This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/ by-nc-nd/4.0), which permits copy and redistribute the material in any medium or format, provided the original work is properly cited.

sciendo

ENDOCRINE REGULATIONS, Vol. 57, 53–60, 2023

doi:10.2478/enr-2023-0007

Identification of DNA methylation change in TCF7L2 gene in the blood of type 2 diabetes mellitus as a predictive biomarker in Iraq Kurdistan region by using methylation-specific PCR

Harem Othman Smail [1,2], Dlnya Asaad Mohamad [2]

[1]*Department of Biology, Faculty of Science and Health, Koya University, Koya KOY45, Kurdistan Region – F.R. Iraq;*
[2]*Department of Biology, College of Science, University of Sulaimani, Sulaymanyah, Iraq*
E-mail: harem.othman@Koyauniversity.org

Objective. Nowadays, type 2 diabetes mellitus (T2D) is the most common chronic endocrine disorder affecting an estimated 5–10% of adults worldwide, and this disease also rapidly increased among the population in the Kurdistan region. This research aims to identify DNA methylation change in the TCF7L2 gene as a possible predictive T2D biomarker.

Methods. One hundred and thirteen participants were divided into three groups: diabetic (47), prediabetic (36), and control (30). The study was carried out in patients who visited the private clinical sector between August and December 2021 in Koya city (Iraq Kurdistan region) to determine DNA methylation status using a methylation-specific PCR (MSP) with paired primers for each methylated and non-methylated region. In addition, the X2 Kruskal–Wallis statistical and Wilcoxon signed-rank tests were used, $p<0.05$ was considered significant.

Results. The results showed hypermethylation of DNA in the promoter region in diabetic and prediabetic groups compared to the healthy controls. Different factors affected the DNA methylation level, including body max index, alcohol consumption, family history, and physical activity with the positive Coronavirus.

Conclusion. The results obtained indicate that DNA methylation changes in the TCF7L2 promoter region may be used as a potential predictive biomarker of the T2D diagnosis. However, the findings obtained in this study should be supported by additional data.

Key words: type 2 diabetes mellitus, methylation-specific PCR, TCF7L2, ROC curve, bisulfite-converted DNA

DNA methylation is an epigenetic signature that received the most attention when a methyl group (CH_3) was added to the DNA nucleotide cytosine. A strictly regulated DNA pattern is essential for normal development, and it plays a crucial role in the life of tissue-specific gene regulation and transcription (Ronn and Ling 2015; Smail 2016). Therefore, identifying individuals in the asymptomatic period would not only allow for early intervention to prevent the development of overt diabetes but also have better disease management.

A significant area of current research is to search for robust, sensitive, and readily accessible biomarkers of type 2 diabetes (T2D). A considerable body of evidence in this circumstance demonstrates that changes in the epigenetic landscape play an important role in insulin-responsive tissues. An essential role in the pathogenesis of obesity, insulin

Corresponding author: Harem Othman Smail, Department of Biology, Faculty of Science and Health, Koya University, Koya KOY45, Kurdistan Region – F.R. Iraq; Department of Biology, College of Science, University of Sulaimani, Sulaymanyah, Iraq; e-mail: harem.othman@Koyauniversity.org.

resistance, and T2D, if reflected in the blood, may represent a potential biomarker candidate (Geach 2016; Kwak and Park 2016; Smail 2019).

The *TCF7L2* (transcription factor 7 like 2) gene is closely associated with an increased risk of T2D, but the association of TCF7L2 with weight-related traits in humans is unclear (Li et al. 2020). Regardless the age, the *TCF7L2* gene polymorphism and a positive family history of diabetes are highly linked to other risk factors among diabetes subjects (Juttada et al. 2020). *TCF7L2* gene codes for transcription factor and is expressed in many human tissues such as fat, liver, and pancreatic islets of Langerhans. TCF7L2 is involved in the Wnt signaling pathway, and the strong connection of single nucleotide polymorphisms in the *TCF7L2* gene is associated with T2D (Verma et al. 2020). Data indicate that rs7908486 single nucleotide polymorphism is strongly associated with obesity. The study, based on genotyping, suggests implementing *TCF7L2* rs7908486- in the early obesity detection in the Iraqi population (Mohammed et al. 2021).

Methylation-specific PCR (MSP) is a PCR tool serving to study CpG island methylation patterns (Ku et al. 2011). MSP is a valuable tool with many advantages for qualitative DNA methylation analysis, including ease of design and execution, sensitivity to the detection of small amounts of methylated DNA, and the ability to scan a massive number of samples fast without the need for costly laboratory equipment to be purchased (Huang et al. 2013). Two pairs of primers are required for an MSP experiment. One pair is methylated DNA (M) specific, and the other is non-methylated (U) specific. One or more CpG sites are included in each primer (or at least one of the pair) sequence to discriminate the methylated and non-methylated DNAs (Li and Dahiya 2002).

Material and Methods

Samples collection. After obtaining approval from the institutional Ethics Committee, a total of 113 blood samples were collected from both men and women, including 47 diabetics, 36 prediabetics, and 30 healthy individuals in Koya city via a sterile disposable syringe from the arm vein. All blood samples were collected in a test tube containing anti-coagulant ethylene diamine tetraacetic acid (EDTA) to prevent blood clotting. These were diagnosed by the hemoglobin A1C (HbA1c) analyzer, since HbA1c is a parameter for the prediabetic and diabetic group determination. In people without diabetes, the normal range for the HbA1c level ranged between 4% and 5.6%. HbA1c levels between 5.7% and 6.4% predispose a higher chance of getting diabetes, while over 6.5% is a mark of diabetes. Besides the blood collection, data on age, gender, body mass index (BMI), smoking, alcohol consumption, family history and measured random blood sugar levels were also collected from the participants.

DNA extraction. DNA extraction was performed by The ReliaPrep™ Blood gDNA Miniprep System (A5081; Promega, Madison, USA) according to the manufacturer's instructions, with the isolated DNA samples kept at –80 °C for further analysis. This process consisted of four essential steps: homogenizing the whole blood to release the DNA, binding DNA to the ReliaPrep™ Binding Column, removing the impurities from the samples by washing them three times, and eluting the purified DNA.

Bisulfite DNA conversion. MethylEdge™ Bisulfite Conversion System (N1301; Promega, Madison, USA) was performed according to manufactures instructions. In summary, in the bisulfite-converted reaction, the non-methylated cytosine was converted to uracil, while methylated cytosine was left unaffected.

Determination of DNA quality and quantity. The amounts of bisulfite-converted DNA were determined using the typical nucleic acid concentration ranges for direct A280 absorbance measurements with the NanoDrop spectrophotometer (JANEWAY, U.K.) Because bisulfite-converted DNA contains uracil and is typically single-stranded, the sample type was

Table 1
Primer sequence for methylation-specific PCR.

No	Primer	Length	Product size	Sequence (5'->3')
Methylated primer				
1	Forward	25	171	TTTCGGAGTAAGTTTTTGTATTTTC
2	Reverse	24	171	GACTAACAATAATCCTTTCGAACG
Unmethylated primer				
3	Forward	25	170	TTGGAGTAAGTTTTTGTATTTTTGT
4	Reverse	26	170	CAACTAACAATAATCCTTTCAAACAC

chosen as RNA-40. DNA samples were run on a 1% agarose gel and stained with Diamond NucleicTM Acid Dye (100 ng of each sample). Assessing the degree of DNA fragmentation after conversion, NanoDrop was set to DNA-50 to estimate DNA concentration before bisulfite conversion and stained with ethidium bromide.

Methylation-specific PCR primer. MethPrimer is a tool for designing bisulfite-based methylation PCR primers. Currently, MSP can be developed. The PCR results were examined using 2% agarose gel electrophoresis, ethidium bromide staining, and ultraviolet illumination. The methylated band was present at 171 base pairs and the non-methylated one at 170 base pairs (Table 1). 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 runs opposite with samples (N1231; Promega, Madison, USA). Tables 2 and 3 show the MSP master mix for the methylated and unmethylated primer, respectively. Table 4 shows the MSP cycling condition for both sets of primers.

Statistical analysis. MSP 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 tests, the study data were nonparametric (Non-Normal Distribution), and therefore, the Kruskal Wallis and Wilcoxon signed-rank tests were used. For categorical variables, frequencies were chosen. Descriptive statistics (mean, standard deviation) were used for continuous variables. The receiver operating characteristic curve (ROC curve) estimated the biomarker test, where $p<0.05$ was considered statistically significant. In addition, GraphPad Prism 8 was used for statistical data analysis.

Table 2
PCR Master Mix with the primer sets specific to the methylated (M) primer for bisulfite-converted DNA.

Component	Volume (µl)	Final concentration
2X PCR Master Mix	12.5	1X
M forward primer	1	10 pmol/l
M reversed primer	1	10 pmol/l
DNA template	3	10–50 ng
DNA nuclease-free water	7.5	The final volume of 25µl

Table 3
PCR Master Mix with the primer sets specific to the unmethylated (U) primer for bisulfite-converted DNA.

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

Table 4
Cycling conditions for methylation-specific PCR of bisulfite-converted DNA.

Steps	Temperature	Time	Number of cycles
Enzyme inactivation	95°C	5 min	1
Denaturation	95°C	30 s	35
Annealing	54°C	30 s	35
Extension	72°C	30 s	35
Final extension	72°C	5 min	1

Results

General characteristics (age, gender, BMI, family history, smoking, alcohol consumption, Covid 19, physical activity, random blood sugar, HbA1c) of the participants divided into 3 experimental groups (control, prediabetic, diabetic) are presented in Table 5. BMI, random blood sugar, and HbA1c parameters showed highly significant differences ($p=0.000$) between groups. Significant differences ($p<0.05$) between experimental groups were observed for age, gender, and alcohol consumption (Table 5).

Data on the methylation status of *TCF7L2* gene for all experimental groups are demonstrated in Table 6. Regarding the methylation frequency, the methylated *TCF7L2* gene was found in 53.2% (n=25) of patients with diabetes and 50.0% (n=18) of patients with prediabetes in comparison to 26.7% (n=8) in healthy controls. As well partially methylated state of *TCF7L2* gene was found more frequently in diabetic and prediabetic patients (31.9% and 22.2%, respectively) in comparison to controls (13.3.%). The differences in methylation status of *TCF7L2* gene between the groups were extremely significant ($p=0.0009$).

The relationship between DNA methylation status of *TCF7L2* gene and clinical-pathologic characteristics is presented in Table 7. Highly significant differences ($p=0.000$) between DNA methylation

Table 5
General characteristics of experimental groups.

Parameters		Group			p-value
		Diabetes (n=47)	Prediabetes (n=36)	Control (n=30)	
Age (years)		49.4±12.5	46.4±11.4	41.0±10.6	0.012
Gender	Male	18 (38.3%)	16 (44.4%)	21 (70.0%)	0.022
	Female	29 (61.7%)	20 (50.6%)	9 (30.0%)	
BMI (kg/m^2)		30.5±2.8	26.0±4.1	23.8±4.3	0.000
Smoking		5 (10.6%)	5 (13.9%)	5 (16.7%)	0.740
Family history		7 (14.9%)	2 (5.6%)	5 (16.7%)	0.316
Covid 19		7 (14.9%)	9 (25.0%)	3 (10.0%)	0.244
Alcohol		1 (2.1%)	0 (0.0%)	4 (13.3%)	0.020
Physical Activity		13 (27.7%)	13 (36.1%)	15 (50.0%)	0.141
RBC (mg/dL)		248.7±84.6	145.8±26.8	113.1±23.7	0.000
HBA1C (%)		9.5±2.0	6.1±0.3	4.7±0.4	0.000

Data are presented as mean±standard deviation or as a number (%) of participants. Kruskal Wallis test was used for statistical analysis. Abbreviations: BMI – body mass index; HbA1c – hemoglobin A1c; RBS – random blood sugar; p – statistical significance.

Table 6
Correlation of methylation state in the TCF7L2 gene between experimental groups.

Group	Methylation state			Total	p-value
	Unmethylated (100%)	Methylated (100%)	Methylated and unmethylated (Partially methylated)		
Control	18 (60.0%)	8 (26.7%)	4 (13.3%)	30 (100%)	0.0009
Diabetes	7 (14.9%)	25 (53.2%)	15 (31.9%)	47 (100%)	
Prediabetes	10 (27.8%)	18 (50.0%)	8 (22.2%)	36 (100%)	

Kruskal Wallis test was used for statistical analysis, p – statistical significance.

and age, BMI, HbA1c, random blood sugar were found in all groups. In addition, highly significant differences (p=0.000) between DNA methylation and gender and physical activity were demonstrated in diabetic patients. Other parametric variables, such as smoking, family history, Covid 19 were significantly (p<0.05) related in the diabetic group.

Figure 1 shows the ROC curve of DNA methylation status of *TCF7L2* gene between diabetes and control groups, and Figure 2 represents the ROC curve of DNA methylation status of *TCF7L2* gene between prediabetes and control groups. The area under the curve (AUC) was higher in the diabetes group (0.7326) than in the prediabetes group (0.6574) (p=0.006 and p=0.0286, respectively).

Figures 3 shows the results of the MSP analysis of *TCF7L2* gene using 2% agarose gel electrophoresis. The expected product size was 170 bp for unmethylated primer and 171 bp for methylated primer. The use of unconverted DNA and distilled water instead of converted DNA demonstrated that the primer was only specific for the converted DNA, no PCR product either methylated or unmethylated for *TCF7L2* gene was observed (Figure 4).

Discussion

In the present study, diabetes-related *TCF7L2* gene methylation in blood samples using MSP was qualitatively investigated. *TCF7L2* methylation was detected at a high frequency: in 25 (53.2%) out of 47 diabetes samples, 18 (50.0%) of 36 prediabetes samples, and in 8 (26.7%) of 30 control samples. To the best of our knowledge, this is the first study identifying *TCF7L2* gene methylation in the Kurdistan region of Iraq.

Bisulfite conversion is a prevalent technique in analyzing DNA cytosine methylation (Ahmad et al. 2021). There are many methods used in methylation

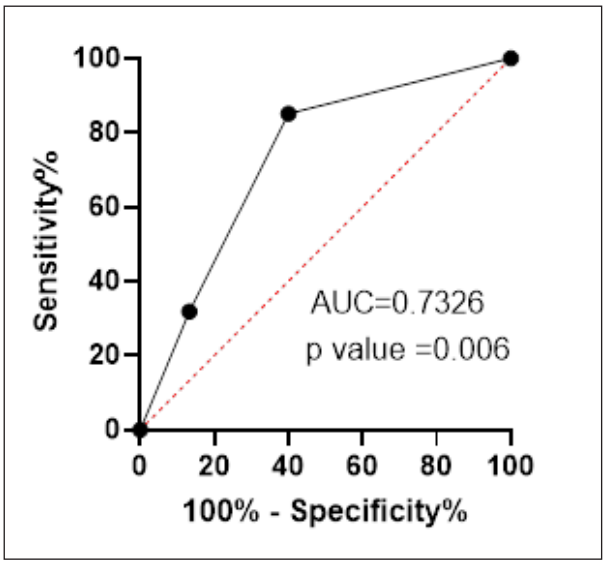


Figure 1. Receiver operating characteristic curve (ROC) for DNA methylation status of *TCF7L2* gene quantified by methylation-specific PCR (MSP) for diabetes group. Abbreviations: AUC – area under the curve.

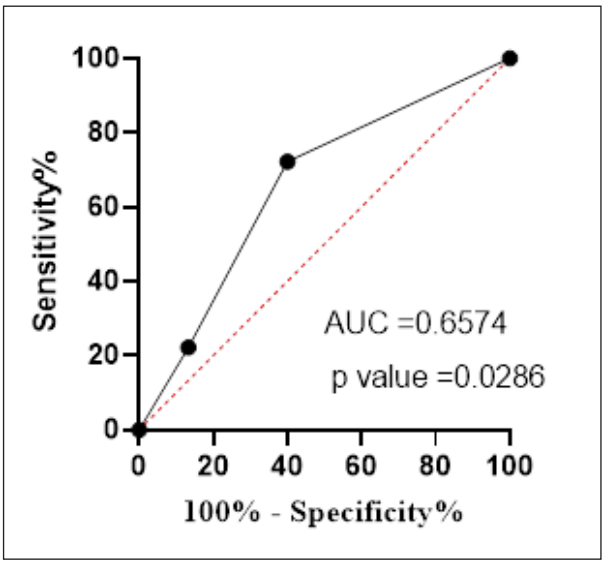


Figure 2. Receiver operating characteristic curve (ROC) for DNA methylation status of *TCF7L2* gene quantified by methylation-specific PCR (MSP) for prediabetes group. Abbreviations: AUC – area under the curve.

studies. Due to its high sensitivity and specificity, MSP is the most widely used technique. The procedure does not require a large DNA amount (Fraga and Esteller 2002). The current study showed significant differences between DNA methylation changes in diabetes and prediabetes vs. control samples, as shown in Table 6. There was a strong relationship between DNA methylation in all three groups (p=0.0009).

Various studies have shown a DNA methylation increase in males compared to females (Fuke et al. 2004; Hsiung et al. 2007). Decreased DNA methylation in females is thought to be due to reduced folate, the key supplier of methyl groups, which is also involved in erythrocyte formation and development. Since menstruation regularly depletes the supply of erythrocytes, females tend to have lower circulating folate levels (Terry et al. 2011). On the other hand, other studies have reported higher methylation status in females than males (Moore et al. 2008), while another study has reported no difference between them (Maghbooli et al. 2014). Our data detected higher DNA methylation in diabetes and prediabetes female groups.

In contrast, minimum DNA methylation was recorded in the healthy female group (Table 7). The current review identified 37 articles investigating DNA markers for T2D detection or risk evaluation using DNA isolated from the blood. Based on reproducible findings from the reviewed studies, differentially methylated sites in *TCF7L2* gene have been found in different population groups (Willmer et al. 2018).

Based on the statistical analysis, there was an association between MSP results and BMI, HbA1c, and random blood sugar (p=0.000), as shown in Table 7. DNA methylation is highly relevant to serve as a diagnostic tool for T2D. Many epigenetic changes are seen before the disease development, such as increased BMI or HbA1c in healthy individuals or detected in prospective studies. The finding of altered DNA methylation in genes relevant for T2D and also in blood suggests that some of these epigenetic alterations are established in early development (Ronn and Ling 2015). Taken together, newly diagnosed drug-naive T2D patients display specific epigenetic changes at the *TCF7L2* promoter as compared to age- and BMI-matched controls. Methylation in the *TCF7L2* promoter is further correlated with fasting glucose in peripheral blood DNA, which sheds new light on the role of epigenetic regulation of *TCF7L2* in T2D (Canivell et al. 2014). Findings suggest that epigenetic age measurements with DNA methylation can potentially be utilized as a risk factor or biomarker associated with T2D development (Kim et al. 2021).

Changes in DNA methylation have been suggested to be a biological mechanism behind the beneficial effects of physical activity (Ronn et al. 2013). In

Table 7
Association of promoter region methylation and clinical pathologic characteristics within the same groups.

Variable		Group											
		Diabetes				Prediabetes				Control			
		M	U	UM	p-value	M	U	UM	p-value	M	U	UM	p-value
Age (years)		48.7±13.0	50.4±10.7	50.0±12.9	0.000	47.0±11.6	50.5±12.4	40.1±7.7	0.000	43.7±16.8	40.5±7.6	37.7±7.4	0.000
Gender	Male	11	5	5	0.000	8	5	3	0.010	4	14	3	0.117
	Female	14	2	10		10	5	5		4	4	1	
BMI (kg/m^2)		30.3±3.5	30.0±2.8	31.2±1.6	0.000	27.2±4.1	23.2±4.0	27.0±2.6	0.000	22.8±6.1	24.2±3.5	24.4±3.8	0.000
Smoking		3	1	1	0.012	3	0	2	0.554	2	3	0	0.500
Family history		5	0	2	0.009	11	1	0	1.000	0	4	1	0.500
Covid 19		5	1	1	0.005	6	1	2	0.191	0	2	1	0.220
Alcohol		0	0	1	0.072	0	0	0	0.637	1	2	0	0.250
Physical activity		6	5	2	0.000	6	4	3	0.400	2	12	1	0.796
RBC (mg/dL)		265.0±100.0	227.0±53.4	231.0±64.1	0.000	148.3±34.2	142.2±7.1	143.2±25.3	0.000	103.6±19.9	120.9±23.9	96.7±11.3	0.000
HbA1c (%)		9.7±2.2	8.7±0.7	9.5±2.1	0.000	6.1±0.3	6.1±0.2	6.1±0.2	0.000	4.7±0.5	4.9±0.3	5.0±0.2	0.000

Data are presented as mean±standard deviation or as a number of participants. Abbreviations: BMI – body mass index; HbA1c – hemoglobin A1C; RBC – random blood sugar; M – methylated state; U – unmethylated state; UM – unmethylated and methylated state; p – statistical significance.

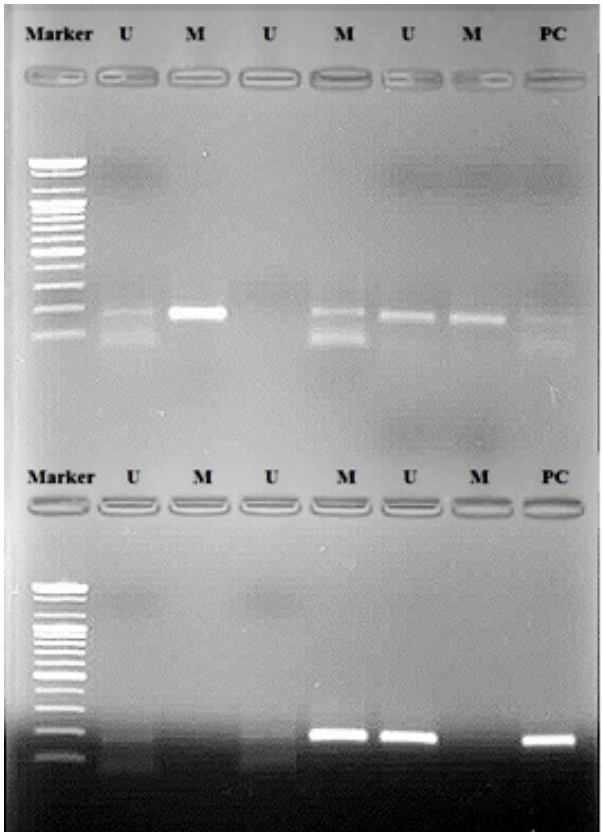


Figure 3. Visualization of methylation-specific PCR (MSP) products for *TCF7L2* gene using 2% agarose gel electrophoresis. Lane 1 represents a DNA marker (3kb DNA ladder). The expected product size was 170 bp for unmethylated primer and 171 bp for methylated primer. Abbreviations: M – methylated specific primer; U – unmethylated-specific primer; P.C. – positive control (methylated human control).

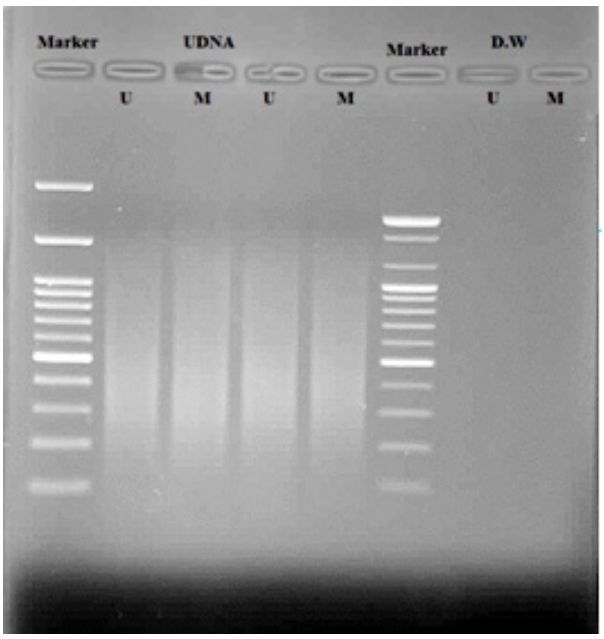


Figure 4. Visualization of methylation-specific PCR (MSP) products for *TCF7L2* gene using 2% agarose gel electrophoresis. Lanes 1 and 6 represent a DNA marker (3kb DNA ladder). Using unconverted DNA and distilled water instead of converted DNA demonstrated that the primer was only specific for the converted DNA and no PCR products, either methylated or unmethylated, for *TCF7L2* were found. Abbreviations: M – methylated-specific primer; U – unmethylated-specific primer; D.W. – distilled water; UDNA – unconverted DNA.

addition, MSP results were affected by hypermethylation of the *TCF7L2* gene in diabetes groups, and the p value was significant. In contrast, the p-value was insignificant in prediabetic and healthy groups, as shown in Table 7. Smoking, family history, and Covid 19 affected the change in DNA methylation. In addition, alcohol consumption had any affect. ROC curve analysis suggests that 25% methylation for the two key CCNL1 and ZNF187 markers is reasonably good at differentiating individuals in case and control groups in diabetic kidney disease (Smyth et al. 2020).

In addition, CpG sites associated with *SMTNL2* and *G6PC* genes were identified as methylation markers, and methylation levels of the features were determined in the urine sediments of patients with diabetes (Marumo et al. 2020). The ROC curves of *TCF7L2* methylation status revealed statistical significance for both diabetes and prediabetes groups, and the AUC was higher in the diabetes group than in the prediabetes group.

Conclusion

Increased DNA methylation in T2D patients may be correlated with the pathogenesis and the developed symptoms of diabetes. The results of this study demonstrate that the hypermethylation of the *TCF7L2* gene is associated with BMI, HbA1c, and random blood sugar in diabetic patients. Future research on the *TCF7L2* gene and its methylation status is needed, including design of a different set of primers, sequencing of the promoter regions, as well as other types of histone modification studies.

Acknowledgments

We would like to thank the Faculty of Science and Health Research Center staff, especially their laboratory technicians, Mr. Hiwa Abdurahman and Mr. Shwan Salih. Without their help and support, this study would not be so successfully performed.

References

Ahmad KK, Kareem ZS, Nabi Q. A new hrm qpcr assay for evaluation of dna methylation rates in the p15 gene among acute lymphoblastic leukemia patients. Acta Medica Mediterranea 37, 2485–2491, 2021.

Canivell S, Ruano EG, Siso-Almirall A, Kostov B, Gonzalez-de Paz L, Fernandez-Rebollo E, Hanzu FA, Parrizas M, Novials A, Gomis R. Differential methylation of TCF7L2 promoter in peripheral blood DNA in newly diagnosed, drug-naive patients with type 2 diabetes. PLoS One 9, e99310, 2014.

Fraga MF, Esteller M. DNA methylation: a profile of methods and applications. Biotechniques 33, 632–649, 2002.

Fuke C, Shimabukuro M, Petronis A, Sugimoto J, Oda T, Miura K, Miyazaki T, Ogura C, Okazaki Y, Jinno Y. Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: an HPLC-based study. Ann Hum Genet 68, 196–204, 2004.

Geach T. Epigenetics: Blood-based markers for T2DM. Nat Rev Endocrinol 12, 311, 2016.

Hsiung DT, Marsit CJ, Houseman EA, Eddy K, Furniss CS, McClean MD, Kelsey KT. Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. Cancer Epidemiol Biomarcers Prev 16, 108–114, 2007.

Huang Z, Bassil CF, Murphy SK. Methylation-specific PCR. Methods Mol Biol 1049, 75–82, 2013.

Juttada U, Kumpatla S, Parveen R, Viswanathan V. TCF7L2 polymorphism a prominent marker among subjects with type-2-diabetes with a positive family history of diabetes. Int J Biol Macromol 159, 402–405, 2020.

Kim K, Joyce B, Zheng Y, Schreiner PJ, Jacobs DR, Catov JM, Shikany JM, Carnethon MR, Greenland P, Van Horn L, Allen NB. DNA methylation GrimAge and Incident Diabetes: The Coronary Artery Risk Development in Young Adults (CARDIA) Study. Diabetes 70, 1404–141413, 2021.

Ku JL, Jeon YK, Park JG. Methylation-specific PCR. Methods Mol Biol 791, 23–32, 2011.

Kwak SH, Park KS. Recent progress in genetic and epigenetic research on type 2 diabetes. Exp Mol Med 48, e220, 2016.

Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics 18, 1427–1431, 2002.

Li L, Wang J, Ping Z, Li Y, Wang C, Shi Y, Zhou W, Zhang L. Interaction analysis of gene variants of TCF7L2 and body mass index and waist circumference on type 2 diabetes. Clin Nutr 39, 192–197, 2020.

Maghbooli Z, Larijani B, Emamgholipour S, Amini M, Keshtkar A, Pasalar P. Aberrant DNA methylation patterns in diabetic nephropathy. J Diabetes Metab Disord 13, 64, 2014.

Marumo T, Hoshino J, Kawarazaki W, Nishimoto M, Ayuzawa N, Hirohama D, Yamanouchi M, Ubara Y, Okaneya T, Fujii T, Yuki K. Methylation pattern of urinary DNA as a marker of kidney function decline in diabetes. BMJ Open Diabetes Res Care 8, e001501, 2020.

Mohammed AK, Al-Thuwaini TM, Al-Shuhaib MBS. Single nucleotide polymorphism rs7908486 of the tcf7l2 gene is highly associated with obesity in the Iraqi population. Arch Biol Sci 73, 39–45, 2021.

Moore LE, Pfeiffer RM, Poscablo C, Real FX, Kogevinas M, Silverman D, Garcia-Closas R, Chanock S, Tardon A, Serra C, Carrato A. Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case–control study. Lancet Oncol 9, 359–366, 2008.

Ronn T, Volkov P, Davegardh C, Dayeh T, Hall E, Olsson AH, Nilsson E, Tornberg A, Dekker Nitert M, Eriksson KF, Jones HA. A six months' exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue. PLoS Genet 9, e1003572, 2013.

Ronn T, Ling C. DNA methylation as a diagnostic and therapeutic target in the battle against Type 2 diabetes. Epigenomics 7, 451–460, 2015.

Smail HO. Qualitative and quantitative identification of DNA methylation changes in blood of the breast cancer patients (MSC thesis, University of Sulaimani), 2016.

Smail HO. The epigenetics of diabetes, obesity, overweight and cardiovascular disease. AIMS Genet 6, 36–45, 2019.

Smyth LJ, Patterson CC, Swan EJ, Maxwell AP, McKnight AJ. DNA methylation associated with diabetic kidney disease in blood-derived DNA. Front Cell Dev Biol 8, 561907, 2020.

Terry MB, Delgado-Cruzata L, Vin-Raviv N, Wu HC, Santella RM. DNA methylation in white blood cells: association with risk factors in epidemiologic studies. Epigenetics 6, 828–837, 2011.

Verma AK, Beg MMA, Saleem M, Al Reshidi F, Ahmad H, Alenazi F, Khan NA, Akther J, Joshi PC. Cell free TCF7L2 gene alteration and their association with Type 2 diabetes mellitus in North Indian population. Meta Gene 25, 100727, 2020.

Willmer T, Johnson R, Louw J, Pheiffer C. Blood-based DNA methylation biomarkers for type 2 diabetes: potential for clinical applications. Front Endocrinol 9, 744, 2018.