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The promoter methylations of the *autoimmune regulator (AIRE)* gene and *matrix metalloproteinase-3 (MMP-3)* gene may have a role in gestational diabetes mellitus

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Abstract. – OBJECTIVE: Gestational diabetes mellitus (GDM) is characterized by new-onset glucose intolerance and is most common in the second and third trimesters of pregnancy. Epigenetic modifications regulate glucose and its cellular interactions with metabolic pathways. Emerging evidence suggests that epigenetic changes contribute to the pathophysiology of GDM. Since these patients have high glucose levels, the metabolic profiles of the fetus and the mother can affect these epigenetic changes. Therefore, we aimed to examine the potential alterations in the methylation profiles of three gene promoters: the *autoimmune regulator (AIRE)* gene*, matrix metalloproteinase-3 (MMP-3),* and *calcium voltage-gated channel subunit alpha1 G (CACNA1G)*.

PATIENTS AND METHODS: A total of 44 patients diagnosed with GDM and 20 controls were involved in the study. DNA isolation and bisulfite modification were performed from peripheral blood samples of all patients. Then, the promoter methylation status of the *AIRE, MMP-3*, and *CACNA1G* genes was determined by methylation-specific polymerase chain reaction (PCR) methylation-specific (MSP).

RESULTS: Our results demonstrated that the methylation status of *AIRE* and *MMP-3* changed to unmethylated in the *GDM* patients compared to healthy pregnant women (p<0.001). However, *CACNA1G* promoter methylation status failed to show a significant change between experimental groups (p>0.05).

CONCLUSIONS: Our results indicated that *AIRE* and *MMP-3* are the genes affected by epigenetic modification, which could be one of the causes of the long-term metabolic effects in maternal and fetal health and can be a target for prevention, diagnosis, or treatment for GDM in future studies.

Key Words:
Gestational diabetes mellitus, Methylation, Epigenetic, Pregnancy, Autoimmune regulator gene, Matrix metalloproteinase-3.

Introduction

Gestational diabetes mellitus (GDM), which has an increasing prevalence worldwide, has contributed to metabolic dysregulation and several diseases in fetuses *via* epigenetic modifications[1]. Fundamentally, GDM is characterized by new-onset glucose intolerance, generally seen in the second and third trimesters and diagnosed in about 7% of pregnant women and can cause macrosomia, pregnancy-induced hypertension, and increase cesarean delivery as negative outcomes of pregnancy[2,3]. Predisposition to obesity, metabolic syndrome, and diabetes in later life are only a few of the devastating and long-lasting effects of intrauterine hyperglycemia on both the fetuses and their mothers[4,5]. According to a recent study[6], women with GDM are more likely to develop type 2 diabetes than women without GDM. Genetic variables may be a confounding element in such intrauterine exposure. Although several mechanisms are claimed to be related to the pathophysiology of GDM, many studies[7] provided strong evidence that overnutrition and obesity, influence metabolic phenotype with epigenetic mechanisms in later life.

Epigenetic mechanisms directly regulate gene expression patterns without changing the DNA sequence. DNA methylation is mainly studied as modification mediated through methylation of the C-5 position of the cytosine at cytosine phosphate guanine (CpG) dinucleotides[8]. Because methylation patterns are also transmitted to the fetus, promoter methylation during development and differentiation leads to an inactive chromatin structure and silencing, resulting in long-term consequences. Thus, investigation of these mechanisms and identifying possible genes that could result in

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the fetus's long-term changes is essential. Several studies[9] demonstrated changes in the methylation profile in GDM patients, and even different results in the various tissues. Mass spectrophotometry and genome-wide methylome studies[10] showed changes in several gene promoters in gestational diabetes mellitus patients. Nevertheless, there is still a need to investigate these possible genes in different patient populations to understand long-term metabolic consequences in the fetus.

The methylation profile of *AIRE*, *MMP-3*, and *CACNA1* are some of the genes that showed to be changed during GDM. However, knowledge about possible changes in their methylation profile in different patients is still limited. Additionally, based on the understanding that *AIRE* is one of the primary regulators of the autoimmune modulation in human fetuses, *MMP-3* and *CACNA1A* are well-known regulators between maternal and fetus blood supply[11]. Therefore, this study aimed to investigate the methylation profile of the GDM patients of *AIRE, MMP-3*, and *CACNA1G* genes with methylation-specific polymerase chain reaction (MS-PCR).

Patients and Methods

Sample Collection

Forty-four patients diagnosed with GDM, and twenty healthy controls were consecutively selected from those who applied to the obstetrics clinic. The study protocol was approved by the Ethics Committee of the Ondokuz Mayıs University (No: 2021/368), and written informed consent was obtained from all participants. Diabetes mellitus diagnosis has been performed between 24-28 weeks of pregnancy with a 75 gr oral glucose tolerance test as described in 2010 statement from the International Association of Diabetes and Pregnancy Study Groups (IADPSG)[12]. If minimum one of the three values is equal to or higher than the described criteria (fasting plasma glucose ≥ 92 mg/dL, one-hour plasma glucose levels ≥ 180 mg/dL, and two-hour plasma glucose levels ≥ 153 mg/dL) then GDM is diagnosed. A peripheral 3 mL blood was collected for DNA isolation and methylation analyses in the third trimester before delivery from each group of patients. It was also collected to another tube for fasting glucose and Hemoglobin A1c levels. Body mass index (BMI) values were calculated. Patients diagnosed with insulin resistance or active smokers before pregnancy were excluded from the study. Control patients were randomly selected from healthy pregnant women who applied for routine pregnancy follow-up. All experiments were reported according to the STROBE guidelines[13].

Analysis of Specimens

Blood samples were collected from all patients and carefully stored at -80°C until the day of the experiments. DNA was extracted from leukocytes with a commercially available DNA isolation kit (#GB300, Geneaid, New Taipei, Taiwan). The purity and concentrations of DNA samples were determined by a nanodrop spectrophotometer, and DNA samples which contained impurities were excluded from the study.

Bisulfite Modification and Methylation-Specific PCR

Following DNA isolation, bisulfite modification of DNA samples was carried out with EpiJET Bisulfite Conversion Kit (Thermo Fisher Scientific, Waltham, MA, USA), strictly following the manufacturer's instructions.

Methylation-specific PCR was performed for *AIRE*, *CACNA1G*, and *MMP-3* promoter regions with methylation and unmethylation-specific oligonucleotide primer pairs. The primers were designed *via* http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi, and sequences and amplicon lengths were provided in **Supplementary Table I**. MS-PCR was carried out in a final volume of 25 µl containing DreamTaq™ Hot Start DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and under the following cycling conditions for *AIRE*: initial denaturation at 95ºC for 3 minutes, followed by 40 cycles at 95ºC for the 40 seconds; 51ºC for 40 seconds and 72ºC for 70 seconds; *CACNA1G*: initial denaturation at 95ºC for 3 minutes, followed by 40 cycles at 95ºC for 40 seconds; 50ºC for 40 seconds and 72 ºC for 70 seconds; *MMP-3*: initial denaturation at 95ºC for 3 minutes, followed by 40 cycles at 95 ºC for 40 seconds; 57ºC for 40 seconds and 72ºC for 70 seconds. Final extensions were carried out at 72°C for 7 minutes and then maintained at 4ºC. Cells-to-CpG™ Methylated and Unmethylated gDNA Controls (Applied Biosystems, Waltham, MA, USA) were used as positive controls. The methylation status of genes was determined as methylated when amplification products were detected in the reactions performed with primers M or both M and U. When amplification products were seen in the reactions performed with primers, U only was determined as unmethylated.

Statistical Analysis

GraphPad Prism version 9.0 (La Jolla, CA, USA) was used to collect and analyze all experimental data. Gene methylation/unmethylation ratios were regarded as continuous variables. Mean, standard deviation (SD), and median-interquartile range (IQR) were used to express quantitative variables. When the variables were normally distributed, according to the Kolmogorov-Smirnoff test, parametric tests were performed for data analysis. Levene's test was used to determine variance homogeneity, and Welch's correction was utilized when the variances were not homogeneously distributed. Non-parametric tests were used, and the variables were not normally distributed. The independent samples *t*-test and Mann-Whitney U test were used to compare groups. The significance level was established at $p<0.05$.

Results

Characteristics of the Study Population

Laboratory results and demographic information are summarized in Table I. Between the 20 control and 44 GDM patients, there was no significant difference in terms of age (29.2±6.1); (32.1±6.5), ($p=0.103$) respectively. GDM group had higher BMI and HbA1c values than the control group (*p*-values <0.001). Blood glucose values did not differ between groups ($p=0.275$).

AIRE and MMP-3 Promoters Strongly Unmethylated in the GDM

In both groups, the methylation status of the *AIRE* and *MMP-3* promoters was analyzed (Figures 1 and 2). Our results indicated that the methylation level of the *AIRE* promoter was significantly lower in patients with GDM (median-IQR=1.98-3.75) compared to the control group (85.5-12.5) ($p<0.001$) (Figures 1A and 2). Similarly, the methylation status of *MMP-3* promoter was discovered to be significantly lower in gestational diabetes patients (median-IQR=1.10-0.22) compared to controls (4.72-2.27) ($p<0.001$, Figures 1B and 2) (Table II).

CACNA1G promoter methylation levels were found similar between groups. Methylation-specific PCR results demonstrated that there was no significant difference in GDM (mean±SD=1.02±0.68) compared to the control group (mean±SD 1.03±0.14) in *CACNA1* promoter methylation levels ($p=0.930$) (Figures 1C and 2).

Discussion

This study investigated possible changes in the methylation profile of three predefined genes, *AIRE*, *MMP-3*, and *CACNA1G*, in GDM patients. Our results demonstrated that *AIRE* and *MMP-3* are strongly unmethylated in GDM patients. However, in contrast to current knowledge, we did not find any significant difference between experimental groups in the context of *CACNA1G* methylation.

Several studies[1,14,15] indicated that changes in the DNA methylation profile in pregnant women significantly impact the fetus's health. Additionally, metabolic changes suddenly occur during pregnancy, such as GDM, which is also a significant concern in the long-term effects on fetus health[14]. Epigenetic modifications are suspicious elements in the sudden changes seen in pregnancy[15]. Therefore, identifying these possible genes or modifications is crucial for the treatment and follow-up of the health of patients and fetuses. Genome-wide association studies[1] demonstrated that several genes are epigenetically modified, and these modifications were also shown to be changed depending on the tissue type. Previous studies[16-18] demonstrated increased global DNA methylation in the placenta of GDM patients.

Table I. Demographic and clinical features of study and control groups.

	Control (n=20) Mean ± SD	GDM (n=44) Mean ± SD	*t*†	*p*-value
Age	29.2±6.1	32.1±6.5	-1.66	0.103
BMI	26.4±3.5	32.8±5.8	-5.43	**<0.001**
HbA1c	4.7±0.4	4.9±0.5	-21.8	**<0.001**
Glucose	120.1±10.1	193.9±13.6	-1.10	0.275

†Independent samples *t*-test. Bold prints in the *p*-values column indicate a statistically significant difference between groups. n, number; SD, standard deviation; GDM, Gestational Diabetes Mellitus; BMI, Body Mass Index, HbA1c, glycosylated hemoglobin.

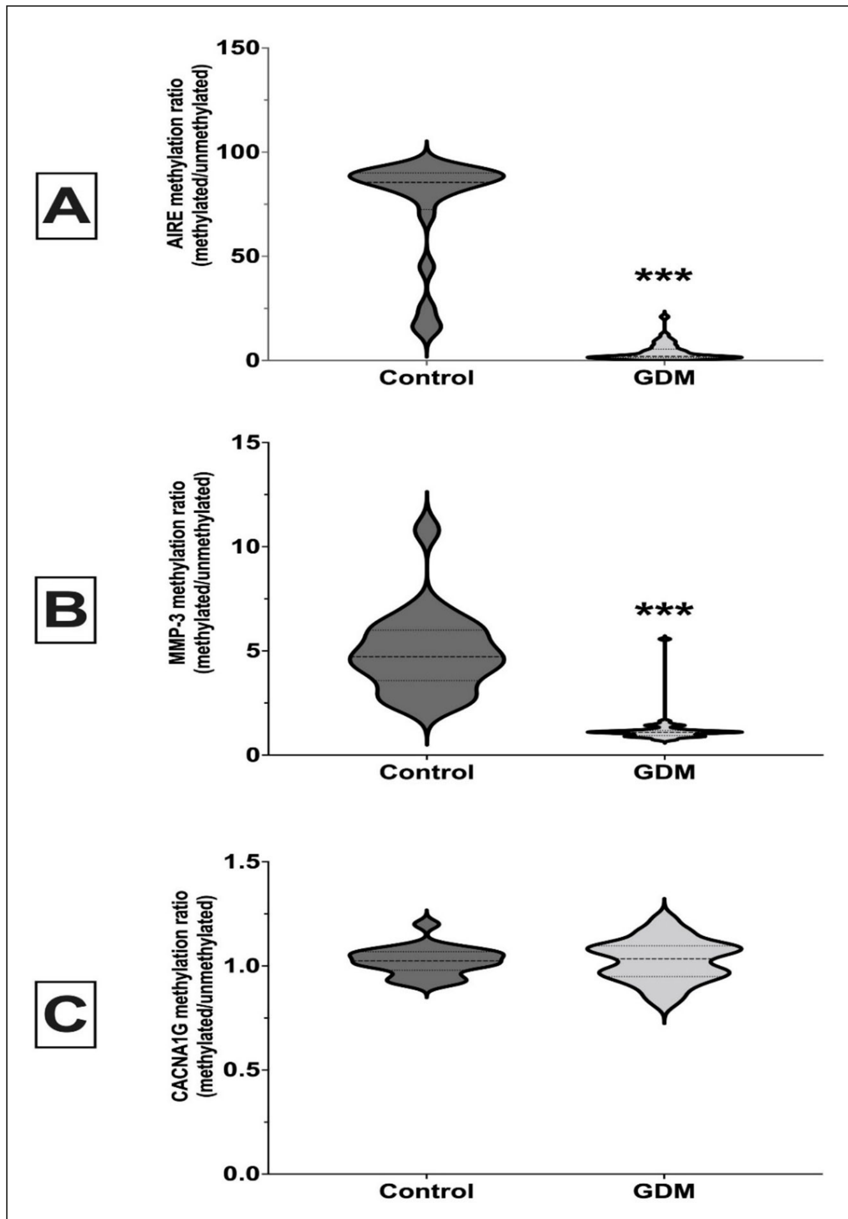

Figure 1. Methylation profile of AIRE (**A**), MMP-3 (**B**), and CACNA1G (**C**) promoter in healthy pregnant and gestational diabetes patients. Data were expressed as a violin box plot. ***p<0.001 *vs.* the control group. bp, base pairs; M, methylated PCR product; U, unmethylated PCR product; m, size marker.

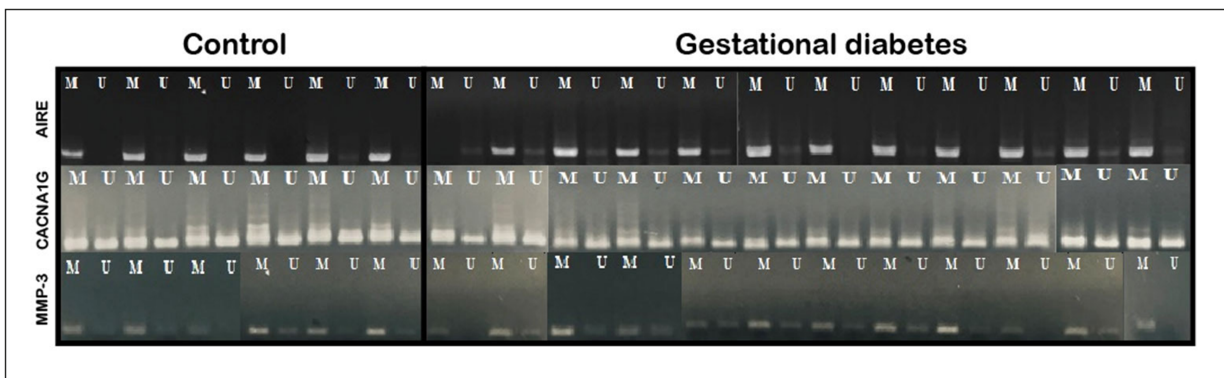

Figure 2. The gel picture stands as representative of all groups. *AIRE*, the *autoimmune regulator* gene; *MMP-3, matrix metalloproteinase-3; CACNA1G, calcium voltage-gated channel subunit alpha1*; M, methylated PCR product; U, unmethylated PCR product.

Table II. Comparison of methylated/unmethylated ratios of *AIRE*, *MMP-3* and *CACNA1G* promoter between groups.

	Control (n=20)		GDM (n=44)			
	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)	Statistic	*p*-value
AIRE	74.2±26.0	85.5 (12.5)	3.99+4.06	1.98 (3.75)	2.0‡	**<0.001**
MMP-3	4.93±1.94	4.72 (2.27)	1.20+0.70	1.10 (0.22)	14.0‡	**<0.001**
CACNA1G	1.02±0.68	1.02 (0.71)	1.03±1.01	1.03 (0.14)	-0.09§	0.930

‡Mann-Whitney U test, §independent samples *t*-test with Welch adjustment. Bold prints in the *p*-values column indicate a statistically significant difference between groups. n, number; SD, standard deviation; IQR, Inter quartile range; GDM, Gestational Diabetes Mellitus; *AIRE*, autoimmune regulator gene; *MMP-3*, matrix metalloproteinase-3; *CACNA1G*, calcium voltage-gated channel subunit alpha1G.

In these studies, several candidate genes were identified, such as fat-cell hormones (leptin and adiponectin), which are involved in the regulation of energy metabolism and body weight, the ATP-binding cassette transporter ABCA1, a primary regulator of cellular cholesterol, the glucose transporters SLC2A1/GLUT1 and SLC2A3/GLUT3, and the imprinted gene *MEST*, which plays a role in adiposity development. Dias et al[19] demonstrated that the *calmodulin-binding transcription activator 1 (CAMTA1)* gene, which regulates insulin metabolism, is strongly regulated by methylation in GDM patients. They also indicated that although methylation is a tissue-specific process, recent studies[20] also demonstrated that peripheral blood reflects several pregnancy-associated changes in DNA methylation. Kang et al[20] also investigated genome-wide changes in the methylation status of diabetes mellitus patients and identified 200 differentially methylated loci mapped to 151 genes. Because these patterns are also affected by the patient population, diet, and phenotype, there is still a need for further studies on the different patient populations.

The *autoimmune regulator (AIRE)* gene is located on chromosome 21 (21q22.3) and is crucial to the development of central tolerance. The medulla (inner region) of the thymus expresses the transcription factor *AIRE*. It is a component of the mechanism that destroys T cells with autoimmune tendencies. It makes normal, healthy proteins from every part of the body available to T cells, and any T cells that react to those proteins are eliminated. *AIRE* gene mutations cause a peripheral Treg deficiency and hinder the central elimination of self-reactive T cells. Therefore, it has an essential role in autoimmune diseases, especially in the endocrine system leading to the development even at young ages[21].

Autoimmune Polyglandular Syndrome (APS) type 1 is a rare recessive hereditary disease caused by the *AIRE* gene mutation and is characterized by three principal manifestations: chronic mucocutaneous candidiasis, chronic hypoparathyroidism, and Addison's syndrome. APS has been linked to various autoimmune illnesses and can affect several endocrine glands[22]. *AIRE* is a well-known component of tolerance to self-antigens. Our results suggest that GDM patients with unmethylated *AIRE* may be predisposed to other multiple autoimmune endocrinopathies.

Based on its role in autoimmunity, the *AIRE* gene is also related to diabetes mellitus, and its pathophysiology is associated with the self-destructing antigens of insulin-secreting cells in the pancreas[23]. Based on this knowledge, the possible role of the *AIRE* gene and its epigenetic modifications could give us clues about the progress of GDM and its long-term metabolic effects on the fetus. Best of our knowledge, the methylation status of the *AIRE* gene is not investigated in GDM patients. So, our study demonstrated that the *AIRE* gene is strongly regulated by methylation and found to be unmethylated in GDM patients. To demonstrate the changes in the methylation status of *AIRE* caused by diabetes or pregnancy, the methylation status of healthy pregnant women was also studied and as predicted, was found to be highly methylated.

As a second objective, we also investigated the methylation status of the *MMP-3* gene, which is a well-known regulator of vascular changes between mother and fetus. Our findings indicate that *MMP-3* is highly unmethylated in patients with diabetes. Baugh et al[24] evaluated the role of *MMP-3*, among other parameters, in the process of coronary plaque rupture caused by hyperglycemia associated with type 2 diabetes. Peeters et al[25] discovered a greater level of *MMP-3* in individuals with albuminuria

during the course of type 1 diabetes, which may imply a role in the etiology of problems caused by impairments in the regulation of extracellular matrix remodeling. There are publications that correlate *MMP-3* expression with microvascular events in patients with type 2 diabetes[26,27]. Since *MMP-3* methylation was impaired in the GDM group in our study, these patients may be prone to microvascular complications and abortion.

We also investigated the possible change in the methylation status of the *CACNA1G* (*calcium voltage-gated channel subunit alpha1 G*) promoter. The *CACNA1G* is located on chromosome 19p13, which encodes the main subunit (1A) of the neuronal P/Q type voltage-gated calcium-ion channel[28]. Even though *CACNA1G* has been linked to both GDM[29], we were unable to find a significant difference in methylation status between GDM patients and controls. This gene can be studied again in larger series and further studies.

Conclusions

In conclusion, our study demonstrated the alteration of *AIRE* and *MMP-3* promoter methylation profiles in GDM. These results suggest that changes in the methylation profile of *AIRE* and *MMP-3* might play a role in the long-term consequence of GDM. Therefore, additional research is required to uncover mechanisms and pathways that modulate *AIRE* and *MMP-3* genes as potential therapeutic targets.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Informed Consent

Informed consent was obtained from all individual participants included in the study.

Ethical Approval

Ethics approval was obtained from the Ethic Committee of Ondokuz Mayıs University (No.: 2021/368). The research was performed in accordance with relevant guidelines/regulations of the Helsinki Declaration.

Authors' Contributions

UC designed the study, collected, analyzed, and interpreted data, and draft the main manuscript. ZBC conducted all experiments, analyzed and interpreted data, and reviewed the manuscript. The authors read and approved the final manuscript.

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Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

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