

Identification DNA Methylation Change of ABCC8 Gene in Type 2 Diabetes Mellitus as Predictive Biomarkers

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Abstract—Type 2 diabetes mellitus is the most common chronic endocrine disorder that affecting 5%–10% of adults globally. Recently, the disease has rapidly spread throughout the Kurdistan Region. This study investigates DNA methylation status in the ABCC8 gene among the study population, and it possibly used as a biomarker. One hundred and thirteen individuals were included in this study, and they were divided into three categories (47 diabetes, 36 prediabetic, and 30 controls). Blood samples were collected to investigate DNA methylation status in patients who attended private clinical sectors in Koya city, Kurdistan Region of Iraq, between August and December 2021. Methylation-specific PCR (MSP) uses paired primers for each methylated and unmethylated region. In addition, the X2 Kruskal–Wallis statistical and Wilcoxon signed-rank tests were run with a significance level of $p < 0.05$. In comparison to the healthy group, hypermethylation of DNA is detected in the promoter region of diabetes and prediabetes. In addition, age, gender, BMI, alcohol use, family history, and physical activity all influence the degree of DNA methylation in people who have had coronavirus illness. The above-mentioned findings suggest that DNA methylation alterations in the ABCC8 promoter region might be exploited as a possible predictive biomarker for type 2 diabetes mellitus diagnosis.

Index Terms—ABCC8 gene, Diabetes mellitus, DNA methylation, Methylated primer, Unmethylated primer.

I. INTRODUCTION

Diabetes mellitus (DM) is a leading cause of death and disability worldwide, affecting 415 million people in 2017, and this is expected to increase to 592 million by 2035 (Willmer, et al., 2018). The disease is the most common chronic endocrine disorder, affecting 5%–10% of adults worldwide. Type 2 diabetes mellitus (T2DM) is associated with resistance to

insulin action and inadequate compensatory insulin secretory response (ADA 2019). Type 2 diabetes affects both the old and the youth (Asimwe, Mauti and Kiconco, 2020).

The study of epigenetic mechanisms can provide novel insights into the pathophysiology of diabetes and its complications, which may result in the identification of new drug targets. In addition, the investigation of associations with DNA methylation (DNAm) in peripheral blood may identify novel biomarkers for noninvasive early disease detection since P.B. (Florath, et al., 2016). Multiple genes and their interactions are involved in the insulin secretion pathway. For example, sulfonylurea receptor encoded by ABCC8 (ATP Binding Cassette Subfamily C Member 8) gene, together with inward-rectifier potassium ion channel (Kir6.2), regulates insulin secretion by ATP-sensitive K⁺ (KATP) channel located in the plasma membranes (Haghverdizadeh, et al., 2014). In T2D, the ABCC8 gene is an essential target for candidate gene association studies. Single nucleotide polymorphism (SNP) in these genes has been associated with metabolic syndrome features across various populations (Matharoo, Arora and Bhanwer, 2013).

II. MATERIAL AND METHODS

A. Study Design and Sample Collection

A cross-sectional study consisting of 113 blood samples was collected from both men and women who visited clinical sectors in the Koya city Kurdistan area of Iraq between August and December 2021 after taking approval and instructions institutional of the scientific Committee. The study population was divided into 47 diabetes, 36 prediabetes, and 30 apparently healthy individuals according to medical history and clinical and laboratory examination. All blood samples were placed in a test tube containing anticoagulant ethylene diamine tetraacetic acid (EDTA) to prevent clotting of the blood (Smail, 2016), which were diagnosed by the HbA1c analyzer, (HbA1c) parameter to determine the prediabetes and diabetic groups. For people without diabetes, the normal range for the hemoglobin A1C level is between 4% and 5.6%. Hemoglobin A1C levels between 5.7% and 6.4% mean you have a higher chance of getting

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diabetes. Levels of 6.5% or higher mean you have diabetes. Besides the blood collection, also asked the participants about age, gender, body mass index, smoking, family history, and alcohol with measured random blood sugar (RBS).

B. DNA Extraction

The ReliaPrep™ Blood gDNA Miniprep System performed DNA extraction (Cat. No. A5081, Promega Compromega, USA) according to the manufacturer's instructions, briefly included four essential steps, homogenizing the whole blood to release. The DNA, Binding DNA to the ReliaPrep™ Binding Column, Removing impurities with wash solution repeated three times, and Eluting purified DNA

C. Bisulfite DNA Conversion

MethylEdge™ Bisulfite Conversion System (Promega Compromega, USA) was performed according to manufacturer's instructions (N1301). The bisulfite-converted reaction was based on the unmethylated cytosine converted to uracil whereas methylated cytosine was unaffected.

D. Determination DNA Quality and Quantities

Typical nucleic acid concentration ranges for direct A280 absorbance measurements using nanodrops were used to determine quantities of bisulfite-converted DNA. The sample type was set to RNA-40 because bisulfite-converted DNA contains uracil and is widely single-stranded. DNA samples (100 ng of each sample) were run on the 1% agarose gel and stained with Diamond Nucleic™ Acid Dye. To evaluate the level of fragmentation following conversion, but nanodrop was set to DNA-50 to estimate DNA concentration before bisulfite conversion and stained with ethidium bromide

E. Methylation Specific PCR Primer

MethPrimer is a tool for creating methylation PCR primers based on bisulfite conversion. It is currently possible to develop methylation-specific PCR, as it is shown in Table I.

PCR products were analyzed on 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under U.V. illumination, as shown in Tables II and III. The methylation band was present at 135 base pairs and the unmethylation band at 136 base pairs. Control reactions were prepared using pre-qualified methylated DNA from a source similar to the source of the experimental samples. Converted Methylated Human Control has been bisulfite-converted. High percent methylation of CpG sites run opposite with samples (N1231 Promega Compromega, USA), as demonstrated in Table IV.

F. Statistical Analysis

Methylation-specific PCR results were determined by deviation from the Normal pattern. Next, the difference in methylation percentage expression between all groups and normal ones was analyzed. According to Shapiro–Wilk and Kolmogorov–Smirnov's nonparametric test, the study data were nonparametric (Non-Normal Distribution) using the Kruskal–Wallis and Wilcoxon signed-rank test. For categorical variables, frequencies were chosen. Descriptive

statistics (mean and standard deviation) are used for continuous variables. The receiver operating characteristic curve (ROC curve) estimated the biomarker test. $P < 0.05$ was considered statistically significant. In addition, GraphPad Prism 8 was used for statistical data analysis.

III. RESULTS

Several variables were studied; body mass index random blood sugar and hemoglobin A1c showed highly significant differences ($P = 0.000$), significant differences were noticed within age, gender, and alcohol, while non-significant with others as shown in Table V.

TABLE I
PRIMER SEQUENCE FOR METHYLATION-SPECIFIC PCR

NoPrimer	Length	Product size	Sequence (5'→3')
1 Forward methylated primer	24	135	TTTGTGTGAAAGTGTTAGATACGT
2 Reverse methylated primer	25	135	AATAAAAAAAAAATAAACAAAACGTT
3 Forward un methylated primer	25	136	TTTTGTGTGAAAGTGTTAGATATGT
4 Reverse un methylated primer	25	136	AATAAAAAAAAAATAAACAAAACATT

TABLE II
PREPARE THE PCR MASTER MIX WITH THE PRIMER SETS SPECIFIC TO THE METHYLATED PRIMER FOR BISULFITE CONVERTED DNA

Component	Volume (μl)	Final concentration
2X PCR master mix	12.5	1X
M forward primer	1	10 picomole
M reverse primer	1	10 picomole
DNA template	3	10-50 ng
DNA nuclease-free water	7.5	The final volume of 25 μl

TABLE III
PREPARE THE PCR MASTER MIX WITH THE PRIMER SETS SPECIFIC TO THE UNMETHYLATED PRIMER FOR BISULFITE CONVERTED DNA

Component	Volume (μl)	Final concentration
2X PCR Master Mix	12.5	1X
U forward primer	1	10 picomole
U reverse primer	1	10 picomole
DNA template	3	10-50 ng
DNA nuclease-free water	7.5	The final volume of 25 μl

TABLE IV
CYCLING CONDITIONS FOR METHYLATION-SPECIFIC PCR OF BISULFITE-CONVERTED DNA

Steps	Temperature	Time	Number of cycle
Enzyme inactivation	95°C	5 min	1
Denaturation	95°C	30 s	35
Annealing	52°C	30 s	35
Extension	72°C	30 s	35
Holder time	72°C	5 min	1

Regarding the methylation status, methylated was more frequent in diabetes and prediabetes than in the control group with 23, 23, and 10 cases, respectively, while 10, 7, and 17 cases in unmethylated. On the other hand, the methylated and methylated as 14 and 6 then 3 cases in control with highly significant differences ($P = 0.000$) as shown in Table VI.

In the same group association between DNA methylation status and some clinicopathologic characteristics showed that there was highly significant between DNA methylation and HbA1c and as well as blood sugar with highly significant differences ($P=0.000$), whereas less significant was observed among other parametric such as smoking, age, and alcohol as shown in Table VII.

IV. DISCUSSION

The present study has investigated diabetes-related ABCC8 methylation qualitatively using methylation-specific PCR on blood. Interestingly, ABCC8 methylation was detected at a high frequency, which was 23 (48.9%)out of 47 samples of diabetes, 23(63.9%) prediabetes out of 36 samples, and 10 (33.3%) of control out of 30 samples limited studies have done regards identifying ABCC8 gene methylation in Kurdistan region of Iraq. DNAm marks mainly occur at the 5' position of the cytosine residues of cytidine-guanine dinucleotides (CpG). Clustered CpG dinucleotides forming dense repeat sequences in the genome are termed CpG islands. Islands are located especially in promoter regions. However, they can also be found in intragenic and enhancer

regions (Jones 2012). Many studies have revealed that DNAm also occurs at sites other than the CpG sequences (non-CpG methylation). Non-CpG methylation has been suggested to be prevalent in human embryonic stem cells and the brain. (Jang, et al., 2017). Methylation of cytosine residues in DNA (DNA methylation) are the most studied epigenetic trait. Recent data show significant influences of age and lifestyle-related risk factors such as overweight and physical activity on site-specific DNAm in blood and tissues relevant to T2D (Dayeh, et al., 2014; Smail, 2019).

Methylation-specific PCR can rapidly determine virtually any cytosine methylation status from CpG sites within a CpG island. MSP requires very small amounts of DNA, is susceptible to 0.1% of a given CpG island locus methylated alleles and can be carried out in paraffin-embedded DNA extracted samples (Herman, et al., 1996). Nested MSP can be conducted if an experiment could not amplify the product abundantly for analysis by direct MSP. A different primer set is required for Nested MSP, which covers the amplified product sequence with two selected pairs of primers. A second PCR with two pairs of primers (each primer set for different methylation states) is carried out after the first PCR with nested MSP primers using the amplified products from the first PCR (Hanaei, et al., 2020). In the current study, there were significant differences between DNA methylation changes in diabetes and prediabetes with control, as shown in Table VI; there was a strong relationship between DNA methylation in all three groups; the p-value was 0.002. Our data demonstrated that higher DNA methylation was detected in females in diabetes and prediabetes groups.

In contrast, minimum DNA methylation was recorded in the male of the healthy group (Table VII). Identifying CpG loci of which DNAm levels are under genetic control or are related to age or gender will facilitate further studies into the role of DNAm and disease (Boks, et al., 2009). Associations between age acceleration and body mass index, total cholesterol to high-density lipoprotein cholesterol ratios, socioeconomic status, high blood pressure, and smoking behavior (McCartney, et al., 2018). With the advent of microarray technology, it became possible to assess many specific genomic sites for age-related changes in DNAm. Microarray studies confirmed a decrease in DNAmwith age, whereas site-specific analysis indicated an increase in variability of DNA methylation with age (Jones, et al., 2015). Furthermore, demonstrate that genotype state at methylation quantitative trait loci (meQTLs) can affect some age-associated CpG sites (Gopalan, et al., 2017).

The statistical analysis showed an association between MSP results with BMI, HbA1c, and random blood sugar, and the p-value was 0.000, as analyzed in Table VII. Moreover, recent studies have shown that changes in body weight and increased physical activity seem to impact the DNAm of certain genes related to T2D (de Mello, et al., 2014). Thus, the search for new biomarkers is ongoing. There is great interest in epigenetic biomarkers such as DNAm, which can influence the environment and potentially improve T2D prediction (Dayeh, et al., 2016). Data demonstrate highly significant correlations between DNAm and the most important risk

TABLE V
GENERAL CHARACTERISTICS OF THE STUDIED POPULATION

Variable	Diabetes (47)	Prediabetes (36)	Control (30)	P value
Age	49.4±12.5	46.4±11.4	41±10.6	0.012
Gender				
Male	(18) 38.3%	16 (44.4%)	21 (70%)	0.022
Female	(29) 61.7	20 (50.6%)	9 (30%)	
BMI (body max index)	30.5±2.8	26±4.1	23.8±4.3	0.000
Smoking	5 (10.6)	5 (13.9%)	5 (16.7)	0.74
Family history	7 (14.9%)	2 (5.6%)	5 (16.7%)	0.316
Covid 19	7 (14.9%)	9 (25%)	3 (10%)	0.244
alcohol	1 (2.1%)	0 (0%)	4 (13.3%)	0.020
Physical Activity	13 (27.7%)	13 (36.1%)	15 (50%)	0.141
RBC (random blood sugar) mg/dL	248.7±84.6	145.8±26.8	113.1±23.7	0.000
HBA1C (hemoglobin A1c)%	9.5±2	6.1±0.3	4.7±0.4	0.000

Sig=($P\leq 0.05$), NS: ($P\geq 0.05$) using Kruskal–Wallis test.

TABLE VI
CORRELATION METHYLATION STATUS WITH THE CONTROL GROUPS

Groups	Methylated state			Total (%)	P value
	Unmethylated (100%)	Methylated (100%)	Methylated and methylated (partially methylated) (%)		
Diabetes	10 (21.3)	23 (48.9)	14 (29.8)	47 (100)	0.002
Prediabests	7 (19.4)	23 (63.9)	6 (16.7)	36 (100)	
Control	17 (56.7)	10 (33.3)	3 (10)	30 (100)	

Sig= ($P\leq 0.05$) using Kruskal–Wallis test.

TABLE VII
ASSOCIATION OF PROMOTER REGION METHYLATION CLINIC PATHOLOGIC CHARACTERISTICS WITHIN SAME GROUPS

	Diabetes				Prediabetes				Control			
	U	M	UM	P value	U	M	Um	P value	U	M	UM	P value
Age	54.1±10.8	44.4±8.7	54.1±15.9	0.000	52.2±11.8	45.3±11.9	43.8±7.9	0.000	40.9±7.2	36.5±7.4	56.6±22.1	0.000
Gender												
Male	1	9	8	0.003	2	11	3	0.007	13	0	0	0.106
Female	9	14	6		5	12	3		4	10	3	
BMI (body max index)	18±4.2	30.7±1.8	32.2±1.8	0.000	25.4±2.2	26.7±4.5	24.3±4.2	0.000	25.0±15.8	21.7±3.3	24.2±7.5	0.000
Smoking	0	3	2	0.106	1	3	1	0.346	2	2	1	0.061
Family history	1	4	2	0.055	0	2	0	0.796	3	2	0	0.039
Covid 19	1	3	3	0.061	1	6	2	0.101	2	1	0	0.012
Alcohol	0	1	0	0.317	0	0	0	0.782	3	1	0	0.018
Physical activity	1	10	2	0.006	3	8	2	0.014	12	1	2	0.755
RBC (random blood sugar) mg/dL	233.9±91.5	243.3±91.8	268.2±67.9	0.000	148.8±5.7	144.8±31	146±274	0.000	114.6±22.6	102.7±21.4	139±13.0	0.000
HbA1c (hemoglobin A1c)%	9.1±2.5	8.9±1.2	10.6±5.7	0.000	6.3±0.1	6.0±0.0	6.0±0.2	0.000	4.9±0.3	4.7±0.2	4.8±0.2	0.000

M: Methylated, U: Unmethylated, UM: Unmethylated and methylated (partial), significant difference Wilcoxon signed-rank test

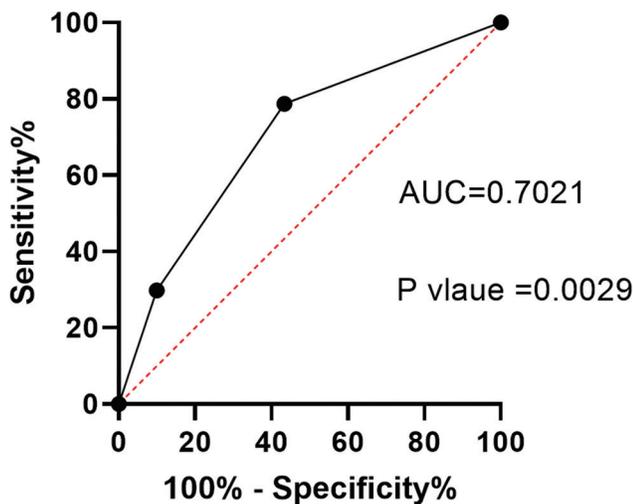


Fig. 1: ROC curve of MSP results in diabetes.

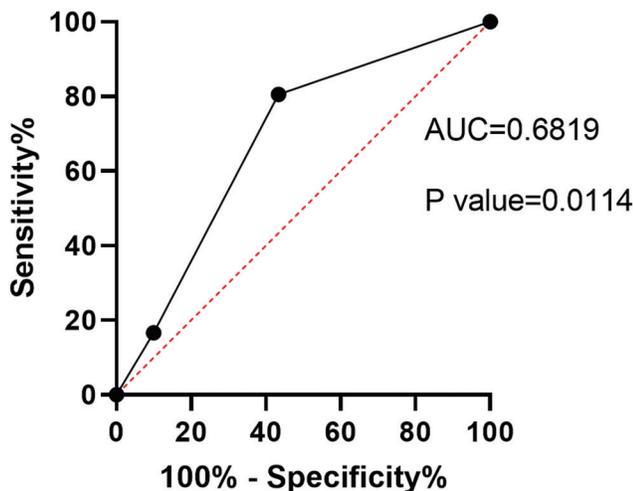


Fig. 2: ROC curve of MSP results in prediabetes.

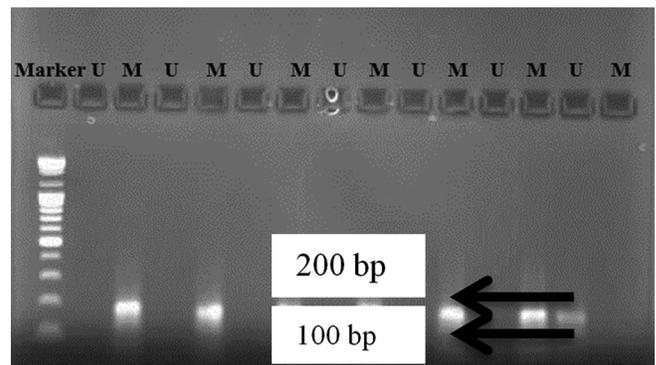


Fig. 3: Methylation-specific PCR (MSP) analysis of ABCC8 gene. *Lane 1: DNA Marker ((3kb. DNA ladder). Agarose gel electrophoresis (2%) of MSP Products are both an excellent demonstration. M refers to a methylated specific primer; U refers to an unmethylated-specific primer. The expected product size was 136 bp for unmethylated primer and 135 bp for methylated primer.

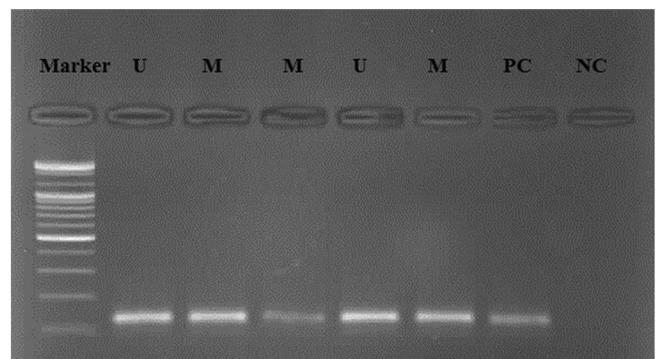


Fig. 4: Methylation-specific PCR (MSP) analysis of ABCC8 gene. *Lane 1: DNA Marker ((3kb. DNA ladder). Agarose gel electrophoresis (2%) of MSP Products is both an excellent demonstration. M refers as a methylated specific primer; U refers to unmethylated-specific primer. P.C.: Positive control(Methylated Human Control) and NC: negative control (Distil water) The expected product size was 136 bp for unmethylated primer and 135 bp for methylated primer.

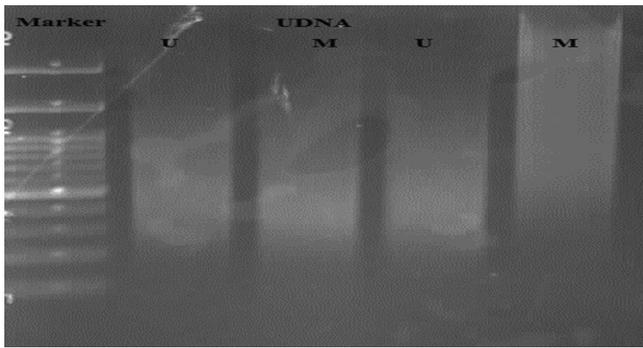


Fig. 5: Methylation-specific PCR (MSP) analysis of ABCC8 gene.

*Lanes 1 and 6: DNA marker ((3kb. DNA ladder). Agarose gel electrophoresis (2%) MSP products are an excellent demonstration. M refers to methylated-specific primer; U refers to unmethylated-specific primer. Using unconverted DNA (UDNA) instead of converted DNA demonstrated that the primer was only specific for the converted DNA; the agarose gel showed not any PCR product either for methylated or unmethylated ABCC8 gene.

factors of T2D, including age and body mass index, in blood and human tissues relevant to insulin resistance and T2D. Furthermore, T2D patients and individuals with increased risk of the disease display differential DNAm profiles and plasticity compared to controls (Gillberg and Ling, 2015). ABCC8 ROC curve showed that both are under the curve and the p-value was significant for both diabetes and prediabetes groups, as illustrated in Figs. 1 and 2. The methylation-specific PCR primer, demonstrated in Figs. 3-5, shows that the size of the product is 136 bp for unmethylated primer and 135 bp for a methylated primer in addition to the methylated control. However, the MSP primers have not been amplified unconverted DNA, and negative control (distill water).

V. CONCLUSION

Increased DNA methylation in type 2 diabetes patients may have correlated with pathogenesis and developed symptoms of diabetes, and the results of this study demonstrated that the hypermethylation of the ABCC8 gene is associated with BMI, HbA1c, and Random blood sugar. Industrially may be used commercial kits to diagnose diabetes in its early stages.

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