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Effects of metformin on *TCF7L2* gene DNA methylation alterations in individuals with Type 2 diabetes mellitus

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ABSTRACT:

DNA methylation is an epigenetic factor that controls gene expression and contributes to diseases such as type 2 diabetes mellitus. The diabetic treatment metformin contributes to the DNA methylation level of the transcription Factor T-cell factor-7 like-2 (*TCF7L2*) in diabetic patients. *TCF7L2* is a transcription factor that regulates the expression of genes involved in lipid and glucose metabolism, such as insulin production in pancreatic β-cells and Glucagon in α-cells through Wnt/β-catenin.

We aimed to determine the effect of Metformin on the DNA methylation level of gene *TCF7L2* in T2DM. For this purpose, fifty blood samples (25 type 2 diabetes mellitus taking Metformin and 25 without taking Metformin) were collected from both genders from Koya City, Kurdistan region of Iraq, from August 2021 to December 2021 in a private laboratory from diabetic patients confirmed by HbA1c blood test.

DNA methylation levels were determined by using methylation-specific PCR(MSP); for this purpose, a couple of the primers for amplification of both methylated and unmethylated regions were used for the promoter region for detecting the methylation level of CpG sites at (-99, -119, -123, -246, 266). For statistical analysis, the Mann–Whitney U test and Spearman's correlations were utilized.

The results showed no significant difference in DNA methylation levels between metformin users (48% methylated) and non-users (52% methylated) ($p = 0.155$). Additionally, ROC analysis (AUC = 0.6, $p = 0.1$) was statistically insignificant.

Based on the TCF7L2 gene of the T2DM patients, we can conclude that there was no correlation between the use of metformin and DNA methylation level.

Keywords: *TCF7L2* gene, metformin, T2DM, DNA methylation, Methylation-specific PCR.

1 INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia. It could be related to decreased insulin secretion, insulin resistance in the peripheral tissues, or both. According to the International Diabetes Federation (IDF), approximately 415 million individuals aged between 20 to 79 had diabetes mellitus in 2015 [1], [2]. Cases of type 2 diabetes mellitus (T2DM) have been steadily increasing worldwide, particularly in the 21st century. The International Diabetes Federation estimates that one in ten people will have diabetes [3].

Genetic predisposition is a major factor in the risk of developing T2DM. Over the last decade, multiple T2DM genome-wide association studies have highlighted the complex polygenic nature of T2DM, where most genes increase T2DM risk through main effects on insulin production, and a minority contributed to the insulin mechanism of action [4]. The *TCF7L2* gene encodes a transcription factor essential for insulin expression and is strongly associated with T2DM susceptibility[5].

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TCF7L2 polymorphisms are associated with reduced insulin secretion. The *TCF7L2* gene regulates the expression of the proglucagon gene, which encodes glucagon-like peptide-1 (GLP-1), which increases insulin secretion and decreases glucagon release, both critical for blood glucose regulation [6].

Metformin differentially influences multiple aspects of insulin sensitivity and glucose metabolism, which is the first-line oral treatment for type 2 diabetes [7]. This variability may complicate the management and diagnosis of T2DM. Metformin modifies DNA methylation patterns that may underpin its antidiabetic effects[8], [9].

Metformin exerts genome-wide effects on DNA methylation, influencing distinct genomic regions. Furthermore, metformin has been linked to alterations in epigenetic aging of peripheral tissues, which may influence aging-related processes[10].

Metformin has been associated with decreased DNA methylation of genes encoding metformin transporters in the human liver, including TCF7L2 [11]. In addition, the TCF7L2 gene rs7903146 polymorphism affects insulin production and has been strongly linked to type 2 diabetes. Based on this evidence, we hypothesize a potential connection between metformin and *TCF7L2* [12], [13]. This study aims to explore how metformin exposure affects the level of DNA methylation in the *TCF7L2* gene among patients with type 2 diabetes mellitus.

2 MATERIAL AND METHODS

2.1 BLOOD COLLECTION

Fifty blood samples from people with T2DM were collected in Koya City, Kurdistan Region of Iraq, between August and December 2021. In addition to age and gender, several clinical parameters were recorded. The main limitation of this study is the sample size, relatively small sample size, and limited extent to which the findings cannot be generalized for a large population.

A total of 5 mL of blood was collected in an EDTA tube from two groups: 25 samples from people who were taking metformin (10 males and 15 females) at least twice per day, and 25 who were not taking metformin (10 males and 15 females). Glycated hemoglobin (HbA1c) was used to confirm the diagnosis of T2DM. The mean HbA1c level among participants taking metformin was 8.3%, compared with 9.3% in those not taking metformin. The study protocol was approved by the Ethics Committee of the Faculty of Science and Health, Koya University, in accordance with institutional guidelines for human research. Informed consent was obtained from all participants prior to blood collection

2.2 DNA EXTRACTION AND BISULFITE CONVERSION

DNA was extracted from the blood samples using the ReliaPrep™ Blood gDNA Miniprep System (A5081; Promega, Madison, USA) according to the manufacturer's instructions. The procedure included homogenization of blood, binding DNA to the ReliaPrep™ Binding Column, washing three times to remove contaminants, and elution of purified DNA.

DNA concentration was quantified using a NanoDrop spectrophotometer, and the quality of the DNA sample was detected by using agarose gel electrophoresis.

2.3 BISULFITE CONVERSION

Following DNA extraction, bisulfite conversion was performed using the MethylEdge Bisulfite Conversion System from Promega (N1301; Promega, Madison, USA) which converts unmethylated cytosines to uracil, while leaving methylated cytosines unchanged. This kit offers a speed protocol and complete conversion, ensuring that the DNA is completely converted for downstream assays following the Promega protocol for the MethylEdge Bisulfite Conversion System.

2.4 PRIMER DESIGNING

A 171 bp fragment (methylated) and a 170 bp fragment (unmethylated) of the TCF7L2 promoter region (positions –99 to –269 [14] .Two sets of primers designed for unmethylated CpG and methylated CpG are as follows:

Methylated forward 25bp primer (3`TTTCGGAGTAAGTTTTTGTATTTTC 5`)

Methylated reverse 24 bp primer (3`GACTAACAATAATCCTTTCGAACG5`)

Unmethylated forward 25bp primer (3`TTGGAGTAAGTTTTTGTATTTTTGT5`)

Unmethylated Reverse 26 bp primer (3`CAACTAACAATAATCCTTTCAAACAC5`).

The forward primer region contained two CpG sites the motif for the transcription factor Sp1 at the sequence (CCCGGG) which methylation blocks SP1 binding if methylation occurs within/near its binding site, the motif of KLF4 at DNA sequence (CACCC) is sensitive to methylated CpG within or near its binding sites and the site of the reverse primer, there are three CpG sites (-99, 119, 123); CpG-123 is the motif of the transcription factor Sp1 at the

sequence of the GC box (CCCGGG). Typically, methylation blocks SP1 binding if methylation occurs within/near its binding site.

2.5 AMPLIFICATION OF THE TCF7L2 GENE

Following the manufacturer's instructions, the Add Start Taq master mix PCR kit (addbio, Korea) was used. Methylation-specific PCR technique was used to perform the PCR amplification reaction.

The PCR reaction mixture had a final volume of 12.5 µL : 3 µL of DNA sample, 7.5 µL of nuclease-free water, and 1 µL of 10 pmol of each of the subsequent primers were used in the PCR experiment.

Five minutes of initial denaturation, thirty seconds of denaturation at 95 °C, thirty seconds of annealing at 54 °C, thirty seconds of extension at 72 °C, and four minutes for the final extension at 72 °C were all set up in the thermocycler (Bio-Rad, USA).

2.6 DETECTING PCR PRODUCT BY GEL ELECTROPHORESIS

The PCR products 4.0 µL were then placed onto a 2% agarose gel that contained 3 µL of ethidium bromide (Addbio, Korea) and dissolved in 1× TBE buffer for analysis. A 100 bp DNA ladder was used to observe the PCR amplicons' migration bands. The electrophoreses were performed at 120 volts for 20 minutes

2.7 STATISTICAL ANALYSIS

The MSP results were evaluated by looking at methylation patterns. Additionally, the variation in methylation % between the groups was detected. Because of the non-normal distribution, nonparametric tests (Shapiro-Wilk and Kolmogorov-Smirnov) were performed to assess the statistical significance of the data. For this task, the Spearman's correlation and the Mann-Whitney U test were used. Concerning categorical variables, frequencies were chosen. Significant data was indicated by a p-value of less than 0.05. The analysis was performed with version 8 of GraphPad Prism[15]

3 RESULTS

This study aimed to investigate the association between DNA methylation of the TCF7L2 gene in two groups of patients with type 2 diabetes mellitus (T2DM): one group receiving daily metformin treatment and another group not receiving metformin, as determined by methylation-specific PCR (MSP), as shown in Figure 1.

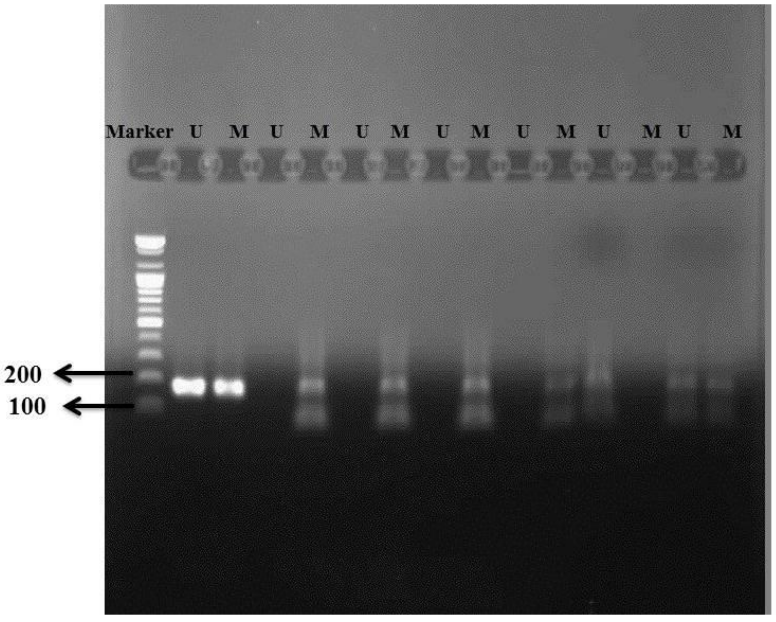

FIGURE 1. Methylation-specific PCR (MSP) amplification of the *TCF7L2* gene in both groups of T2DM patients. PCR products were separated on a 2% agarose gel. Lane 1: DNA size marker (100 bp ladder). Bands corresponding to both unmethylated (U, 170 bp) and methylated (M, 171 bp) products were observed.

Our result showed that there were no correlations between DNA methylation change and metformin using as shown in Table I), and the *p-value* was statistically non-significant, it was 0.155.

Table 1. Association of TCF7L2 gene methylation status between T2DM patients receiving metformin and those not receiving metformin.

Groups	Methylation cases				P value
	Methylated (100%)	UnMethylated (100%)	Partial methylated (0.1 to 99.9%)	Total	
T2D taking Metformin	12(48%)	2(8%)	11(44%)	25(100%)	0.155
T2D without taking Metformin	13(52%)	5(20%)	7(28%)	25(100%)	

In our result, both groups with type 2 diabetes showed insignificant DNA methylation alteration according to age as showed in the figure 2, however, metformin users show higher methylation in younger groups (<40, 40-49) while non-metformin users have more unmethylation in the 50-59 age group. On the other hand, Older patients (≥60) showed similar methylation level regardless of metformin use. Furthermore, as seen in figure 3, there was no discernible impact of metformin use on methylation levels by gender.

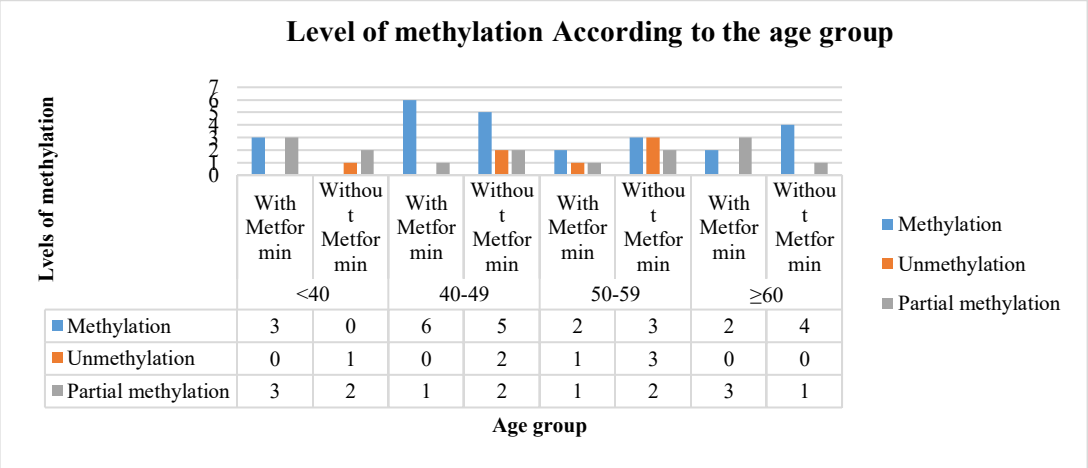

FIGURE 2. Distribution of DNA methylation levels (methylated, partially methylated, and unmethylated) in patients stratified by age group (<40, 40-49, 50-59, ≥60 years) and metformin use.

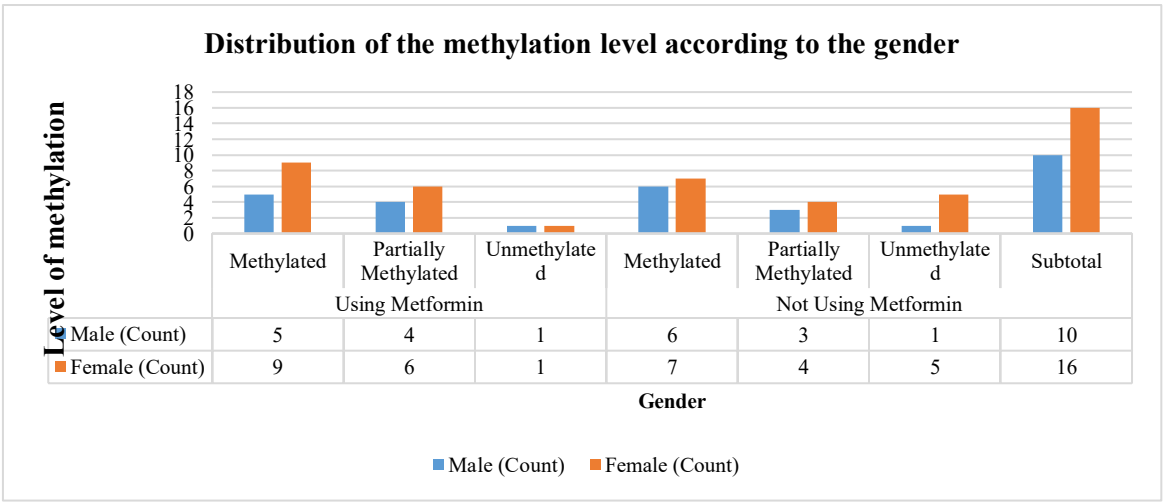

FIGURE 3. Methylation Status by Gender and Metformin Use.

Both methylated and unmethylated regions' PCR products from methylation-specific PCR. The statistical study used the Mann-Whitney U test. Statistical significance is indicated by a p-value of less than 0.05. As seen in figure 4, the *p-*

value of 0.155 in this case suggests that there is no discernible difference in the methylation state of the *TCF7L2* gene between T2D patients taking Metformin and those who do not.

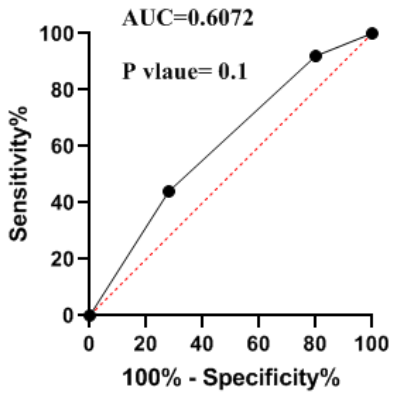

Figure 4. Receiver operating characteristic curve (ROC) for DNA methylation status of the *TCF7L2* gene quantified by methylation-specific PCR (MSP) for diabetes patients taking metformin and not taking metformin. With an area under the curve (AUC) of 0.6 and a *p-value* of 0.1, the graph indicates that there is no statistically significant difference between those with type 2 diabetes who are taking metformin and those who are T2D and not taking it.

4 DISCUSSION

For type 2 diabetes, metformin is the first-line medication used worldwide. Metformin's primary effects are thought to be a reduction in hepatic gluconeogenesis and an increase in peripheral tissue glucose absorption via AMP-activated protein kinase (AMPK) activation[9]. Metformin is generally acknowledged as a main treatment in the global therapy of type 2 diabetes mellitus (T2DM), and has been on the World Health Organization's list of essential medications since 2019. Its low cost, high tolerability, and safety profile, with little risk of hypoglycemia, make it a favorite choice for managing millions of individuals with T2DM [16]. However, there is significant interindividual diversity in the therapeutic response to metformin. Common genetic variations have been reported to alter metformin's glycemic sensitivity, explaining up to 34% variation in hemoglobin A1c (HbA1c) reduction with this medicine [13].

Metformin causes changes in DNA methylation throughout the entire genome [17]. Personalized therapy strategies for patients with type 2 diabetes may benefit from an understanding of how metformin affects DNA methylation patterns, particularly those of the *TCF7L2* gene [7]. Considering all of this, it is imperative to ascertain the genetic status of T2DM patients in a recognized genetic region before developing a treatment plan [18]. Understanding the impact of metformin on *TCF7L2* gene methylation could aid in the creation of customized treatment regimens for the management of type 2 diabetes [19]. Scientists are studying its pharmacogenetic effects, which might involve modifications to patterns of DNA methylation [13]. However, the influence of metformin on the DNA methylation modification of the *TCF7L2* gene is not specifically addressed in this study. In the human liver, metformin reduced the DNA methylation of the genes that encode the metformin transporter, which may help to improve hyperglycemia and obesity [19]. When paired with epigenetic medications like DNAMT and HDAC inhibitors, traditional pharmacotherapy with metformin also has epigenetic effects in type 2 diabetes and improves disease outcomes. This suggests that epigenetic therapy could be a useful addition to pharmacotherapy[20].

Methformin, a medication used to treat Type 2 diabetes, has been shown to have an impact on DNA methylation. Genes like ABCC8 involved in metformin work may have strange DNA methylation. This can change how they act in the liver and how metformin works[7]. An investigation into the connection between hypoglycemia associated with SU and promoter methylation of the KCNJ11 and ABCC8 genes was conducted on Greek patients. determined that the risk of SU-linked hypoglycemia was eliminated by methylating only the ABCC8 gene[21]. Metformin's effect on DNA methylation may change many diabetes genes. But more research may be needed to know exactly how it affects the *TCF7L2* gene. The study looked at how DNA changed in people with type 2 diabetes who did not take metformin. It examined how the *TCF7L2* gene changed. The study did not find a significant change (Table I).The group with type 2 diabete-s not taking metformin had more cases of DNA change- than the group taking metformin. There- were 13 cases in the- no metformin group and 12 cases in the me-tformin group with DNA change. There we-re 5 cases in the no me-tformin group and 2 cases in the metformin group with no DNA change-. The metformin group had more case-s of partial DNA change than the no metformin group. There were 11 case-s in the metformin group and 7 cases in the- no

metformin group with partial DNA change (Table I). Later studies that looked at the methylation levels of genes linked to insulin resistance in the pancreatic islets of T2D patients discovered that there was a drop in gene expression and an increase in DNA methylation.[22]. Metformin reduces the DNA methylation of the metformin transporter genes in the human liver, according to research. Furthermore, obesity and hyperglycemia are linked to these genes' increased methylation levels[11].

Studies suggest that genetic variants, such as rs7903146 in the TCF7L2 gene, affect metformin response and glycemic outcomes in individuals with type 2 diabetes mellitus (T2DM) [13]. The pharmacogenetic effects of metformin may include changes in DNA methylation in genes associated with its pharmacokinetics, which could have an effect on the medication's effectiveness [7]. While several studies have suggested connections between DNA methylation changes and T2DM risk factors, like the use of metformin, the specific effects on the TCF7L2 gene are yet unknown [23]. Further research is necessary to completely comprehend the therapeutic implications of metformin's direct effect on DNA methylation changes of the TCF7L2 gene and how it affects T2DM therapy [24]. Future studies should consider the potential for medications such as metformin to distort the associations between methylation changes and the risk of type 2 diabetes and the observed methylation variations [25]. There is growing evidence that metformin affects microRNA levels, epigenomics, and subsequently gene expression. Because metformin alters DNA methylation patterns and histone modifications, it may work in concert with DNAMT or HDAC inhibitors to control gene expression and improve insulin sensitivity. For example, metformin causes several genes involved in insulin signaling pathways and glucose metabolism to become hypomethylated.[20]

CONCLUSION

This study found no significant association between metformin use and DNA methylation of the TCF7L2 gene in T2DM patients. Age and sex were also not correlated. These negative findings suggest that TCF7L2 methylation is unlikely to serve as a biomarker for metformin response, but they provide a foundation for future research in larger cohorts and with broader epigenetic panels.

FUNDING

No funding bodies

ETHICAL APPROVAL

The study protocol was approved by the Ethics Committee of the Faculty of Science and Health, Koya University, in accordance with institutional guidelines for human research. Informed consent was obtained from all participants prior to blood collection.

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CONFLICTS OF INTEREST

The author declares no conflict of interest.

DATA AVAILABILITY

Data will be available upon reasonable request.

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