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Evaluation of H19, Mest, Meg3, and Peg3 genes affecting growth and metabolism in umbilical cord blood cells of infants born to mothers with gestational diabetes and healthy mothers in Rafsanjan City, Iran

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Abstract

Hyperglycemia during the first trimester leads to an increased risk of innate malformations as well as death at times close to delivery dates. The methylated genes include those from paternal H19 and PEG3 and those from maternal MEST and MEG3 that are necessary for the growth and regulation of the human fetus and its placenta. The aim of this study was to evaluate and compare the expression of these genes in the cord blood of healthy infants born to mothers with gestational diabetes mellitus (GDM) and healthy mothers.

This case-control study was conducted on the cord blood of 40 infants born to mothers with GDM and 35 infants born to healthy mothers. Mothers were identified by measuring oral glucose tolerance in the 24th–26th week of pregnancy. Cord blood was obtained post-delivery, and cord blood mononuclear cells were immediately extracted, using Ficoll solution. Then, RNA extraction and cDNA synthesis were performed, and gene expression of MEG3, PEG3, H19, and MEST was assessed through quantitative real-time PCR.

Findings show that the expression levels of MEG3, PEG3, H19, and MEST genes were significantly decreased in mononuclear cord blood cells of infants born to mothers with GDM when compared to those of the healthy control group.

These findings reveal that the reduction of imprinted genes in mothers with GDM is most likely due to changes in their methylation by an epigenetic process. Considering the importance of GDM due to its high prevalence and its side effects both for mother and fetus, recognizing their exact mechanisms is of high importance. This has to be studied more widely.

Introduction

Gestational diabetes mellitus (GDM) is defined as glucose intolerance of variable degree with onset or first recognition during pregnancy.[1] In Iran, the result of a meta-analysis showed that the prevalence of GDM is 3.4% (the highest and lowest incidence rates were 18.6% and 1.3%, respectively).[2] GDM increases the probability of occurrence of additional complications in the mother and fetus during and after pregnancy. Some of these complications are preeclampsia, polyhydramnios, infant macrosomia, difficult delivery, and metabolic complications in infants including hypoglycemia, hyperbilirubinemia, hypoglycemia, and prenatal death.[3] Also, in these mothers and their infants, there is a greater risk of type II diabetes after delivery.[4] Macrosomia, the most prevailed GDM complication, occurs in 20% of cases. In macrosomia, some organs including the liver, heart, pancreatic islets, and adrenal glands become enlarged due to hypertrophy and cell hyperplasia.[5] The exact mechanism is not clear, but undesirable conditions in the uterus may change the placenta's gene regulation and physiological dimensions, and the growth of the fetus. A high-glucose environment during pregnancy disrupts the endothelial cell cycle,[6] changes inflammatory patterns,[7] promotes vasculopathy,[8] and affects vascular permeability of the placenta.[9]

Epigenetics is a potential molecular mechanism underlying long-term exposure to a destructive fetus environment. Epigenetics refers to gene expression regulation that preserves nucleic acid sequence. Deoxyribonucleic acid methylation is one of the most widely studied epigenetic

mechanisms. Abnormal DNA methylation with the intermediation of the DNA methyltransferase enzyme can lead to inappropriate gene silencing; regulating gene expression and performances of neighboring cells. Gene imprinting is an epigenetic modification resulting in the unequal expression of the paternal gene increasing its inheritance. Gene imprinting ensures only one allele will be expressed and the other silenced defying Mendelian Laws and may play a role in mammalian fetal growth.[10]

Imprinted genes play an important role in fetal development and placental performance.[10] There are some hypotheses set forth concerning the point that environmental changes before birth can disrupt fetal programming and can lead to obesity and chronic disease during life.[11,12] A fetus of mothers with GDM will grow in an environment completely different from that of healthy mothers. Glucose, alanine, and free fatty acids are transferred to the fetus via the mother's blood circulation.[13] As a result, the concentration of insulin in the amniotic fluid increases demonstrating the compensating response of the fetus to the increase of such materials.[14,15] Studies of animal models showed that fetal hyperglycemia can change the expression of an imprinted gene from the placenta.[16.] This study was designed to uncover genes that have been imprinted by the mother's diabetic condition in healthy children.

After reviewing previous studies, we selected four genes that have been implicated in glucose metabolism and diabetes to examine for epigenetic modification in diabetes. H19 gene is one of the first identified imprinted genes. H19 is imprinted in the H19/IGF2 gene cluster on chromosome 7 in mice; its orthologous region in humans is located on chromosome 11. Once the paternal allele is silenced by CpG promoter methylation, H19 is expressed from the maternal allele. It has been shown that H19IncRNA controls fetal growth through epigenetic regulation of the IGN imprinted gene network.[17] Lack of H19 expression is implicated in 70% of liver cancers and plays a role in several liver diseases including NASH, NAFLD, PLD, liver fibrosis, steatosis, cirrhosis, and diabetic hyperglycemia.[18,19] Maternally expressed gene 3 (MEG3) is an imprinted gene located in the 14q32.3 human chromosome in DLK1-MEG3. The gene codes long non-coding ribonucleic acid.[9] Expression reduction of MEG3 is associated with many diseases such as hepatocellular carcinoma, acute myeloid leukemia, ovarian cancer, and Huntington's disease. MEG3 expression levels are higher in pancreatic beta cells than in alpha cells; and, the gene is one of the initial transcription factors that regulate insulin secretion in beta cells; suppression of MEG3 expression leads to a lack of performance regulation and an increase in the death of beta cells, as well as type II diabetes pathogenesis.[11]

MEST or PEG1 codes for a member of the alpha/beta hydrolase superfamily and is an imprinted gene located on chromosome 7p31.3. Losing the paternal form of the gene is related to certain types of cancer and has been attributed to changes in the promoter. The protein coded by this gene plays a role in growth and development.[7] Peg13/Pw1 is one of the genes on human chromosome 19 that is necessary for the development of muscles; it plays a role in the cell cycle and metabolic regulation. Peg13/Pw1 expression is observed in brain tissues, skeletal muscle, intestine, bone marrow, and hypothalamus; it is recognized as an intermediary in P53 regulation of apoptosis.[12]

The present study examines gene expression levels of methylated paternal H19 and PEG2 and maternal MEST and MEG3 to determine if these genes – required for fetal and placental growth in humans, cell differentiation, and neurological behaviors – are differentially expressed in cord blood of healthy infants born to mothers with GDM and healthy mothers.

Materials and methods

This case-control study was conducted with two groups of healthy infants – those born to mothers with GDM (case group) and those born to healthy mothers (control group) – in Niknafs Maternity Hospital in Rafsanjan City, Iran, in 2021. Mothers were selected with an oral glucose tolerance test during the 24th–26th week of pregnancy. Those mothers with fasting blood sugar levels greater than 95 mg/dl, a 1-h blood sugar above 180 mg/dl, and a 2-h blood sugar greater than 155 mg/dl were selected for the case group. Blood glucose in all mothers with GDM was controlled by diet and insulin. Mothers in the control group had normal blood sugar during pregnancy. The groups were individually matched for age, type of delivery, the number of children delivered, gender, weight, and height of their infants. Those mothers diagnosed with underlying diseases like hypertension and cardiovascular diseases were excluded from the study. Written informed consent and demographic data checklist were completed for all research participants.

Separating mononuclear cells of cord blood

Cord blood collection is performed by a trained umbilical cord blood bank nurse. Once the placenta is delivered, it is transported quickly to the collection area, which contains the necessary supplies and equipment. There was no time limit set between placental delivery and the cord blood collection. The placenta was placed on a specially designed tray with a central hole that allowed the umbilical cord to hang down. After stringently cleaning the umbilical cord with 70% alcohol and an iodine swab, cord blood was collected from the umbilical vein by gravity in a sterile collection 350 ml double bag (R 1315, Baxter) containing EDTA anticoagulant. Cord blood collection was stored at 4°C until processing. Peripheral blood mononuclear cells (PBMC) or granulocytes were separated using the density difference created by the Ficoll solution. Total RNA extraction and purification were conducted on 200 µl of granulocyte cells.

RNA extraction

RNA extraction from granulocyte cells was conducted using a kit protocol provided by Pars Toos Co. To do so, 750 µl of RL solution containing triazole was added to the sample. Then, after pipetting and incubating at ambient temperature, chloroform solution was added and the solution was centrifuged for 12 min (12,000 rpm) under 4°C. After the formation of three phases in the microtube, 400 µl of the upper phase containing RNA was removed and ethanol (70%) of the same volume was added. The solution was centrifuged, transferred to the filtered microtube available in the kit, and washed twice with PW solution. The filter was transferred to the new microtube to dissolve sediments containing RNA and 30 µl DEPC water was added. The resulting transparent solution containing RNA was used for cDNA synthesis.

The concentration of the extracted RNA was assessed using a NanoDrop device. A ratio of optical absorption in 260/280 wavelengths was used as the desirable purification. This ratio was 1.8–2 and the RNA concentration of the samples was increased to 100 ng/ul.

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cDNA synthesis

After RNA extraction, complementary DNA was made from mRNA strand through a reaction by reverse transcriptase. In this respect, the kit provided by Pars Toos Co. was used. As instructed by the kit, 0.2 cc, 2 µl oligo dt, 1 µl primer, 5 µl DEPC water, and finally 3 µl of RNA were added to the microtube in stages. Then and following the thermal protocol of thermocycler, samples were held for 10 min at 25°C, for 60 min at 47°C, and 5 min at 85°C temperature. After the end of these stages, DNA samples were held at −20°C for real-time PCR reaction.

Real-time PCR reaction

To determine the expression levels of genes studied, Real-Time PCR was performed and the amounts of materials added to 0.2 ml vials were as follows: 2 µl of exclusive primers (forward and reverse) + 1 µl +cDNA + 5 µl SYBER Green I Master Mix bought from Biosystem Co.; and, 10 µl Dnase free water was added to reach final volume. The mixture resulted was added to special strips and the strips were placed in the Bio-Rad device.

Table 1 provides the exclusively designed primers and thermal conditions for the primers. The design and blasting of the primers were done through the NCBI database. It should be noted that primers specific to the gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were used in real-time PCR as an internal control. As shown, different temperatures and times for each gene were tested and those producing the optimum results were used.

Statistical analysis

The continuous variables were expressed as the mean ± SD, and the categorical variables were presented as a percentage. An independent t-test was used if normality was established; and, a Mann–Whitney U test was used if normality was not established to compare the expression levels of various genes in two groups. All statistical analyses were performed with SPSS (version 18.0, SPSS Inc., Chicago, IL, USA). A "*P*-value" less than 0.05 was considered significant.

Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki. Institutional Review Board approval (code: IR.RUMS.REC.1398.020) was obtained. The present study did not interfere with the process of diagnosis and treatment of patients.

Results

Demographic data related to healthy infants born to mothers with GDM (case group; $n = 40$) and those born to healthy mothers (control group; $n = 35$) are shown in Table 2. No significant difference was observed between the demographic data between case and control groups on any of the measures.

Quantitative real-time PCR findings

To find expression changes in H19, Mest, Meg3, and Peg3 in cord blood cells from infants born to mothers with GDM, gene expression was studied via quantitative real-time PCR. In all samples, the GAPDH gene was used as an internal control gene.

To be assured of the production of an exclusive product, melt curve plots were determined in all samples. Two samples were run for each gene. The melt curve plots for each primer and the proliferation diagram are provided in Figures 1-4.

Analyzing qRT-PCR data

H19 gene expression in cord blood cells of infants born to mothers with GDM was significantly reduced when compared to controls using a Wilcoxon test (log2FC: −2.2, *P*-Value < 0.0001, SEM (Control group): 0.1824, SEM (case group): 0.2158; Figure 5-A).

Peg3 gene expression in cord blood samples from infants born to mothers with GDM was significantly reduced when compared to controls using a Paired t-test (log2FC: −1.869, *P*-Value: 0.0023, SEM (Control group): 0.6238, SEM (case group): 0.2995: Figure 5-B).

MEG3 gene expression in cord blood samples obtained from infants born to mothers with GDM was significantly reduced when compared to infants born to healthy mothers via Wilcoxon statistical test (log2FC: −1.8, *P*-Value: 0.0041, SEM (Control group): 0.5581, SEM (case group): 0.0.6584; Figure 5-C).

Mest expression in cord blood cells from infants born to mothers with GDM was significantly reduced when compared to those born to healthy mothers via Wilcoxon statistical test (log2FC: −2.5, *P*-Value: 0.0172, SEM (Control group): 0.6504, SEM (case group): 0.2782; Figure 5-D).

Discussion

In this case-control study, the levels of PEG3 and H19 genes were compared in the cord blood obtained from healthy infants born to mothers with GDM (case group; $n = 40$) and healthy mothers (control group; $n = 35$). Findings suggest that the expressions of these genes were lower in the cord blood of infants born to mothers with GDM which may be attributed to DNA methylation a regulatory mechanism for gene expression.

In a study on diabetic and healthy groups of mothers, it was shown that the MEST methylation difference between the two groups was significant; and, the gene has been recommended as the main candidate for reprogramming body metabolism. Moreover, the findings also showed that obese mothers have lower methylation levels of MEST compared to the control group with normal weights. These findings support the hypothesis set forth regarding changes in MEST expression and the lifetime risk of obesity and other metabolic disorders.[7] Our findings also show a significant decrease in MEST expression regulation in the cord blood of infants born to mothers with GDM when compared to healthy ones.

According to the studies, effective genes on growth and metabolism studied here are all from among imprinted genes that do not follow Mendelian Laws. Such a mechanism is called genomic imprinting which is one of the candidate mechanisms helping fetal development regulation in mammals. Imprinted genes play an important role in fetus development and growth as well as placental growth factors.[20] Studies of animal models showed that fetal hyperglycemia can change the expression of an imprinted gene from the placenta.[16]

It has been reported that MEG3 plays a role in various types of human diseases. MEG3 promoter region is filled with CpG islands and MEG3 expression is regulated in two different methylated regions (DMR1), IG-DMR2, and MEG3-DMR.[21] MEG3-DMR methylation status plays an important role in MEG3 expression regulation, average cell proliferation, migration, and apoptosis in pituitary tumors,[22] retinoblastomas,[23] cervical cancer,[24] and severe

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Table 1. The sequence, temperature, and concentrations of primers used in the real-time PCR reaction

Primer concentration	Temperature	Primer 5' to 3'	Gene
2 nm	60°	TACGTCCGGCCTTCCTGAA	F(H19)
		CAGCCATAGTGTGCCGACT	R(H19)
		CCCATCTACACCTCACGAGG	F(MEG3)
5 nm	62°	GGCTGGTCAGTTCCGGTC	R(MEG3)
		AGAACCGCAGGATCAACCTT	F(MEST)
10 nm	58°	TCAGTCGTGTGAGGATGGGT	R(MEST)
		AGAGAGGACGACAGGGACTC	F(PEG3)
10 nm	58°	ATCTTCACAAATCCCCCGCC	R(PEG3)
		GAAGGTGAAGGTCGGAGTC	F(GAPDH)
5 nm	58°	GAAGATGGTGATGGGATTTC	R(GAPDH)

Table 2. Demographic data of healthy infants born to mothers with GDM (case group; n = 40) and healthy mothers (control group; n = 35)

Variable	Control group ($n = 35$)	Case group ($n = 40$)	*p*-value
Infant's weight (g)	3350 ± 347.5	3536.5 ± 199.5	0.49
Infant's height (cm)	44.99 ± 1.81	48.94 ± 1.78	0.8
Baby's head circumference (cm)	34.56 ± 1.06	35.40 ± 1.52	0.28
Mother's height (cm)	162.47 ± 6.77	161.68 ± 5.64	0.63
Mother's age (years)	35.7 ± 4.6	36.6 ± 5.1	0.89
Mother's weight before child delivery (kg)	75.07 ± 11.00	88.36 ± 11.27	0.46
Mother's weight before pregnancy (kg)	63.56 ± 10.85	73.00 ± 15.29	0.41
Gender of infants			1
Male	18(51.0)	19(48.0)	
Female	17(49.0)	21(52.0)	
Type of child delivery			0.135
C-section	20(58.0)	32(80)	
Vaginal delivery	15(42.0)	8(20)	

acute myeloid leukemia.[25-27] Also, epigenetic correction in the DLK1-MEG3 region is changed in type I and II diabetes. Excessive increases in the methylation of MEG3-DMR in mice with diabetes lead to the reduction of mRNA MEG3. Studies performed on genomic imprinting have shown that D1K1-Dio3 is responsible for fetal growth in mice. Therefore, the DNA MEG3 methylation profile seems to play a potential role in developmental programming related to GDM. It has been reported that MEG3 inactivates PI3k/Akt signaling and causes disruption in retinal arterial performance with diabetes in mice.[27] Moreover, MEG3 regulates Wnt/β-certain 48 signaling against podocyte damage in diabetic nephropathy.[28] Studies on diabetic patients also showed that increasing methylation in MEG3-DMR is enough to suppress MEG3 expression and affects the sensitivity of β cells to oxidative stress with the intermediation of cytokine.[29] In studies performed to assess MEG3 expression in blood cells of healthy and diabetic mothers, it was found that reduction of gene expression occurs at childbirth in mothers with GDM. More research is needed to determine if this is due to MEG3 methylation under hypoglycemia conditions.

Growth factor II (IGF2) gene like imprinted maternal insulin on the 11p15.5 chromosome is one of the most regulated regions from a genetics perspective. H19 in 90 KB 3'originates in IGF2, is imprinted mutually through IGF2; and regulates its imprinting and expression.[29] Maternal factors, including gestational diabetes and obesity, regulate DNA methylation in IGF2 and H19 DMR.[17,28,29] Methylation increase in H19 and its reduction in IGF2 may be accompanied by the high weight of infants at birth. Also, a study investigating the relationship of methylation in the IGF2/H19 region to body weight showed that European and African American children with obesity or overweight have had higher levels of methylation in H19DMR and lower levels at IGF2 DMR at birth.[30]

Heijmans et al. showed that mRNA levels of H19 and Peg3 in diabetic mothers' placenta compared to control groups were considerably changed.[22] These genes are usually located in clusters specified genetically by DNA methylation in DMRs; and, regulate relative gene expressions. Also, considerable changes in methylation levels in DMRs H19 and Peg3 in diabetic mice placenta have been observed.[31] In another study abnormal methylation level was

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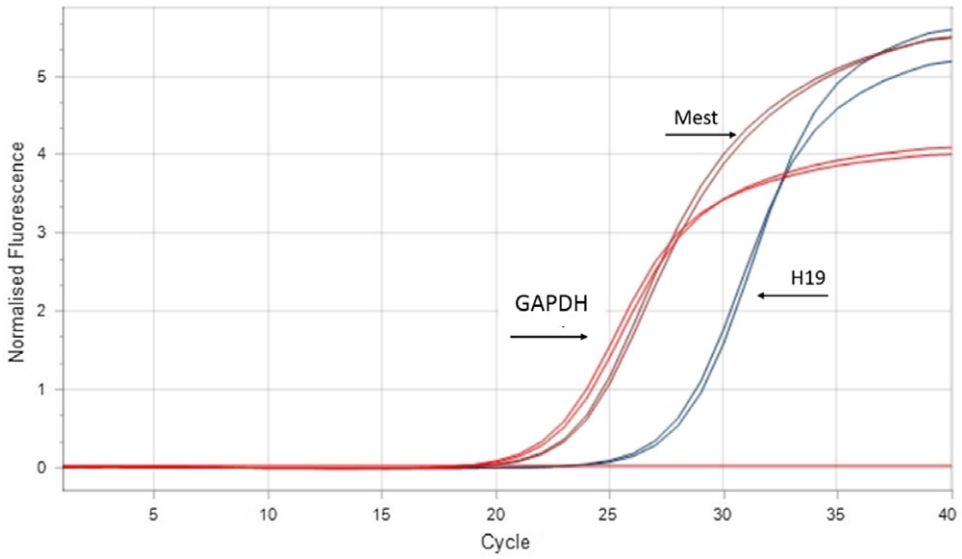

Fig. 1. Evaluation CT of H19 and MEST genes with GAPDH gene in healthy infants born to mothers with GDM (case group; $n = 40$). Two samples were run for each gene.

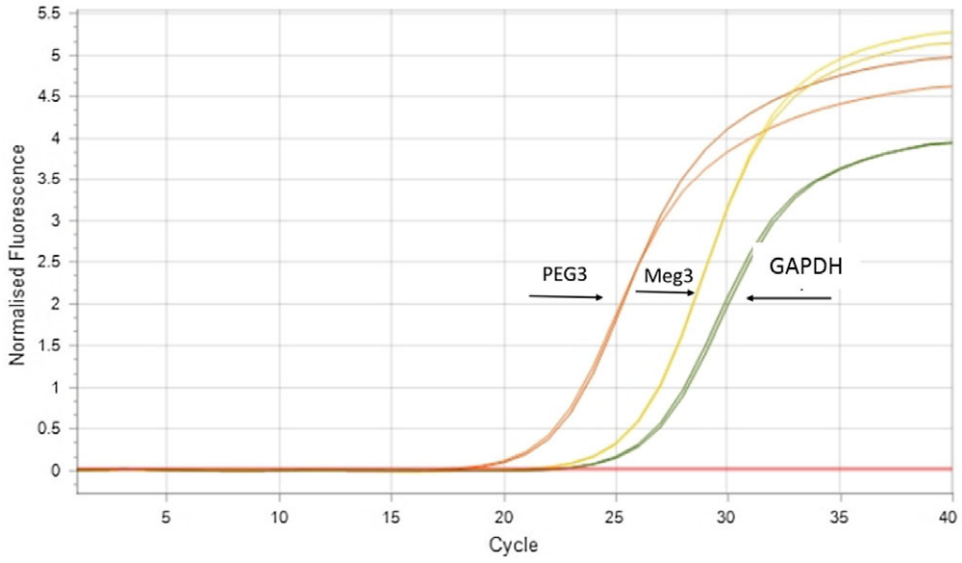

Fig. 2. Evaluation CT of PEG3 and MEG3 genes with GAPDH gene in healthy infants born to mothers with GDM (case group; $n = 40$). Two samples were run for each gene.

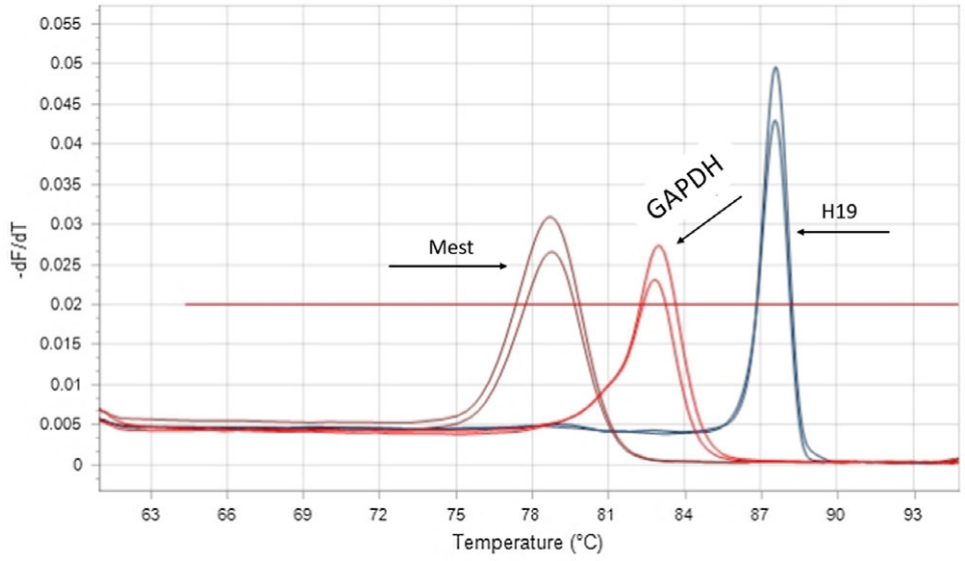

Fig. 3. Comparison of the melting curves of H19 and MEST genes with GAPDH gene in healthy infants born to mothers with GDM (case group; $n = 40$). Two samples were run for each gene.

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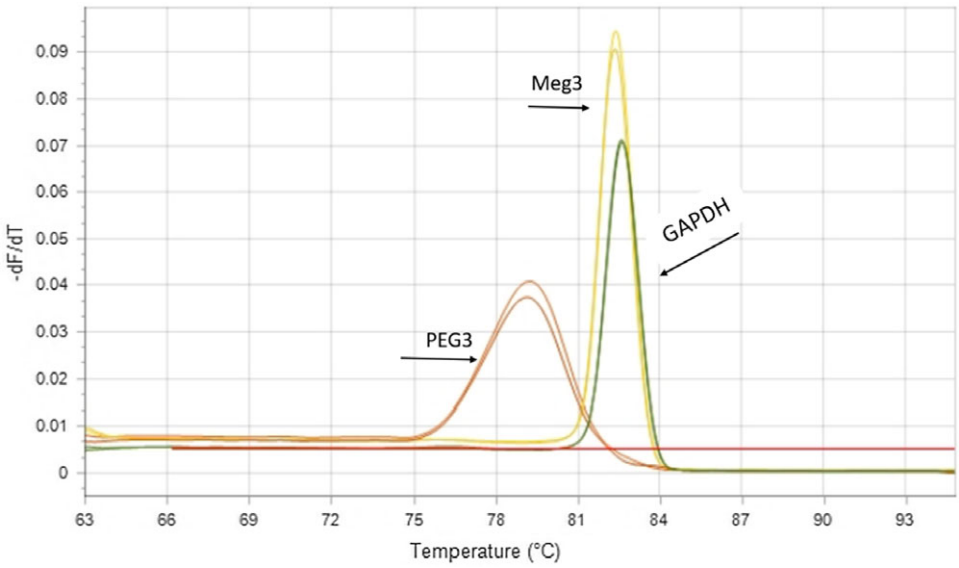

Fig. 4. Comparison of the melting curves of PEG3 and MEG3 genes with GAPDH gene in healthy infants born to mothers with GDM (case group; $n = 40$). Two samples were run for each gene.

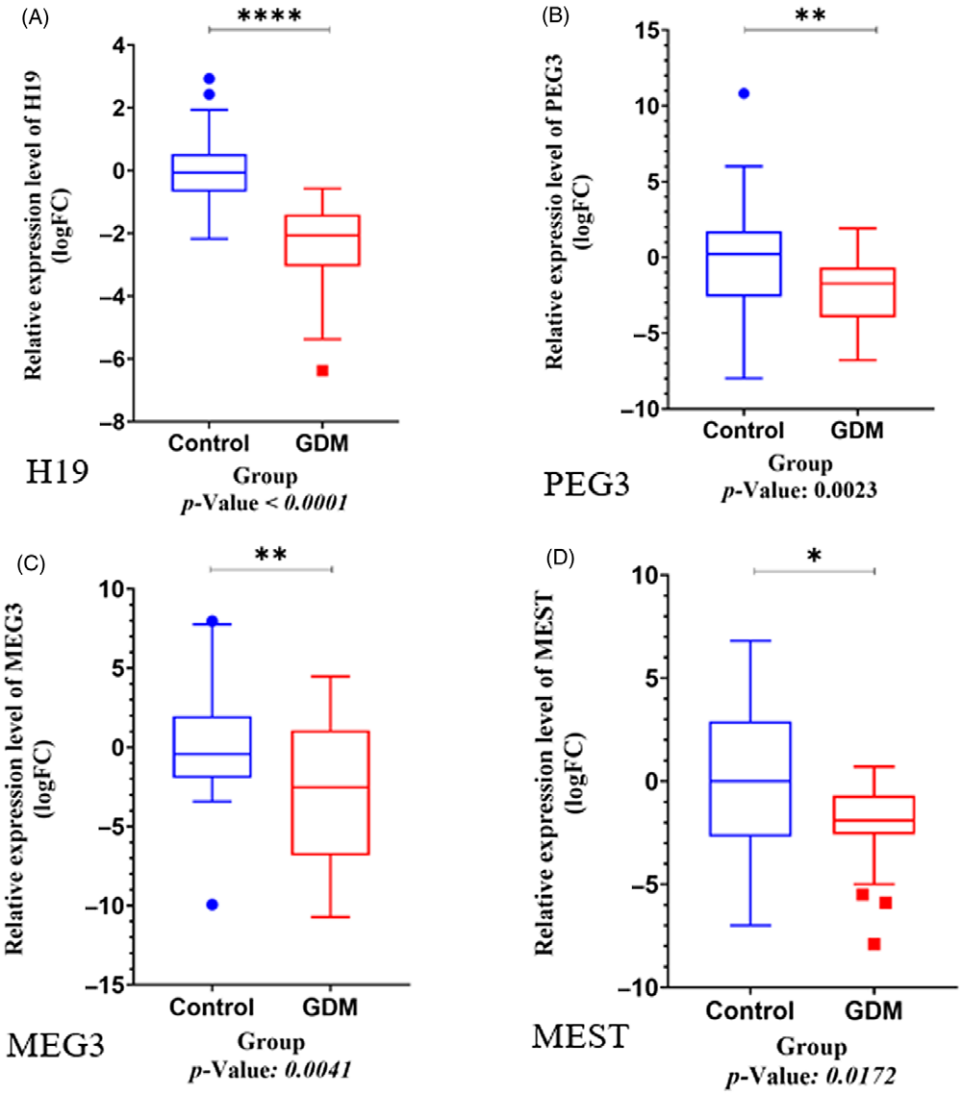

Fig. 5. (A) H19, (B) PEG3, (C) MEG3 and (D) MEST gene expression in cord blood from healthy infants born to mothers with GDM (case group; n = 40) and healthy mothers (control group; $n = 35$). logFC: log2FoldChange: log2 fold change gene expression of group 2 compared to group 1.

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assumed to be a reason for changes of expression in these genes. However, these changes may have been created by various Peg3 distributions in various cell populations in the placenta.[32-34]

Studies of the changes in the expression of genes affected by PEG3, MEG3, and H19 IncRNAs are recommended to identify the regulation mechanisms of the gene network involved in GDM and to clarify treatment procedures. Studies of these genes' methylation process would provide valuable information regarding the disease and its method of treatment.

Limitations of the study

There were limitations in sampling, due to delays in receiving samples. Some samples were not included in the study if they were clotted or damaged. Thus the sample size is small and originates from a small municipality. Larger studies with more diversity are required to determine the efficacy of our results.

Conclusion

Based on the current findings, expression levels of H19, PEG3, MEG3, and MEST are significantly lower in cord blood cells of infants born to mothers with GDM compared to infants born to healthy mothers. Considering that GDM is one of the greatest contributors to pregnancy complications and produces severe side effects in mother and fetus during pregnancy and the child's life, early diagnosis of the disease and a deeper understanding of the factors that contribute to its development can help prevent complications and pave the way for treatment. Therefore precise identification of these imprinting genes are of high importance in the treatment of and in recognizing the mechanisms that lead to the disease.

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Conflicts of interest. The authors of this study declare no conflict of interest.

Ethics approval and consent to participate. The study was conducted in accordance with the Declaration of Helsinki and Institutional Review Board approval has been obtained (IR.RUMS.REC.1398.020).

Abbreviations. Growth factor II: IGF2; Maternally expressed gene 3: MEG3; Differently methylated regions: DMR1.

Authors' contributions. FRM and MRH were responsible for the study concept and design. MM, ZJ and FNK led data collection. JS, RH, MNK, and MRH were responsible for the analysis and interpretation of data. FRM and MM wrote the first draft. FNK, JS, RH, and MNK contributed to the writing of the second and third drafts. JS provided comments on initial drafts and coordinated the final draft. All authors read and approved the final manuscript. All authors take responsibility for the integrity of the data and the accuracy of the data analysis.

Role of the funding source. The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Availability of data and materials. The data used in this study are available from the corresponding author on request.

Consent for publication. By submitting this document, the authors declare their consent for the final accepted version of the manuscript to be considered for publication.

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