Hypermethylation of the VTRNA2-1 promoter in maternal blood is associated with preterm birth

Young-Ah You
EWHA Medical Research Institute

Eun Jin Kwon
EWHA Medical Research Institute

Han-Sung Hwang
Konkuk University School of Medicine

Suk-Joo Choi
Samsung Medical Center

Sae Kyung Choi
Catholic University of Korea School of Medicine

Young Ju Kim (✉ kkyj@ewha.ac.kr)
https://orcid.org/0000-0002-3153-3008

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Abstract

**Background:** Preterm birth is associated with an increased risk of neonatal complications and death, as well as poor health and disease later in life. Epigenetics could contribute to the mechanism underlying preterm birth.

**Results:** Genome-wide DNA methylation analysis of whole blood cells from ten women was performed using an Illumina Infinium HumanMethylation450 BeadChips array. We identified 1,581 differentially methylated CpG sites in promoter regions between term and preterm birth. Although the differences were not significant after correcting for multiple tests, seven CpGs on the genomically imprinted VTRNA2-1 showed the largest differences (range: 26–39%). Pyrosequencing verification was performed with blood samples from pregnant women recruited additionally (n = 82). In total, 28 (34.1%) cases showed hypomethylation of the VTRNA2-1 promoter (< 13% methylation), while 54 cases (65.9%) showed a methylation level of 30–60%. Hypermethylation of VTRNA2-1 was associated with an increased risk of preterm birth after adjusting for maternal age, season of delivery, parity and white blood cell count. The mRNA expression of VTRNA2-1 was 0.51-fold lower in women with preterm deliveries (n = 20) compared with women with term deliveries (n = 20).

**Conclusions:** Our results suggest that changes in VTRNA2-1 methylation in maternal blood are related to preterm birth. Further studies are needed to confirm the association of VTRNA2-1 methylation with preterm birth in a large population, and to elucidate the underlying mechanism.

Background

Preterm birth (PTB) is defined as parturition before 37 weeks of gestation and approximately 15 million babies are born prematurely each year [1]. In Korea, the rate of PTB has continuously increased, from 4.8% in 2008 to 7.8% in 2018 [2]. To date, clinicians and researchers have made great efforts to improve the identification of women at risk for PTB before its occurrence, as well as to develop therapeutics for its prevention. However, the early identification of PTB and therapy to mitigate its risk remain controversial [3, 4].

PTB is associated with an increased risk of complications and neonatal death, as well as poor health and disease later in life [5–8]. Spontaneous PTB has a range of contributing risk factors, including infection, undernutrition, stress, and substance use [9], which are linked by two common pathways [10]. First, inflammatory and neuroendocrine pathways are activated in response to stress or stress-related behaviours, such as smoking [11], causing the upregulation of inflammatory cytokine production [11]. Second, cytokine-prostaglandin cascades are activated in response to infection [10]. Due to the involvement of inflammatory mechanisms in both pathways, previous studies investigating potential biomarkers for PTB have focused on inflammatory mediators [12, 13].

Genome-wide DNA methylation analysis may provide information on the mechanism underlying PTB and represents a new approach for biomarker discovery [14-16]. DNA methylation in the blood may change
according to conditions such as inflammation, and multiple studies have shown that differential DNA methylation is related to smoking status, as well as to obesity and various other diseases [17]. Moreover, the ability to assess the epigenome has resulted in the identification of epigenetic signatures of the intrauterine environment, which are affected by smoking, stress, nutrition, body mass index (BMI), and medication use over several years [18–22]. Many studies have suggested that differentially methylated genes are involved in the genetic and environmental contributions to PTB and chronic disease risk. DNA methylation changes in the amnion or foetal tissue were determined to be partially involved in the physiological process of PTB and in foetal development [23, 24]. Thus, the evaluation of genes containing these differentially methylated sites may be useful to identify biological pathways involved in PTB, thereby facilitating the identification of clinically informative biomarkers for the prediction of PTB.

In this study, we investigated the DNA methylation profiles of maternal blood collected from pregnant women with term and PTBs. We also analysed the epigenetic modifications to determine the underlying mechanism of PTB.

**Results**

**Methylation array in the peripheral blood of women delivering at term and preterm**

A genome-wide analysis of DNA methylation was performed to search for candidate differentially methylated genes in maternal blood at the time of delivery. A total of 87,507 CpG sites were tested on the CpG island promotors (except SNP-affected CpG sites) and the results showed that 1,581 sites had significantly different levels of methylation ($p < 0.05$) between term and preterm groups (Table S1). Although we found no differential methylation (DM) of these CpGs after correcting for multiple testing, seven VTRNA2-1 CpG sites in the promoter showed the largest differences (26–39%) between PTB and term samples ($p < 0.05$) (Table 1). In addition, these seven CpG sites were all hypermethylated (range: 25.0–55.1%, $n = 5$) or hypomethylated (range: 2.6–14.1%, $n = 5$) in every sample (Figure S1).

**Validation of VTRNA2-1 methylation**

Subsequently, we performed pyrosequencing to investigate the association between VTRNA2-1 methylation (cg04481923) and PTB in maternal blood samples (term, $n = 39$; preterm, $n = 43$). The primer sets for the VTRNA2-1 CpG sites were designed using PSQ Assay Design software (Biotage AB, Uppsala, Sweden). Table 2 shows the clinical characteristics of the 82 pregnant women. Maternal age ranged from 27–39 years in women with term deliveries, and from 22–43 years in women with PTB. The mean gestational age and BMI of women with term births were 39 weeks, 4 days and 26.0, respectively, compared to 29 weeks, 1 day and 23.8, respectively, in women with PTB. The incidence of C-section did not differ significantly between the term and preterm groups according to the chi-square test ($p > 0.05$).

Table 3 shows the levels of DNA methylation of three CpG sites on VTRNA2-1 following pyrosequencing; two CpG sites differed significantly between pregnant women with term births and PTBs ($p < 0.05$). These three CpG sites on VTRNA2-1, identified in all samples, were concordant with hypomethylation (range: 0–13%, $n = 28$) or hypermethylation (range: 30–60%, $n = 54$) (Figure 1a). In addition, women who delivered
Preterm infants were more likely to exhibit hypermethylation (> 30%) of VTRNA2-1 than women who delivered at term. To explore the association between methylation status and mRNA expression, the mRNA expression of VTRNA2-1 in maternal blood was analysed. The relative expression level of VTRNA2-1 was 0.51-fold lower ($p < 0.05$) in PTB women ($n = 20$) compared to those with term deliveries ($n = 20$) (Figure 1b).

**Preterm birth-related DNA methylation changes in maternal blood**

Table 4 shows the association between DNA methylation sites and PTB, as determined by logistic regression analysis. We analysed the association between DNA methylation changes in VTRNA2-1 and PTB. In total, 28 (34.1%) cases showed hypomethylation of the VTRNA2-1 promoter (< 13% methylation by pyrosequencing), while 54 cases (65.9%) showed a methylation level of 30–60%. Based on these results, the patients were divided into hypomethylation and hypermethylation groups. Hypermethylation of VTRNA2-1 was associated with a significantly increased risk of PTB compared with hypomethylation of VTRNA2-1 (adjusted OR = 3.358, 95% CI 1.114–10.126). Interestingly, younger women in the VTRNA2-1 hypomethylation group were more likely to have preterm deliveries ($p < 0.05$, Table 5). Moreover, women with preterm deliveries in the hypermethylation group had lower BMIs and higher WBC counts and were also more likely to deliver during spring or summer than autumn or winter ($p < 0.05$).

**Discussion**

We examined PTB-related DNA methylation changes through genome-wide methylation analysis of maternal blood. A total of 1,581 differentially methylated CpG sites in promoter regions were identified and seven CpG sites of the genomically imprinted gene VTRNA2-1 showed the largest differences in methylation between term and preterm blood samples. In addition, VTRNA2-1 hypermethylation was associated with an increased risk of PTB (determined by pyrosequencing) after adjusting for maternal age, parity, season of delivery and WBC count, and was negatively correlated with gene expression. This result sheds light on the mechanism underlying PTB.

PTB remains the leading cause of childhood morbidity and death. Its aetiology remains unclear; however, significant advances have been made in the identification of biomarkers to predict high-risk pregnancies resulting in PTB. Changes in DNA methylation caused by environmental factors regulate gene transcription and can play a role in a variety of diseases [25]. Studies have suggested that maternal factors, including socioeconomic status [11], pre-pregnancy BMI [26], smoking during pregnancy [27] and nutrition status [28] affect the risk of PTB. DNA methylation partially explains the effects many of these factors [19–24, 26-28]. In this study, we found associations of maternal BMI, delivery season and WBC count with PTB; however, these factors did not influence the DNA methylation levels of VTRNA2-1 in maternal blood.

VTRNA2-1, also known as nc886 and miR886, is an environmentally responsive epiallele and its expression is regulated by epigenetic silencing (in turn caused by promoter methylation) [29]. Levels of VTRNA2-1 methylation are similar among normal mature B cells, T cells and granulocytes [30].
addition, VTRNA2-1 is reported to have DM sites in various regions and is associated with several diseases [30, 31]. Although few studies have investigated the relationship between VTRNA2-1 and PTB, its hypermethylation is associated with poor outcomes in patients with acute myeloid leukaemia and small cell lung cancer [30, 31].

VTRNA2-1 is a putative tumour suppressor and modulator of innate immunity [31]. VTRNA2-1 expression is suppressed in clinical tumour samples compared to normal tissues; more importantly, low expression of VTRNA2-1 is associated with poor survival. In addition, inhibition of VTRNA2-1 leads to activation of the cellular antiviral response pathways involving protein kinase-R (PKR) [32-34]. PKR activation can be induced by various stressful stimuli, such as cytotoxic cytokines, growth factor deprivation and DNA damage [34]. Our results showed that expression of VTRNA2-1 was negatively correlates with methylation level, and that VTRNA2-1 hypermethylation was associated with PTB. Therefore, our results suggest that VTRNA2-1 hypermethylation could cause PTB due to PKR activation in response to stressful stimuli.

A study conducted in rural Gambia reported that the methylation level of VTRNA2-1 was associated with the season of conception and maternal nutrition [28]. Specifically, the authors reported that the nutrition status of mothers during pre-conception differed between the dry and rainy seasons. However, although our VTRNA2-1 methylation data were associated with the season of delivery, it is difficult to explain the relationship between PTB and methylation changes by season. Another study reported that alterations in DNA methylation result from inflammatory processes involving high circulating levels of C-reactive protein (CRP) or other inflammatory proteins [35, 36]. In this study, the level of VTRNA2-1 methylation was correlated with the circulating levels of CRP, which were correlated with the WBC count (data now shown). Therefore, our study suggests that VTRNA2-1 hypermethylation can induce PTB in response to PKR activation by inflammation.

We acknowledge several limitations of our study, including the paucity of clinical and demographic data, for example on smoking status, alcohol consumption, and the use of other drugs or medications. We were only able to demonstrate a correlation between methylation and expression of VTRNA2-1 in a small subset of CpG sites; moreover, the sample size was small due to the limited availability of RNA from maternal blood samples. Finally, when we analysed the VTRNA2-1 methylation level using a simple linear regression model that included the cell composition percentages as covariates, the VTRNA2-1 methylation level was not affected by cell composition ($p > 0.05$). However, we did not analyse DNA methylation status according to cellular heterogeneity, because we did not sort the samples by blood cell type at the time of collection.

**Conclusions**

This study suggests that VTRNA2-1 methylation, identified through genome-wide DNA methylation analysis of blood cells, may be associated with PTB. Interestingly, our results suggest an association of PTB with VTRNA2-1 methylation in response to PKR activation by inflammation. Further studies are
required to confirm DNA methylation changes of VTRNA2-1 in a large population, and to better understand the change of VTRNA2-1 methylation in response to PKR activation by inflammation.

**Methods**

**Study population**

We conducted a case-control study of 10 pregnant women with term (n = 5) and preterm deliveries (n = 5) at Ewha Womans University Mokdong Hospital (Seoul, Korea) to screen changes in methylation level. Maternal peripheral blood samples from participants were collected at the time of delivery, and the birth outcome was followed (Table S2). DNA methylation was measured using the Illumina Human Methylation 450 BeadChip. To validate the DM levels, 82 blood samples from women with term (n = 39) and preterm (n = 43) births were examined. All participants gave informed consent, and the study was approved by the Institutional Review Board of Ewha Womans University Mokdong Hospital (Certificate No. EUMC 2014-06-010-003, Samsung Medical Center (SMC 2014-06-094-003), Konkuk University Medical Center (KUH1040034), and Seoul St. Mary’s Hospital (KC14TIMI0591). Women with multiple births, major birth defects, and pregnancy complications were excluded. Gestational age was determined using the first day of the last menstrual period and ultrasound examination.

**DNA preparation and genome-wide DNA methylation analysis**

Maternal blood was collected in EDTA tubes, and the plasma was separated and stored at -80°C. Genomic DNA was extracted from blood samples using the QIAGEN Mini Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's protocol. The quality of the extracted DNA was evaluated using agarose gel electrophoresis. To analyse DNA methylation, ~700 ng genomic DNA was bisulphite-converted using the Zymo EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA), amplified, fragmented, and hybridised to the Illumina Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA, USA) following the manufacturer’s protocol. After washing, the BeadChips were scanned with the HiScan SQ System (Illumina). Scanned images were processed to determine the signal intensity and β-values were calculated using Genome Studio software (Illumina). The β-value, as defined below, was used to measure methylation levels on a scale from 0 to 1:

\[
\text{β-value} = \frac{\text{Max (Signal A,0) } - \text{Max (Signal B,0)}}{\text{Max (Signal A,0) } + \text{Max (Signal B,0) + 100}}
\]

Max (Signal A,0) indicates the signal intensity of the unmethylated allele, and Max (Signal B,0) indicates the signal intensity of the methylated allele. A constant bias of 100 was added to regularise the β-value. The β-values were calculated; normalisation, filtration, and statistical analyses were performed using GeneSpring ver. 7.3 (Agilent Technologies, Santa Clara, CA, USA). The normalised β-value of all CpG sites in the two groups (term vs. preterm) were statistically evaluated using Welch’s t-test (p < 0.05). We
accounted for multiple testing by controlling for the false discovery rate (FDR). The FDR was controlled using the Benjamini–Hochberg correction (q <0.05).

**DM analysis by pyrosequencing**

DM levels measured by the genome-wide methylation array were validated in maternal term (n = 39) and preterm (n = 43) blood by pyrosequencing. The cg04481923 site was amplified using a primer set designed using PSQ Assay Design software (Biotage AB, Uppsala, Sweden) (Table S3). Genomic DNA was bisulphite-converted according to the manufacturer's instructions with an EZ DNA Methylation Kit (ZYMO Research, Irvine, CA, USA). An EpiTect PCR Control DNA Set (Qiagen) was used as a methylated/unmethylated control. The percentage of methylated cells in each region was quantified using the PyroMark ID pyrosequencer (Qiagen) and Pyro Q-CpG Software (Figure S2). The software incorporates controls to check for completed bisulphite conversions, and provides an adequate signal over background noise. All samples were run in duplicate and average values were calculated. The details of the pyrosequencing methodology have previously been reported [37].

**RNA isolation and quantitative real-time polymerase chain reaction**

Total RNA from maternal blood (n = 40) was extracted using the Easy-BLUE™ Kit (iNtRON Biotechnology, Sungnam, Korea) according to the manufacturer’s instructions. RNA was reverse transcribed using 1 µg total RNA in a 25 µL reaction mixture containing 1 µL 10 pM oligonucleotide primer, 5 µL 10× reverse transcription buffer, 5 µL 2.5 mM dNTPs, 1 µL 20 U RNase inhibitor, and 1 µL 200 U Moloney murine leukaemia virus reverse transcriptase (M-MLV RT) (Promega, Madison, WI, USA) for 60 min at 42°C. Real-time quantitative-polymerase chain reaction (qPCR) was performed using synthesised cDNA as a template, gene-specific primers (VTRNA2-1), and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, USA). The reactions (including the no-template controls) were run in duplicate on the ABI PRISM 7000 sequence detection system (Applied BioSystems) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference for normalisation of target gene mRNA expression. The PCR conditions were as follows: denaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. We tested primer specificity by RT-PCR and confirmed it using melting (dissociation) curve analysis. Comparative quantification of each target gene was performed based on the cycle threshold (CT), which was normalised against the CT of GAPDH using the ΔΔCT method. Data are presented as the fold change between groups as the mean ± standard error of the mean (SEM). The primer sets and melting temperature (Tm) for qPCR are described in Table S4.

**Statistical analysis**

The basic characteristics of the study groups were compared using Student’s t-test for continuous variables and the chi-square test for categorical variables. After pyrosequencing, the DNA methylation levels between the two groups were compared using the Mann–Whitney U-test. The DNA methylation
levels of VTRNA2-1 were analysed as two separate groups: hypomethylation (< 13%) and hypermethylation (30–60%) groups by the rank of methylation level. To explore the association between VTRNA2-1 methylation level and PTB, multiple logistic regression was conducted, controlling for maternal age, parity, season, and white blood cell (WBC) count. In addition, the clinical characteristics of the VTRNA2-1 hypo- and hypermethylation groups were analysed using Student’s t-test and the chi-square test. All analyses were two-tailed, and a p-value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS software ver. 21.0 (IBM, Armonk, NY, USA).

**Declarations**

**Ethics approval and consent to participate**

All participants provided informed consent and the study was approved by the Institutional Review Board of Ewha Womans University Mokdong Hospital (Certificate No. EUMC 2014-06-010-003, Samsung Medical Center (SMC 2014-06-094-003), Konkuk University Medical Center (KUH1040034), and Seoul St. Mary’s Hospital (KC14TIMI0591).

**Consent for publication**

Not applicable.

**Availability of data and materials**

**Competing interests**

The authors declare that they have no competing interest.

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**Author’s Contribution**

YAY and KYJ designed the study and wrote the manuscript. YAY and KEJ performed the experiments. YAY, KEJ, and KYJ performed the research and analysed the data. HHS, CSJ, and CSK participated in data analysis. All authors interpreted and discussed the data, reviewed and revised the manuscript, and approved the final version of the manuscript.

**Abbreviations**

VTRNA2-1, vault RNA 2-1

PTB, preterm birth
BMI, body mass index
PKR, protein kinase-R
CRP, C-reactive protein
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
CT, cycle threshold
SEM, standard error of the mean

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Tables

Table 1. The seven CpGs sites of VTRNA2-1 with the largest differences in methylation between term and preterm samples (n = 10).
| Target ID† | Gene symbol | Gene ID‡ | CHR | Functional location | Mean β value in term samples (n = 5) | Mean β value in preterm samples (n = 5) | Difference* | p-valuea | q-valueb |
|-----------|-------------|----------|-----|----------------------|--------------------------------------|----------------------------------------|-------------|----------|----------|
| cg26328633 | VTRNA2-1    | 100126299 | 5   | TSS200               | 0.125                                | 0.520                                  | 0.395       | 0.014    | 0.050    |
| cg25340688 | VTRNA2-1    | 100126299 | 5   | TSS200               | 0.121                                | 0.508                                  | 0.387       | 0.014    | 0.050    |
| cg06536614 | VTRNA2-1    | 100126299 | 5   | TSS200               | 0.129                                | 0.492                                  | 0.363       | 0.020    | 0.050    |
| cg00124993 | VTRNA2-1    | 100126299 | 5   | TSS200               | 0.114                                | 0.471                                  | 0.357       | 0.014    | 0.050    |
| cg26896946 | VTRNA2-1    | 100126299 | 5   | TSS200               | 0.192                                | 0.503                                  | 0.311       | 0.013    | 0.050    |
| cg04481923 | VTRNA2-1    | 100126299 | 5   | 1stExon;3'UTR        | 0.122                                | 0.389                                  | 0.267       | 0.015    | 0.050    |
| cg18678645 | VTRNA2-1    | 100126299 | 5   | TSS200               | 0.115                                | 0.376                                  | 0.261       | 0.051    | 0.050    |

†Name of the probe. ‡GenBank gene ID was updated in May 2016. *Difference between mean β values of preterm births and term births.

aMann–Whitney U-test, p < 0.05. bThe false discovery rate (FDR) was controlled using the Benjamini–Hochberg correction (q < 0.05). CHR, Chromosome

Table 2. Clinical characteristics of the study groups (n = 82).
|                        | Term (≥ 37, n = 39) | Preterm (< 37, n = 43) | p-value |
|------------------------|---------------------|------------------------|---------|
| Maternal age           | 31.7 ± 2.8          | 30.9 ± 4.6             | 0.418   |
| BMI at delivery        | 25.9 ± 3.3          | 24.1 ± 4.2             | 0.037³  |
| Gravidity              |                     |                        | 0.471*  |
| 0                      | 16 (41.0)           | 15 (34.9)              |         |
| 1                      | 23 (59.0)           | 28 (65.1)              |         |
| Parity                 |                     |                        | 0.118*  |
| Nulliparous            | 22 (56.4)           | 22 (51.2)              |         |
| Multiparous            | 17 (43.6)           | 21 (48.8)              |         |
| Delivery season        |                     |                        | 0.021*  |
| Spring, n (%)          | 5 (12.8)            | 12 (27.9)              |         |
| Summer, n (%)          | 3 (7.7)             | 11 (25.6)              |         |
| Autumn, n (%)          | 19 (48.7)           | 13 (30.2)              |         |
| Winter, n (%)          | 12 (30.8)           | 7 (16.3)               |         |
| Mode of delivery       |                     |                        | 0.599*  |
| Vaginal, n (%)         | 24 (61.5)           | 24 (55.8)              |         |
| C-section, n (%)       | 15 (38.5)           | 19 (44.2)              |         |
| Education (n = 68)     |                     |                        | 0.096*  |
| Below high school      | 8 (20.5)            | 13 (44.8)              |         |
| College or more        | 31 (79.5)           | 16 (55.2)              |         |
| Gestational age        | 39.4 ± 1.0          | 29.1 ± 2.8             | <0.001* |
| White blood cell, (cells/µL) | 10101.8 ± 3004.8  | 12209.5 ± 3825.8      | 0.008   |
| Mycoplasma             |                     |                        | 0.702*  |
| Positive, n (%)        | 2 (5.4)             | 3 (8.6)                |         |
| Negative, n (%)        | 35 (94.6)           | 32 (91.4)              |         |
| Ureaplasma (n = 72)    |                     |                        | <0.001* |
| Positive, n (%)        | 0 (0.0)             | 13 (37.1)              |         |
| Negative, n (%)        | 37 (100.0)          | 22 (62.9)              |         |
| Chorioamnionitis (n = 67)|                     |                        | <0.001* |
| Positive, n (%)        | 0 (5.1)             | 17 (56.7)              |         |
| Negative, n (%)        | 37 (100)            | 13 (43.3)              |         |
| Birth weight           | 3310.5 ± 394.7      | 1385.7 ± 504.1         | <0.001  |
| Sex                    |                     |                        | 0.654*  |
| Male, n (%)            | 20 (51.3)           | 24 (55.8)              |         |
| Female, n (%)          | 19 (48.7)           | 19 (44.2)              |         |
| Apgar 1 min            | 9.5 ± 0.8           | 5.9 ± 2.4              | <0.001  |
| Apgar 5 min            | 9.9 ± 0.2           | 7.5 ± 2.1              | <0.001  |

BMI, body mass index. ³Student’s t-test, p < 0.05; *χ² test, p < 0.05. Data are shown as the mean ± SD for continuous variables and as n (%) for categorical data.

Table 3. Comparison of specific DNA methylation sites between women with term and preterm births after pyrosequencing.
### Table 4. Logistic regression analysis of the association between specific DNA methylation sites and preterm births (n = 82).

| Variable          | β-value | SE     | Odds ratio | 95% CI         | p-value |
|-------------------|---------|--------|------------|----------------|---------|
| Age               | -0.043  | 0.035  | 0.958      | 0.894-1.027    | 0.227   |
| Season            | -0.521  | 0.234  | 0.594      | 0.375-0.941    | 0.026   |
| Parity            | 0.353   | 0.315  | 1.423      | 0.767-2.639    | 0.263   |
| VTRNA2-1*         | 1.173   | 0.557  | 3.232      | 1.085-9.633    | 0.035   |
| WBC count         | 0.000   | 0.000  | 1.000      | 1.000-1.000    | 0.026   |

β-values and SE estimated using the multivariable logistic regression model. * VTRNA2-1 had three CG positions and the methylation levels of VTRNA2-1 were grouped as hypomethylation (< 13%) and hypermethylation (30–60%) methylation. VTRNA2-1, vault RNA 2-1.

### Table 5. Clinical characteristics of VTRNA2-1 hypomethylation and hypermethylation groups (n = 82).

| Variable       | Target ID† of differentially methylated site | Term (n = 39) | Preterm (n = 43) | p-value |
|----------------|---------------------------------------------|---------------|------------------|---------|
| VTRNA2-1_pos1  | cg04481923                                  | 25.8 ± 20.9   | 33.9 ± 18.6      | 0.065   |
| VTRNA2-1_pos2  |                                             | 26.3 ± 22.2   | 35.2 ± 19.5      | 0.045a  |
| VTRNA2-1_pos3  |                                             | 27.4 ± 22.3   | 36.4 ± 19.8      | 0.026a  |

†Name of the probe. VTRNA2-1, vault RNA 2-1. a Mann–Whitney U-test, p < 0.05. Data are presented as the mean ± SD.
|                            | Hypomethylation† | p-value | Hypermethylation‡ | p-value |
|-----------------------------|------------------|---------|-------------------|---------|
|                             | Term (n = 17)    | Preterm (n = 11) | Term (n = 22)    | Preterm (n = 32) |
| Maternal age                | 31.7 ± 2.7       | 28.2 ± 2.5 | 31.7 ± 3.1       | 31.9 ± 4.7 | 0.812 |
| BMI                         | 26.3 ± 3.3       | 24.5 ± 5.4 | 25.8 ± 3.3       | 23.5 ± 3.4 | 0.024 |
| Gravidity*                  | 0.954            |          | 0.412            |          |
| 0                           | 6 (35.3)         | 4 (36.4) | 10 (45.5)        | 11 (34.4) |
| 1                           | 11 (64.7)        | 7 (63.6) | 12 (54.5)        | 21 (65.6) |
| Parity*                     | 0.761            |          | 0.026            |          |
| Nulliparous                 | 9 (52.9)         | 5 (45.5) | 13 (59.1)        | 17 (53.1) |
| Multiparous                 | 8 (47.1)         | 6 (54.5) | 9 (40.9)         | 15 (46.9) |
| Delivery season*            | 0.775            |          | 0.009            |          |
| Spring, n (%)               | 4 (23.5)         | 4 (36.4) | 1 (4.5)          | 8 (25.0)  |
| Summer, n (%)               | 1 ( 5.9)         | 0 (0.0)  | 2 (9.1)          | 11 (34.4) |
| Autumn, n (%)               | 9 (52.9)         | 5 (45.5) | 10 (45.5)        | 8 (25.0)  |
| Winter, n (%)               | 3 (17.6)         | 2 (18.2) | 9 (40.9)         | 5 (15.6)  |
| Mode of delivery*           | 0.435            |          | 0.264            |          |
| Vaginal, n (%)              | 9 (52.9)         | 8 (72.7) | 15 (68.2)        | 16 (50.0) |
| C-section, n (%)            | 8 (47.1)         | 3 (27.3) | 7 (31.8)         | 16 (50.0) |
| Education                   | 0.417            |          | 0.226            |          |
| Below high school           | 3 (17.6)         | 3 (37.5) | 5 (22.7)         | 10 (47.6) |
| Above college               | 14 (82.4)        | 5 (62.5) | 17 (77.3)        | 11 (52.4) |
| Gestational age             | 39.4 ± 1.0       | 29.3 ± 2.4 | < 0.001         | 39.4 ± 1.4 | < 0.001 |
| WBC (x10³)                  | 10.3 ± 3.3       | 11.9 ± 3.0 | 0.218          | 9.9 ± 2.9 | 12.3 ± 4.0 | 0.028 |
| Ureaplasma*                 | 0.005            |          | 0.002            |          |
| Negative                    | 15 (88.2)        | 4 (36.4) | 22 (100.0)       | 18 (56.3) |
| Positive                    | 0 (0.0)          | 5 (45.5) | 0 (0.0)          | 8 (25.0)  |
| Birth outcome               | 0.934            |          | 0.783            |          |
| Body weight (kg)            | 3.5 ± 0.4        | 1.2 ± 0.3 | < 0.001         | 3.2 ± 0.3 | 1.4 ± 0.5 | < 0.001 |
| Sex*                        | 0.320            |          | 0.043            |          |
| Male, n (%)                 | 9 (52.9)         | 6 (54.5) | 11 (40.0)        | 18 (56.3) |
| Female, n (%)               | 8 (47.1)         | 5 (45.5) | 11 (60.0)        | 14 (43.8) |
| Apgar 1 min                 | 9.3 ± 0.9        | 6.3 ± 1.8 | < 0.001         | 9.7 ± 0.5 | 5.7 ± 2.5 | < 0.001 |
| Apgar 5 min                 | 9.9 ± 0.3        | 7.8 ± 1.6 | < 0.001         | 10.0 ± 0.0 | 7.4 ± 2.2 | < 0.001 |

†Hypomethylation, < 13% methylation of VTRNA2-1, ‡Hypermethylation, methylation level of 30–60%. BMI, body mass index. *Student's t-test, p < 0.05; *χ² test, p < 0.05. Data are presented as the mean ± SD for continuous variables and as n (%) for categorical variables.

**Figures**
Figure 1

Methylation differences in VTRNA2-1 between term and preterm samples. (a) Rank plot of three methylated sites on VTRNA2-1 by bisulphite pyrosequencing. (b) Relative expression levels of VTRAN2-1 in maternal blood (Term, n = 20; Preterm, n = 20).

Supplementary Files

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- 200623STable1v2.xlsx
- 200623STablesandFigures.docx