ORIGINAL RESEARCH

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Evaluation of the Effect of Delivery Mode on Methylation Changes in the Global DNA and the *PTEN* Gene

Hilal USLU YUVACI[a], Aysel KALAYCI YİĞİN[b], Mehmet Musa ASLAN[c], Filiz ÖZDEMİR[b], Ayşegül ÖZEL[d], Arif Serhan CEVRİOĞLU[a], Mehmet SEVEN[b]

[a]Department of Obstetrics and Gynecology, Sakarya University Medical Faculty, Sakarya, Türkiye
[b]Department of Medical Genetics, İstanbul University Cerrahpaşa Medical Faculty, İstanbul, Türkiye
[c]Department of Obstetrics and Gynecology, Sakarya University Training and Research Hospital, Sakarya, Türkiye
[d]Department of Obstetrics and Gynecology, İstanbul University Cerrahpaşa Medical Faculty, İstanbul, Türkiye

ABSTRACT Objective: This study aimed to investigate the association between global DNA and PTEN gene methylation changes and the type of delivery. **Material and Methods:** The study included a total of 129 pregnant women at the >37th week of pregnancy who had a cesarean (CS) (n=62) or vaginal delivery (VD) (n=67) and their infant's cord blood (n=129). The 5-mC DNA ELISA method performed global DNA methylation analyses, while PTEN gene methylation change analyses were conducted using the methylation-specific polymerase chain reaction method. **Results:** The global DNA methylation changes in the women in the CS (Group 1) and VD (Group 2) groups and their infants were found to be CS 9.15±4.82, VD 10.6±5.09 (p=0.09); CS 11.3±6.78, and VD 11.7±5.69 (p=0.75), respectively. Based on age, when the global DNA methylation changes were examined in the pregnant women under and over 30, and their infants, the differences in both groups were statistically significant (p=0.05, p=0.015). The PTEN gene methylation rates of the pregnant women in Group 1 (22.5%) were higher than in Group 2 (13.4%). The PTEN gene methylation rates of the infants in Group 1 (17.7%) were higher than those in Group 2 (14.9%). **Conclusion:** In this study, a significant increase was determined in mothers and infants regarding their global DNA methylation based on age. Our study is also the first study showing gene-specific methylation changes in the PTEN gene. Our findings are believed to pioneer new studies and positively contribute to scientific research.

Keywords: Cesarean section; delivery; DNA methylation; PTEN gene methylation

Delivery is a physiological event, especially when it occurs spontaneously as vaginal delivery (VD). However, in recent years, it is seen that number of cesarean-section (C-section, CS) operations, which are performed more frequently among women of reproductive age, and the CS rates in both Türkiye and the world are increasing. Studies have shown that the CS rate has increased 4 times worldwide in the last 20 years.[1,2] The CS rate in Türkiye is 53.1%.[3] The World Health Organization reported that a CS rate of 10-15% is ideal in terms of maternal and neonatal outcomes.[4]

Although the short-term outcomes of CS regarding fetal/maternal mortality and morbidity are known, its long-term outcomes are still under debate. Several recent studies have reported an association between CS and increased risk of Type I diabetes mellitus, asthma, allergies, stomach-bowel disorders, childhood leukemia, and testicular cancer in infants born with CS. Although there are abundant data on these diseases, the underlying mechanisms are still uncertain.[5-9]

Epigenetic modifications have an important role in the growth and development of the fetus.[10] Nutrition and environmental exposures during fetal development may lead to epigenetic changes. Although the developmental process is not disturbed, deleterious effects may trigger various diseases to occur.[11] Environmental effects such as chemicals released into nature irresponsibly, genetically modified foods,

Correspondence: Hilal USLU YUVACI
Department of Obstetrics and Gynecology, Sakarya University Medical Faculty, Sakarya, Türkiye
E-mail: hilaly@sakarya.edu.tr

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thermal changes and other external stresses may lead to growth, developmental, and metabolic changes in future generations by epigenetic regulation on gene expression. Hot topics to be investigated include how environmental factors affect epigenetic regulation on gene expression and how these changes are effective on phenotypes.[12]

Epigenetic modifications like DNA methylation and histone modifications regulate gene expression by altering DNA accessibility and the chromatin structure, while there are different studies showing this relationship.[12-14] It was reported that persistent changes in the epigenome related to regulations in genomic DNA methylation during delivery may lead to life-long phenotypic changes and increase the risk of diseases in relation to the type of delivery.[15-17]

Studies have been carried out on the relationship between DNA methylation in the placenta, cord blood and the leukocytes of the fetus and the intrauterine environment. The study showed that there was a methylation difference between leukocytes they obtained from the umbilical cord after CS and leukocytes obtained from the cord blood of vaginally delivered babies, and methylation was higher in the infants born via CS.[18] On the other hand, there are also studies showing no significant difference in the DNA methylation levels of infants born via elective CS, emergency CS or VD.[19]

The *PTEN* (Protein Tyrosine Phosphatase and Tensin Homologue) gene is a tumor-suppressor gene located on chromosome 10q23.3. It plays a role in basic cellular functions such as cell growth, proliferation, migration, adhesion, and cell survival as well as in epigenetic processes. Epigenetic alterations play an important role in several diseases through hyper/hypomethylation and silencing of genes.[20,21] The prevalence of *PTEN* mutation/deletion is high in various types of cancer including breast, brain, bladder, prostate, and endometrial cancers were reported.[20] Although *PTEN* is defined as a tumor-suppressing gene, recent studies showed that changes that take place in this gene are associated with diseases like autism spectrum disorder.[22,23]

Changes in DNA methylation may lead to permanent damage in the epigenome, show their effects throughout life and increase the risk of diseases associated with the type of delivery. Therefore, this study aimed to investigate the relationship between prenatal and postnatal global DNA and *PTEN* gene promoter region methylation changes and the type of delivery.

While there are numerous studies showing the association between the type of delivery and DNA methylation levels, among epigenetic mechanisms, a consensus has not yet been reached about the results. Additionally, the issue has not been clarified with all its aspects, and therefore, changes in the global DNA methylation levels have not been associated with a certain gene. The literature review did not reveal any study on the gene with which global DNA methylation level changes are associated. However, the effect of PTEN promoter methylation on the type of delivery outcomes has not been fully elucidated. To the best of our knowledge, this is the first study that examined global DNA and *PTEN* gene methylation changes in both mothers and infants in cases of elective CS and VD.

MATERIAL AND METHODS

Approval for the study was obtained from the Clinical Research Ethics Committee of Sakarya University (date: March 29, 2017; no: 25), and all participants signed informed consent. This study was conducted in accordance with the principles of the Declaration of Helsinki.

The study included 129 pregnant women who visited Sakarya University Research and Training Hospital between March and October 2019 who were in the >37th week of their pregnancy and their infants. Molecular analyses were performed at the Department of Medical Genetics, Cerrahpaşa Medical Faculty, İstanbul University-Cerrahpaşa. The pregnant women were divided into 2 groups those delivered via CS and those delivered via the normal vaginal course. The women who delivered via CS were included in Group 1 (n=62), and the women who had spontaneous VD were included in Group 2 (n=67). Both groups consisted of mothers and their infants.

SAMPLE SELECTION

The inclusion criteria of participants were being in week >37 of pregnancy. Those with any obstetric risk factors such as multiple pregnancies, diabetes mellitus, hypertension, preeclampsia, congenital malformation, congenital infection, intrauterine development retardation, neonatal asphyxia, early membrane rupture, and chorioamnionitis and those who smoked and drank alcohol, as well as their infants, were excluded.

MATERNAL AND UMBILICAL CORD BLOOD COLLECTION AND DNA ISOLATION

From the pregnant women included in the study, 2 mL blood samples were collected from the peripheral brachial vein during delivery and from the umbilical cord after delivery. Genomic DNA isolation was performed on the blood samples by using a commercial kit (Quick-DNA/RNA Blood Tube Kit, Zymo Research, USA). DNA concentration was measured by using a Nanodrop 2000 spectrophotometer (Thermo Scientific, ABD).

DNA METHYLATION ANALYSIS

Determination of global 5-methylcytosine (5-mC) in the DNA was carried out by using a 5-mC DNA ELISA kit (Zymoresearch, USA). A standardization curve was created by using a negative control (unmethylated) and positive control (methylated). 100 ng from each gDNA was added into different polymerase chain reaction (PCR) tubes, and the final volume was completed to 100 uL with the 5-mC coating buffer.

The DNA specimens were heated at 98°C for 5 minutes in a thermal cycle device (Thermo Fisher Scientific, USA) and denatured. After the heating procedure, the specimens were kept on ice for 10 minutes. The following stages were carried out in compliance with the protocol of the commercial kit (5-mC DNA Elisa Kit, Zymo Research, USA). Absorbance values were measured at 450 nm by using an ELISA plate reader (Multiskan FC, Thermo, USA). All experiments were performed in duplicates.

BISULFITE MODIFICATION AND METHYLATION-SPECIFIC PCR (MSP)

Bisulfite modification from the DNA specimens was performed based on the manufacturer's protocol using a DNA methylation kit (Zymo Research, USA). Following bisulfite modification, to examine *PTEN* gene promoter region methylation level changes, methylation-specific PCR (MS-PCR), which is able to distinguish methylated and unmethylated cytosine bases, was carried out. The *PTEN* gene-specific methylated and unmethylated primers in the promoter region were checked with the MethPrimer software (Table 1).[24]

For MS-PCR reactions, PCR for *PTEN*-UM and *PTEN*-M were carried out in a 50-µL volume (Hot Start PCR Master Mix, GeneMark) containing 10 pM of each primer and 100 ng of bisulfite-modified DNA. Amplification was performed in a thermocycler with the following conditions: 95°C for 10 minutes, cycled at 94°C for 45 seconds, 62°C for 30 minutes, and 72°C for 30 seconds (40 cycles) followed by extension at 72°C for 10 minutes. The reaction samples were then resolved in 2% agarose gel visualize the products.

STATISTICAL ANALYSIS

All statistical analyses were carried out using SPSS 21.0 (IBM Statistics, USA) and in a 95% confidence

TABLE 1: Primer sequences used in the study.

Primers	Sequence	Sequence product size	Annealing temperature
	Methylated		
PTEN-forward	5'-TTCGTTCGTCGTCGTCGTATTT-3'	207 bp	58˚C
PTEN-reverse	5'-GCCGCTTAACTCTAAACCGCAA-3'		
	Unmethylated		
PTEN-forward	5'-GTGTTGGTGGAGGTAGTTGTTT-3'	163 bp	58˚C
PTEN-reverse	5'-ACCACTTAACTCTAAACCACAACCA-3'		

interval. All categorical data are expressed as frequencies and percentages. A chi-squared test was used to compare the demographic and risk factors among the cases and controls. The percentage of genomic DNA methylation is expressed as mean±SD. Independent-samples t-test was used to evaluate the difference in 5%-mC between the groups. The level of statistical significance was set as $p<0.05$.

RESULTS

The demographic characteristics of the pregnant women and their infants in the study are demonstrated in Table 2. The maternal and gestational age, gravida, parity, abortus, newborn weight and gender, and Apgar scores both at 1 and 5 min did not differ significantly between the groups (Table 2).

The global DNA methylation levels in the pregnant women in Group 1 and Group 2 were found as 9.15±4.82 and 10.6±5.09, respectively. While there were noticeable differences in these determined values, the differences were not statistically significant (p=0.09). The global DNA methylation changes in the newborns in Group 1 and Group 2 were found as 11.3±6.78 and 11.7±5.69, respectively. While the values obtained from the groups were different, this difference was not statistically significant (p=0.75) (Table 3). When the methylation level changes in the pregnant women and their infants were evaluated based on the mother's age being under or over 30, the global DNA methylation level changes showed a variation between the mothers over and the mothers under the age of 30 and their infants. This difference was statistically significant (p=0.05, p=0.015) (Table 3). This result may have been caused by time-dependent changes correlated with age in the DNA methylation pattern.

When the *PTEN* gene methylation level changes of Group 1 and Group 2 were examined by the MS-PCR method, and band presence was analyzed, it was seen that the *PTEN* gene of 14 of the 62 mothers in Group 1 (22.5%) was methylated, while the *PTEN* gene of 48 (77.5%) was unmethylated. The *PTEN* gene of 9 out of the 67 mothers in Group 2 (13.4%) was methylated, while the *PTEN* gene of 58 (86.6%) was unmethylated. Accordingly, the *PTEN* gene promoter region methylation rate of the pregnant women in Group 1 was higher than that in Group 2.

Considering the *PTEN* gene methylation patterns of the infants, the gene was methylated in 11 of the 62 infants in Group 1 (17.7%) and unmethylated in 51 (82.3%). The gene was methylated in 10 of the 67 infants in Group 2 (14.9%) and unmethylated in 57 (85.1%). Accordingly, the *PTEN* gene promoter region methylation rate of the infants in Group 1 was higher than that in Group 2 (Table 4).

TABLE 2: Demographic characteristics of pregnant women and infants based on groups.

		Group 1 (n=62)	Group 2 (n=67)	p value
Maternal age		30.5 ±3.89	29.8±6.48	0.45
Gravida, n		2.6±1.31	3.4±1.12	0.35
Parity, n		0.89±0.94	1.27±1.88	0.14
Abortus, n		0.2±0.60	0.2±0.59	0.95
Gestational age (week)		38.5±1.0	39.0±1.61	0.02
1-min APGAR		8.7±0.61	8.6±0.65	0.43
5-min APGAR		9.7±0.60	9.7±0.45	0.88
Birthweight (g)		3410.63±474.46	3237.39±516.44	0.37
Infant gender	Girls, n %	24 (38.7)	35 (52.2)	0.12
	Boys, n %	38 (61.3)	32 (47.8)	

CS: Cesarean section; VD: Vaginal delivery.

TABLE 3: DNA methylation levels based on the types of delivery and age.

		Mothers DNA methylations	p-value	Infants DNA methylations	p-value
Group 1 (n=62)		9.15±4.82	0.09	11.3±6.78	0.75
Group 2 (n= 67)		10.6±5.09		11.7±5.69	
Age	≥30 (n=80)	10.3960±5.45	0.05	11.6506±6.97	0.015*
	<30 (n=49)	9.1376±4.10		11.3588±4.69	

*Bold values indicate p<0.05; CS: Cesarean section; VD: Vaginal delivery.

TABLE 4: Number of mothers and infants showing *PTEN* gene methylation based on the type of delivery.

PTEN gene methylation	Group 1 (n=62)	Group 2 (n=67)	p value
# of mothers (%)	14 (22.5%)	9 (13.4%)	0.12
# of infants (%)	11 (17.7%)	10 (14.9%)	0.55

CS: Cesarean section; VD: Vaginal delivery.

DISCUSSION

Regulation of gene expression is not just dependent on the changes that take place in the DNA sequence, but it is also dependent on epigenetic mechanisms such as DNA methylation and histone acetylation.[25] DNA methylation has become a field that is most prevalently studied among epigenetic changes as it is affected by environmental factors in critical periods of development. One of the important fields of research is determination of epigenetic modifications in the early period.

Delivery is an important early life event that involves hormonal changes. In comparison to those being born via CS, infants that are born through VD are exposed to high-gravity stress in connection to excessive stimulation of the immune system and an increase in stress hormones as the delivery process is longer.[26,27] Excessive stress and immune activation have a potential to induce adaptive modification of the epigenome. While there are different studies showing the relationship between type of delivery and DNA methylation levels, this remains a topic for further investigation.

The *PTEN* gene plays a role in cell progression and growth in the embryonal/fetal and adult period of development. In humans and animal models, *PTEN* function loss has been held responsible for several cognitive dysfunctions.[28] *PTEN* gene methylation level changes have been associated with several types of cancer and diseases such as autism whose etiology is not precisely known, whereas no study was encountered to show the relationship between the type of delivery and *PTEN* gene methylation level changes. This study investigated the relationship between both global DNA and *PTEN* gene methylation level changes and normal and CS deliveries in the prenatal and postnatal periods.

In our study, when the global DNA methylation changes were examined in the pregnant women who had CS and VD, while there was a numerical difference between the groups, the difference was not statistically significant (p=0.09). A study on this topic examined the relationship between the type of delivery and DNA methylation and found that infants born via elective CS had higher methylation level changes than those born with VD, but this difference disappeared 3-5 days after birth.[18] Another study on this topic, similarly to our study, found no significant difference in DNA methylation levels checked on cord blood based on the type of delivery.[29] The expected risk in terms of birth anomalies starts to increase from the age of 35, while it increases by 10 times in comparison to the general population after the age of 40. This age limit associated with major congenital anomalies is started from the 30s in terms of methylation changes. For this reason, when the methylation level change results were examined by division into 2 groups as over and under 30 years of age, the results of the mothers under 30 and those over 30 and their infants were significantly different (p=0.05, p=0.015). This result of our study was in agreement with the information in the literature showing the relationship between DNA methylation and aging.[30,31] The aging process starts after birth, and in time, changes also appear in methylation patterns. In the antenatal and neonatal periods, growth-development and gene expression-methylation changes are in an interaction. In this time-dependent process, changes defined in the content of 5-methylcytosine appear as hypomethylation in the genome and promoter-specific hypermethylation.[32] In this study, there was a significant increase based on age in the global DNA methylation levels in both the mothers and the infants (p=0.015). On the other hand, age-dependent global DNA hypomethylation was more significant in the under-30 age group. This situation may have been related to mother-independent DNA methylation patterns.

In addition to global DNA methylation, this study also examined *PTEN* gene promoter region methylation level changes. In the cases of CS delivery, the *PTEN* gene was methylated in 14 of the 62 (22.5%) mothers and unmethylated in 48 (77.5%). In

the cases of VD, it was methylated in 9 of the 67 (13.4%) mothers and unmethylated in 58 (86.6%). This situation showed that the *PTEN* gene promoter region is more methylated in CS delivery cases than VD cases. The *PTEN* gene was methylated in 11 of the 66 infants born via CS (17.7%), while it was methylated in 10 of the 67 infants born via VD (14.9%). Similarly, the *PTEN* gene methylation rate in the infants born via CS was higher than those born via VD. There are studies in the literature showing the relationship between *PTEN* gene methylation and various types of cancer.[33,34] An increase in the *PTEN* gene promoter methylation rate was shown to suppress *PTEN* gene expression and activate AKT.[35]

This study has limitations. The number of patients included in the study was small. There is also a need for large, adequately powered trials comparing methylation status between the time at delivery and later life, such as 3 or 6 months later.

CONCLUSION

The rates of CS deliveries are also increasing in Türkiye, as in the world. Moreover, it is reported that there are methylation changes in infants born via CS that may pave the way for some diseases that may emerge in further periods of life. The results of this study were in agreement with data in the literature about global DNA methylation changes. Nevertheless, there is no study on this topic showing gene-specific methylation changes. Considering it from this perspective, this study is the first study showing *PTEN* methylation changes. We believe our findings will be guiding for new studies and contribute positively to scientific research.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: *Hilal Uslu Yuvacı, Aysel Kalaycı Yiğin;* ***Design:*** *Hilal Uslu Yuvacı, Aysel Kalaycı Yiğin;* ***Control/Supervision:*** *Arif Serhan Cevrioğlu, Mehmet Seven;* ***Data Collection and/or Processing:*** *Hilal Uslu Yuvacı, Ayşegül Özel, Mehmet Musa Aslan, Filiz Özdemir;* ***Analysis and/or Interpretation:*** *Hilal Uslu Yuvacı, Mehmet Musa Aslan, Aysel Kalaycı Yiğin;* ***Literature Review:*** *Hilal Uslu Yuvacı, Aysel Kalaycı Yiğin;* ***Writing the Article:*** *Hilal Uslu Yuvacı, Aysel Kalaycı Yiğin, Mehmet Musa Aslan;* ***Critical Review:*** *Mehmet Seven, Arif Serhan Cevrioğlu;* ***References and Fundings:*** *Aysel Kalaycı Yiğin, Filiz Özdemir;* ***Materials:*** *Hilal Uslu Yuvacı, Aysel Kalaycı Yiğin, Filiz Özdemir.*

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