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The Identification of Methylation Profiles of FTO and PPARG Genes in Type 2 Diabetes Mellitus Patients

Tip 2 Diyabet Mellitus Hastalarında FTO ve PPARG Genlerinin Metilasyon Profillerinin Belirlenmesi

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Öz

Tip 2 diyabet (T2DM), genetik yatkınlıklar, çevresel etkileşimler ve çeşitli genler tarafından yönlendirilen karmaşık bir metabolik hastalıktır. Günümüzde, giderek artan sayıda çalışma diyabetes mellitus (DM) ile epigenetik, özellikle DNA metilasyonu arasındaki ilişkiyi göstermektedir. Bu çalışmada, klinik olarak T2DM tanısı almış hastaların periferik kan örneklerinde yağ kütlesi ve obezite ilişkili (FTO) ve peroksizom proliferatör aktive reseptör gama (PPARG) metilasyon düzeylerini ölçmeyi amaçladık. Çalışmamızda, Endokrinoloji Polikliniğine başvuran T2DM hastalarından (n=43) ve yaş-cinsiyet eşleştirilmiş sağlıklı bireylerden (n=42) tam kan alındı. Tam kan örneklerinden izole edilen genomik DNA'ların bisülfit dönüşümünden sonra hedef genlerin metilasyon profilleri metil-spesifik PCR ve jel elektroforezi yöntemleri ile analiz edildi. İstatistiksel analizler sonrası, T2DM ve kontrol grupları arasında FTO metilasyon durumu açısından anlamlı bir fark bulunmadı. T2DM'de PPARG geninin metilasyon seviyesi kontrol grubuna kıyasla önemli ölçüde daha yüksekti. PPARG'nın insülin duyarlılığını artırıcı etkileri göz önüne alındığında, bulgularımız metilasyon aracılı PPARG gen ekspresyonunun baskılanmasının T2DM hastalarında insülin direncinin yükselmesine yol açabileceği olasılığını doğrulamaktadır. T2DM hastalarında PPARG genindeki metilasyonun etkilerini ve hastalıkla ilişkisini daha iyi anlamak için daha fazla hasta ve kantitatif yöntemlerle yürütülen daha fazla gen ekspresyonu çalışması gerekecektir.

Anahtar Kelimeler: DNA Metilasyonu, FTO, PPARG, Tip 2 Diyabet Mellitus

Abstract

Type 2 diabetes (T2DM) is a complex, metabolic disease driven by genetic susceptibilities, environmental interactions, and various genes. Nowadays, increasing number of studies show the relationship between diabetes mellitus (DM) and epigenetics, especially DNA methylation. In this study, we aimed to measure the methylation levels of fat mass and obesity associated (FTO) and peroxisome proliferator activated receptor gamma (PPARG) in the peripheral blood samples of patients with clinical diagnosis of T2DM. In our study, whole blood was taken from T2DM patients (n=43) who applied to the Endocrinology Outpatient Clinic and from age-gender-matched healthy individuals (n=42). After the bisulfide conversion of isolated genomic DNAs from whole blood samples, the methylation profiles of target genes were analyzed with methyl-specific PCR and gel electrophoresis methods. Post-statistical analyses, no significant difference was found between the T2DM and control groups regarding FTO methylation status. The methylation level of PPARG gene in T2DM was significantly higher compared to the control group. Given the insulin sensitizing effects of PPARG, our findings confirm the possibility that methylation-mediated suppression of PPARG gene expression may lead to elevation of insulin resistance in T2DM patients. Further gene expression studies with more patients and quantitative methods will be required to better understand the effects of methylation in the PPARG gene in T2DM patients and its relationship to the disease.

Keywords: DNA Methylation, FTO, PPARG, Type 2 Diabetes Mellitus

Introduction

Type-2 diabetes mellitus (T2DM) is a complex, chronic, and progressive disease which arises due to insufficiency of insulin secretion and/or efficiency and is accompanied with hyperglycemia (1). T2DM is characterized with disruption of diet regulation as a result of dysfunctional carbohydrate, lipid, and protein metabolisms together with controllable or uncontrollable risk factors (2).

Epigenetic is defined as inherited alterations in gene activity and function independent from DNA sequence changes (3). Epigenetic mechanisms play crucial roles in cellular differentiation and maintenance of cell viability, which is essential for a healthy developmental process yet, any deviation from these regulations can lead to propensity for severe pathologies (4). Understanding the underlying mechanism of association between epigenetic alterations and disease progression contributes to enlighten the intricate combinatory effect of genetic and environmental factors in pathogenesis of several complex diseases (5). The most prevalent epigenetic modification in mammals is methylation of DNA from cytosine residues within CpG dinucleotides (6).

Fat mass and obesity-associated (FTO), also called alpha-ketoglutarate dependent dioxygenase, is one of the first genes contributing to polygenic obesity which has been identified with genome-wide association studies (GWAS) (7). FTO is commonly expressed in energy homeostasis and food-intake related regulatory regions in the body (1,8). Previous studies reported that FTO level was upregulated in skeletal muscle and adipose tissues of T2DM

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patients and it showed a strong correlation with T2DM disease that is characterized with dyslipidemia and hyperglycemia (9). Besides, in a clinical epigenetic study conducted with patients bearing impaired glucose metabolism, hypomethylation was detected within a single CpG region of FTO gene's first intron (10).

Peroxisome proliferator activated receptor gamma is a transcription factor which is a member of hormone receptors super family (11). PPARG regulates the expression of various genes involved in the pathogeneses of some metabolic diseases such as hyperlipidemia, diabetes, and obesity (11). PPARG is a type-II nuclear receptor and was initially identified in adipose tissue due to its prominent roles in fatty acid storage and glucose metabolism (12). PPARG supports lipid uptake and adipogenesis by increasing insulin sensitivity and adiponectin secretion (12). Certain mutations and epigenetic alterations in PPARG gene were correlated with obesity and dysfunctional lipid and glucose homeostasis which eventually leads to T2DM (12). In previous clinical case-control cohort studies performed with T2DM patients, various epigenetic alterations including DNA methylation were detected in several candidate genes like PPARG in target tissue and organs for insulin such as skeletal muscle, adipose tissue, and liver (13-16).

In this context, DNA methylation emerges as a promising indicator for complex diseases of which pathogeneses cannot be solely explained with traditional genetic traits (4). The aim of the present study is to investigate the DNA methylation statuses of certain regions containing CpG islands within promoters of PPARG and FTO genes in T2DM patients and healthy controls by using methylation specific PCR method in bisulfite-converted peripheral blood genomic DNA.

Material and Method

Study Population

An approval was obtained for the present study from Hatay Mustafa Kemal University Clinical Research Ethical Board (date 06/09/2021, issue no. 4298783/05045), and all experimental procedures involving human subjects adhered to the Helsinki Declaration. Biological materials were collected after verbal and written consent was obtained from participants who visited the endocrinology polyclinic at Hatay Mustafa Kemal University Research and Training Hospital. We included 43 patients with type-2 diabetes mellitus (T2DM), diagnosed by an expert clinician according to the American Diabetes Association criteria, and 42 age- and gender-matched healthy control subjects without insulin resistance or fasting blood glucose levels exceeding 100 mg/dL. The total sample size was calculated using G-Power analysis software (17) based on a previous study (18). Volunteers who are pregnant, under 18 years of age, or had inflammatory, chronic kidney, oncological, and acute or chronic infectious diseases were excluded from the study.

Sample Collection

Ten mL whole blood samples were collected into EDTA-containing tubes via venipuncture and stored at -20°C until the day of experiments which were performed between September-December 2022 at Hatay Mustafa Kemal University, Department of Medical Biology and Genetics.

DNA Extraction and Concentration Assessment

DNA isolation was performed following instructions of a commercial kit (Thermo Scientific GeneJET Genomic DNA Purification Kit, #K0721). Briefly, a 200 µL whole blood sample was mixed with a lysis solution containing proteinase K. After short vortex, the mixture was incubated at 56°C for 10 min with vortexing every 2 min. Then, pure ethanol was added to the mixture, and after brief vortex it was spun down through a column at 6000×g for 1 min. After two washing steps, the DNA sample was eluted from the column with 200 µL of elution buffer and kept at -20°C for long-term storage. DNA concentration and purity were measured using the µ-Drop tool with a spectrophotometer (MultiScan Go, Thermo Scientific).

Bisulfite Modification

In order to detect methylated cytosine residues with methylation specific PCR, bisulfite conversion of genomic DNA was performed following the manufacturer's instructions of a commercial kit (EpiJET Bisulfite Conversion Kit, Thermo Fisher, #K1461). For this purpose, 400 µg purified genomic DNA was mixed with modification reactive reagent and incubated in a thermal cycler (BioRad Thermal Cycler) with the following settings: 10 min at 98°C, 150 min at 60°C. The converted DNA sample was pipetted into the binding reagent-containing column and spun down for 30 s. After a single wash, the sample on the column was incubated with desulfonation reagent for 30 min and centrifuged briefly. After two washing steps of the column, the bisulfite-converted DNA sample was eluted with 10 µL elution buffer and stored at -20°C.

Methylation Specific Polymerase Chain Reaction (MSP)

We utilized "UCSC Genome Browser" database (UC Santa Cruz California University, http://genome.ucsc.edu) to determine the promoter regions of FTO and PPAR genes (19). Following detection of promoter sequences of FTO (chr16:53,703,706-53,704,323) and PPAR (chr3:12,287,496-12,288,834) genes, we used "Methprimer 2.0" database (http://www.urogene.org/methprimer2) to predict

CpG islands within the marked regions and to design specific primer pairs targeting methylated and unmethylated sequences (20).

The designed primer sequences (Macrogen Europe, Amsterdam, Netherlands) targeting methylated and unmethylated promoter regions of FTO and PPAR genes were listed in Table 1.

For MSP reactions, a commercial PCR master mix (Thermo Fisher PCR Master Mix 2X #K0171) and methylated and unmethylated control DNA kit (EpiTect PCR Control DNA kit, Thermo Fisher, #59695) were used. MSP reaction cycling conditions were summarized in Table 2. Agarose gel electrophoresis (2.5%) was run to visualize the PCR products with a computerized imaging system under ethidium bromide imaging channel (BioRad ChemiDoc XRS+).

Statistical Analysis

Data analysis was performed with Graphpad 8.0.2 package program. Non-categorical variables were expressed as n (sample size) and mean±standard deviation. For categorical data, we reported n (sample size) and percentage (%). To assess the normal distribution of non-categorical data, we employed Shapiro-Wilk test. The comparison of two individual groups was performed with either the independent t-test (for normally distributed data) or the Mann-Whitney U test (for non-normally distributed data). For comparisons involving categorical values, we applied either the Fisher's exact test or Chi-square test for trend.

Results

Demographic Features of Subjects

The demographic characteristics of the participants were summarized in Table 3. The priori test of the power analyses using two-sided Fisher exact test assumption gave the following outputs: Total sample size: 36, actual power: ~0.83, actual α: ~0.03 (with α error probability: 0.05).

We found no significant differences between the subjects in T2DM and control groups concerning age, gender, and smoking habits ($p>0.05$). However, there was a notable difference in BMI values between two groups ($p<0.05$).

Table 1. Methylated and unmethylated primer sequences designed based on the promoter regions of *FTO* and *PPARG* genes.

Gene	Primer type	Primer sequence	Amplicon size (bp)
FTO	Methylated	**F:** 5'-GTTGAGAGAATTATATGTAGGAGGCG-3' **R:** 5'-GTTCCTCGACAATCGAAATACG-3'	**112**
	Unmethylated	**F:** 5'-GTTGAGAGAATTATATGTAGGAGGTGG-3' **R:** 5'-CATTCCTCAACAATCAAAATACACTT-3'	**113**
PPARG	Methylated	**F:** 5'-ATTGACGGGGTTTTAGACGGAT-3' **R:** 5'-CGTCAATCCGAATCCTACCG-3'	**102**
	Unmethylated	**F:** 5'-GGGAATTGATGGGGTTTTAGATG-3' **R:** 5'-CCATCAATCCAAATCCTACCAAAC-3'	**107**

FTO: Fat mass and obesity-associated, PPARG: Peroxisome proliferator activated receptor gamma, bp: base pair.

Modified Profile of Methylated DNA Pattern in PPARG but Not FTO in T2DM Patients

MSP analyses revealed that the designed primers targeting the methylated (M) and unmethylated (UM) promoter regions of FTO and PPAR genes yielded amplicons at expected sizes based on agarose gel monitoring (Figures 1-2).

In figure 1, a representative 26-well gel image of FTO-gene specific methylation reactions is depicted, which consists of 2 DNA markers (1. and 13. wells), 3 control samples (M-DNA, UM-DNA, and non-bisulfite DNA) that were treated with either methylated (wells 2-4 respectively) or unmethylated primers (wells 14-16 respectively). The rest of the wells corresponds to the representative control (K1-K4) and patient (H1-H5) samples treated with M- and UM-primers. As expected, the positive control samples in 2. and 14. wells gave a positive signal with 112 and 113 bp amplicons corresponding to M- and UM-primers respectively. After analyses of all samples regarding FTO methylation status we found that, ~93% of control samples and ~98% of T2DM patient samples were unmethylated and there was so significant difference between the two groups ($p>0.05$, Table 4). The rest of the samples was heterozygous for the methylation status.

Table 2. PCR reaction conditions.

Step	Temperature (°C)	Duration (s)	Cycle (times)
Initial denaturation	95	180	1×
Denaturation	95	45	
Annealing	62^a, 64^b, 63^c, 56^d	30	35×
Extension	72	30	
Final extension	72	300	1×

Annealing temperatures for; a: FTO unmethylated primer, b: FTO methylated primer, c: PPARG unmethylated primer, d: PPARG methylated primer. FTO: Fat mass and obesity-associated, PPARG: Peroxisome proliferator activated receptor gamma.

Table 3. Demographic features of T2DM patients and healthy controls.

Parameters	HC (n=42)	T2DM (n=43)	*p* value
Age (Years, mean±SD)	59.8±10.5	58.1±11.8	0.46
Gender n (%)			
Male	23 (54.8)	17 (39.5)	0.19
Female	19 (45.2)	26 (60.5)	
BMI (kg/m^2, mean±SD)	26.7±4.0	29.6±5.0	***0.0034**
Smoking n (%)			
Smoker	13 (31.0)	14 (32.6)	>0.9999
Non-smoker	29 (69.1)	29 (67.4)	
HA1C (%)	N/A	9.3	N/A

BMI: Body mass index, HC: Healthy control, T2DM: Type-2 Diabetes mellitus, N/A: Not applicable

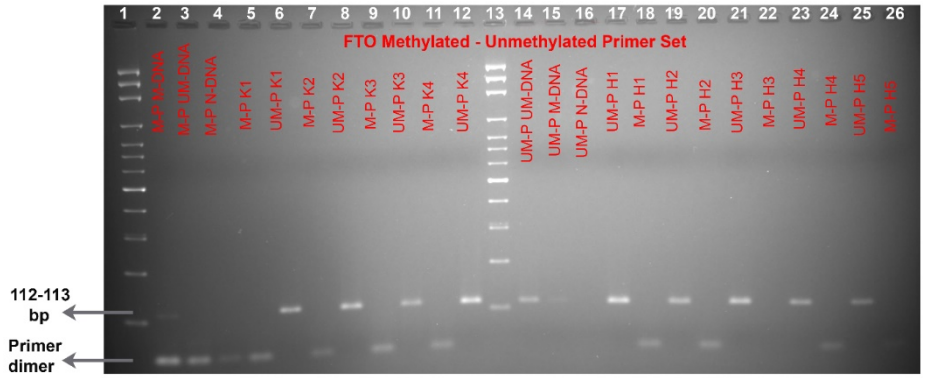

Figure 1. Representative gel images of MSP amplicons amplified with M- and UM-primers targeting promoter region of FTO gene. 1. and 13. wells: DNA marker, 2.-4. and 14.-16. wells: M-DNA, UM-DNA, and non-bisulfite-DNA controls amplified with M- and UM-primer sets respectively, 5.-12. wells: K1-K4 control samples amplified with M- and UM-primer sets, 17.-26. wells: H1-H5 T2DM patient samples amplified with M- and UM-primer sets. MSP: Methyl specific PCR, M: Methylated, UM: Unmethylated, K: Control, H: Patient, FTO: Fat mass and obesity-associated, bp: base pair.

In figure 2, representative gel images are depicted which shows MSP reactions conducted with M- and UM-primer set that is specific to PPAR gene promoter region. In gel image at upper panel indicating M-primer specific reactions, DNA marker at 1. well, positive control (M-DNA) at 2. well, and the rest of the wells with control (K27, K38-40, K50) and patient (H1-H13, H15-H20) samples were located. Methylated samples had a clear band size at 102 bp as the positive control sample. At the lower panel of figure 2, there are MSP reactions conducted with UM-primer set which consists of DNA marker (1. well), positive control (UM-DNA, 2. well), and the other samples between 3-23 wells (K28-K30, K32-K37, K39-50). The positive reactions had a clear band size at 107 bp. Further analyses revealed that (Table 4), the ratio of unmethylated samples was ~86% in the control group while ~67% in T2DM patient group. The heterozygous (M+UM) ratio in control and patient groups was ~12% and ~30% respectively. We found a significant difference between the two groups regarding methylation status ($p<0.05$, Table 4).

Table 4. Methylation status of FTO and PPARG genes in T2DM patients and healthy controls

Genes	HC (n=42)	T2DM (n=43)	*p* value
FTO n (%)			
Methylated	0 (0.0)	0 (0.0)	
Unmethylated	39 (92.9)	42 (97.7)	0.36
Hetero	3 (7.1)	1 (2.3)	
Non-detected	0 (0.0)	0 (0.0)	
PPARG n (%)			
Methylated	1 (2.4)	0 (0.0)	
Unmethylated	36 (85.7)	29 (67.4)	***0.01**
Hetero	5 (11.9)	13 (30.2)	
Non-detected	0 (0.0)	1 (2.3)	

*$p<0.05$. Chi-square test for trend for PPARG. Fisher's exact test for FTO. HC: Healthy control, T2DM: Type 2 diabetes mellitus, FTO: Fat mass and obesity-associated, PPARG: Peroxisome proliferator activated receptor gamma.

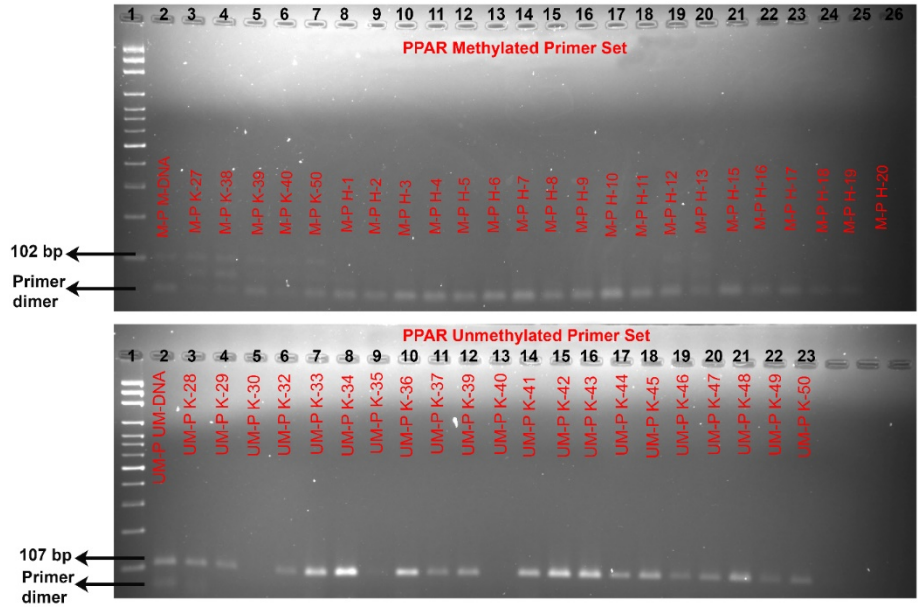

Figure 2. Representative gel images of MSP amplicons amplified with M-primer (upper panel) and UM-primer (lower panel) targeting promoter region of PPARG gene. In the upper panel (M-primer); 1. well: DNA marker, 2. well: Positive control (M-primer + M-DNA), rest of the wells: K27, K38-40, K50, H1-H13, H15-H20. In the lower panel (UM-primer); 1. well: DNA marker, 2. well: Positive control (UM-primer + UM-DNA), 3.-23. wells: K28-K30, K32-K37, K39-K50. MSP: Methyl specific PCR, M: Methylated, UM: Unmethylated, K: Control, H: Patient, PPARG: Peroxisome proliferator activated receptor gamma, bp: base pair.

Discussion

T2DM, which is a metabolic complex disease progressing due to life style, environmental, and genetic factors, is the primary reason of various diseases and disabilities worldwide (21). Since the physiological onset of T2DM occurs far earlier than the emergence of clinical symptoms, seeking early diagnostic and prognostic biomarkers, which would facilitate interference strategies concerning prevention or delay of the disease progression, has lately being gained a pivotal notice (21,22). As a result of gene-environment interaction, the modulation of gene expression can take place through chemical alterations around the genome so called epigenetic mechanisms such as DNA methylation and histone modifications (23). In this manner, the early diagnosis of T2DM via epigenetic indicators can contribute to disease management as well as can prevent the disease progression in people under high risk (24). DNA methylation is one of the most studied epigenetic modifications in several diseases including T2DM due to its stable chemical structure and easy profiling (6,21).

In the present study, we analyzed the methylation statuses of FTO and PPARG genes at their certain promoter regions in peripheral blood genomic DNA samples of T2DM patients and heathy controls using MSP method. Based on our findings, there was a significant difference regarding DNA methylation profile in PPARG between patient and control samples whereas there was no significant alteration in FTO gene.

FTO encodes a 2-oxoglutarate-dependent nucleic acid demethylase and several studies have reported that variants within FTO locus showed a strong correlation with obesity and can be used as a risk predictor for T2DM and cardiovascular diseases (25-27). A previous genome-wide DNA methylation profiling study conducted by Dayeh and Ling in pancreatic islets of T2DM patients analyzed 1649 CpG regions of 853 genes including FTO and PPARG (28). They ascertained that there was a close association between some of the GWAS-analyzed T2DM and obesity-linked candidate genes (CDNK1A, PDE7B, EXOC3L2, HDAC7, FTO) and the level of deteriorated β-cell function. In another clinical study performed with peripheral leucocytes of 25 T2DM patients and 11 healthy controls, a hypermethylation of a CpG region within FTO gene's promoter sequence has been identified and correlated with T2DM and metabolic syndrome (29). More recently, a strong association has been revealed between the methylation profile in a CpG region of FTO gene and T2DM disease (7). The association between FTO methylation status and T2DM disease was analyzed previously with a differential manner comprising 1169 cases and controls in total (10). First, in this study, a pool-based scanning was performed to differentially detect methylated DNA sequences among T2DM-related genomic regions and then a microarray method was applied to confirm and measure methylation status of the regions at upper levels of the list. Finally, it was reported that a CpG region within the first intron of FTO was found to be mildly

(~3.4%) but significantly () hypomethylated in T2D patients compared to controls.

FTO is the first gene that was shown to contribute to non-syndromic human obesity (7). The recent findings demonstrated that fatty diet leads to lipid deposition in several organs and thereby it affects the risk ratios of metabolic diseases namely obesity, insulin resistance and T2DM, and cardiovascular disorders (30). In a previous clinical study conducted by Perfilyev et al. it was ascertained after analyzing 4875 CpG regions that diet can affect DNA methylation profile (30). In the same study, the saturated fatty acid diet altered the DNA methylation status of 1797 genes including FTO whereas polyunsaturated fatty acid diet affected the methylation status of 125 genes excluding FTO. In light of these outcomes, the unaffected methylation profile of FTO in our study could arise from the diet habits of the participants in our study.

The recent multidirectional studies seeking the complex diseases revealed solid evidences regarding how genetic and epigenetic factors are implicated in the etiopathogenesis of these multifactorial diseases. FTO was claimed to be involved in T2DM genotype-epigenotype interactions on the basis of propensity for obesity by increasing its DNA methylation status (31). In the present study, we found no significant difference between the FTO methylation profiles of patient and control groups. The contradicting results between our findings and previous studies can likely stem from the scanning of different regions of FTO gene for possible DNA methylation process. Besides, we analyzed peripheral blood leukocytes for methylation analysis rather than adipose or pancreatic tissue, which can be the main reason for such contrasts between the studies (7). On the other hand, parallel to our findings, in a case-control study, no marked correlation was detected between FTO methylation profile and T2DM disease (29). The contradictions between these studies can rise from the multiple factors such as genetic diversity, life style, environmental effects, and differences in analyzed CpG regions (32). Besides, the DNA methylation variations among the subjects were asserted to change the individual predisposition to T2DM (7,10).

PPARG is another key player involved in metabolic pathways, which is commonly expressed in adipose tissue and acts as a hormone receptor (33). Basically, PPARG is activated upon binding to its ligand that results in its heterodimerization with retinoid X receptor and finally induction of transcriptional activation which leads to adipocyte differentiation and increasing of insulin sensitivity (31,34). Moreover, PPARG agonists were suggested to be utilized as anti-diabetic agent in clinics owing to its adipogenic and insulin-sensitivity actions (29). In the present study, we showed that the DNA methylation level of PPARG in T2DM patients was notably higher compared to the one in the control group. It has been previously reported in line with our findings that, the altered methylation profile in PPARG, KCNQ1, TCF7L2, and IRS1 genes were detected in adipose and pancreatic tissues of T2DM patients (23,24). In addition, a hypermethylated CpG region was detected in promoter sequence of PPARG gene of T2DM patients compared to healthy controls (29).

Epigenetic mechanisms play pivotal roles in gene expression regulations and thus they are crucial for normal and healthy development. Therefore, any deviations in epigenetic modulations can result in severe irregularities in gene expressions and eventually diseases (4). It is of primary importance to elucidate epigenetic modifications with respect to eliciting the intricate nature of gene-environment interactions in complex multifactorial diseases namely diabetes and cancer. In this regard, DNA methylation steps forward as a valuable indicator for complex diseases that cannot solely be explained with genetic traits of individuals (5). For future studies, populations with larger cohort size should be analyzed to find out promising DNA methylation signatures for T2DM pathogenesis by taking into account other confounder factors such as ethnicity and diet (21,24).

Conclusion

In the present study, we analyzed the methylation profile of metabolism-related FTO and PPARG genes' promoter regions in T2DM patients. We observed a marked increase in methylation levels of PPARG gene in patient group compared to healthy controls whereas there was no significant difference in FTO gene's methylation pattern. We suggest that, the methylation profile at the selected target promoter region of PPARG can be a promising indicator for T2DM pathogenesis. Still, further studies with larger population size together with longitudinal research strategy are warranted for implementation of peripheral blood-based DNA methylation profiling into clinical bed-side.

Study limitations

The total number of participants is relatively fewer in our study compared to similar studies in the literature. The current study limited to the methylation profile analysis of a single region within the promoter of target genes. We used a qualitative method for the analysis of methylation status of target genes however, quantitative methods are required for analytical inferences. The present study has also missing data concerning the alterations in mRNA expression levels of target genes depending on methylation patterns.

Acknowledgements

Regarding the limitations of our study, this was a retrospective review with a small sample size in a single center in a restricted region.

Conflict of interest statement

The authors of the present study disclose no financial or non-financial conflict of interest.

Ethics Committee Approval: The present study was approved by Hatay Mustafa Kemal University Clinical Research Ethical Board (Date: 06/09/2021, issue No: 4298783/05045).

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