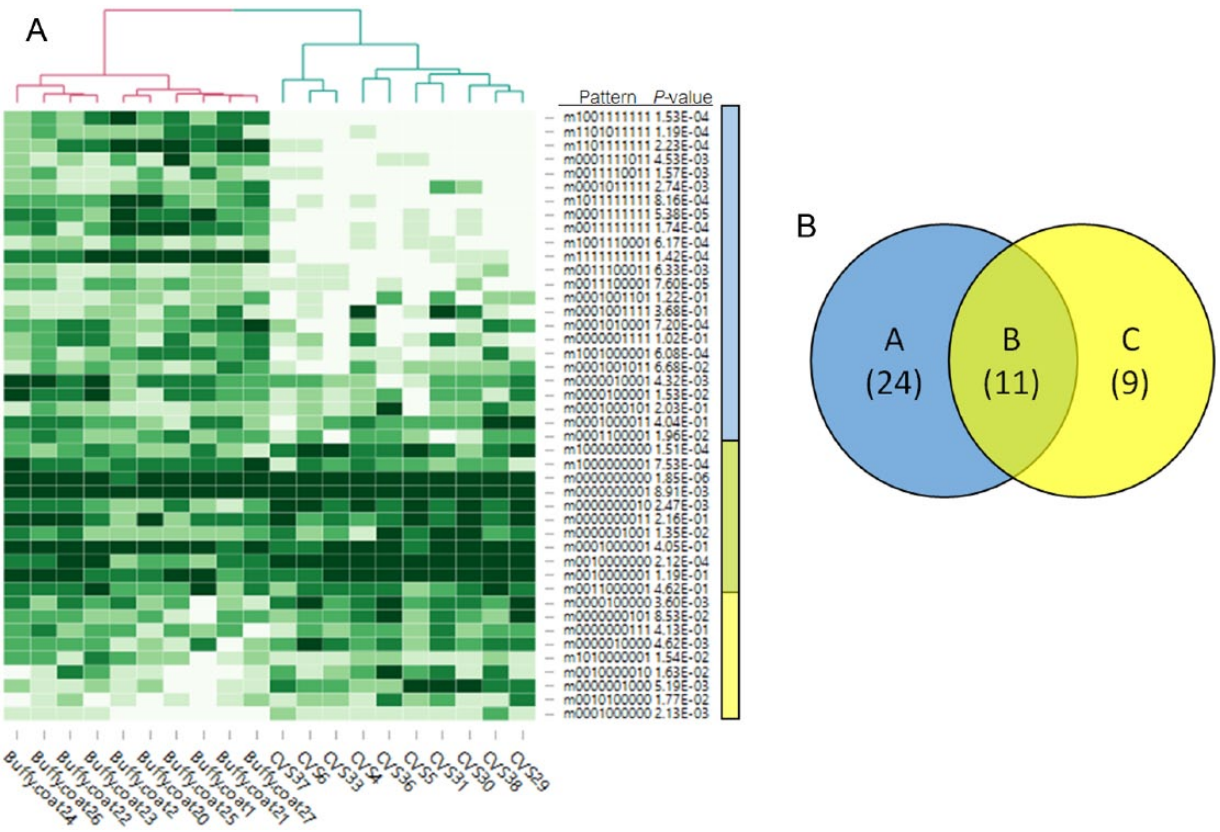

Figure 4. Comparison and classification of the DNA methylation patterns. (A) The DNA methylation pattern in buffy coats (red line) and CVS (blue line), represented by mXXXXXXXXXX; 0 or 1 is the unmethylated or methylated status of a CpG site, respectively. The consensus patterns were used to classify the data into 3 groups: the group of relative complement patterns of CVS in buffy coat (A area), the group of intersecting patterns of CVS and buffy coat (B area), and the group of relative complementary patterns of buffy coat in CVS (C area). (B) The Venn diagram shows the intersecting part of the 2 different types of sample in the green area. CVS indicates chorionic villus samples.

Another interesting point is that CpG10, adjacent to and downstream of rs2541677, had a very high methylation level in both leukocytes and the placenta when compared with the other 9 CpG sites (Figure 3A and B). The use of CpG10 as a hallmark to compare with CpG10 on the wild-type allele indicates that the DNA methylation level from this study is contrary to previous studies. The data reported on the DNA methylation track hub on the UCSC Genome Browser[29] show that DNA methylation levels in placental tissue[23,24] and leukocytes,[21,22] as well as the primary germ layer,[23] tend to nonmethylation (Supplementary Figure B). This indicates that the deletion mutation on the α-globin gene cluster affects epigenetic modification and the alteration of methylation pattern.

The alteration of DNA methylation may be consistent with the findings of previous studies that report upregulation of ζ-globin chain expression in SEA carriers.[38–41] In future, if this association could be elucidated, it could enable better understanding of ζ-globin gene regulation. It might, therefore, lead to the development of therapeutic targets for α-thalassemia by assembling ζ-globin chains with excess β-globin chains into $\zeta_2\beta_2$ (Hb Portland-2), which function normally in adult physiology.[42] Sickle cell anemia may be treated by increasing the level of Hb $\zeta_2\beta^S_2$ to greater than pathological Hb $\alpha_2\beta^S_2$ levels.[43]

In conclusion, this study compared the DNA methylation patterns of the placenta, which is a major source of cffDNA, and leukocytes, which are a major source of cfmDNA, using the 10 CpG sites adjacent to the SEA breakpoint junction. This study shows that the large deletion of α-globin gene clusters can lead to differential DNA methylation patterns between the placenta and leukocytes in the mutation locus. The 9 DNA methylation patterns found might be used as candidate biomarkers for NIPT.

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Author Contributions

TP conceived the study, conducted the molecular genetic studies, participated in the deep sequencing analysis, drafted the

manuscript, participated in the design of the study, and performed the statistical analysis. TSe, NS, MS, and WK helped to draft the manuscript. KM conducted the genotyping. PS participated in the design and the research coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Disclosures and Ethics

The material presented in this paper has not been published before nor has it been submitted for publication to another scientific journal or is being considered for publication elsewhere. This work has been approved by all co-authors.

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