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Original Paper

Association of DNMT3A Polymorphism, Oxidative Stress, and Global DNA Methylation in Type 2 Diabetes Mellitus Patients

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Key Words
DNMT3A • DNA methylation • Epigenetics • Total oxidant status • Oxidative stress • Type 2 diabetes mellitus

Abstract
Background/Aims: DNA methylation is a critical epigenetic mechanism involved in the regulation of gene expression, including genes implicated in glucose metabolism. Despite growing evidence indicating that epigenetic mechanisms are involved in the pathogenesis and progression of type 2 diabetes mellitus (T2DM), available data remain limited. This study analyzed the genetic association of two single-nucleotide polymorphisms (SNPs) in the DNA methyltransferase 3A (DNMT3A) gene, rs11683424 and rs1550117, with T2DM susceptibility and investigated their potential associations with global DNA methylation. ***Methods:*** Total antioxidant status (TAS) and total oxidant status (TOS) were evaluated, the oxidative stress index (OSI) was calculated, and diabetes-related parameters were assessed in T2DM patients and healthy controls, including HbA1c, fasting blood glucose (FBG), insulin, insulin sensitivity (IS), and insulin resistance (IR). Global DNA methylation was determined, and rs11683424 and

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rs1550117 were genotyped. Associations between metabolic and oxidative stress parameters, global DNA methylation, DNMT3A genotypes, and T2DM status were statistically analyzed. ***Results:*** T2DM patients exhibited significantly higher HbA1c, FBG, insulin, and IR levels than healthy controls (p=0.001), whereas IS was significantly lower (p=0.001). TAS, TOS, and OSI were significantly increased in T2DM patients compared with controls (p=0.001), as was global DNA methylation (p=0.001). In patients, a weak but statistically significant inverse correlation was observed between 5mC levels and BMI. Global methylation showed weak inverse associations with TAS and TOS; however, these associations did not reach statistical significance. No significant associations (p>0.05) were observed between 5mC levels and the other studied biomarkers. Neither regression model for predictors of global DNA methylation was statistically significant in T2DM patients (F(5,94)=0.852, p=0.516) or healthy controls (F(4,95)=0.592, p=0.669), and all variance inflation factor (VIF) values were <2.0. For rs11683424, the CT genotype was detected in 15.6% of T2DM patients compared with 2.6% of controls (OR=6.81, 95% CI=1.50–31.25, χ^2=7.902, p=0.005). The T allele was present at frequencies of 7.8% and 1.3%, respectively (χ^2=7.502, p=0.006). In contrast, rs1550117 was not significantly associated with T2DM. Linkage disequilibrium analysis indicated incomplete or moderate linkage disequilibrium between the two SNPs. The CG haplotype was the most common in both groups, whereas the TG and TA haplotypes were enriched among patients and showed increased disease odds, particularly TG (OR=7.89); however, neither association reached statistical significance. Genotype–phenotype analyses revealed only limited differences in global DNA methylation among the investigated genotypes. ***Conclusion:*** T2DM was associated with altered oxidative stress parameters and increased global DNA methylation. The findings further indicate a potential association between DNMT3A rs11683424 and T2DM susceptibility, whereas rs1550117 was not significantly associated with disease status. However, the data do not establish a direct relationship between the investigated DNMT3A variants, oxidative stress, and global DNA methylation. These findings warrant validation in larger, multi-ethnic cohorts.

Introduction

Type 2 diabetes mellitus (T2DM) is a complex disorder influenced by both genetic and environmental determinants. It is estimated to affect 693 million individuals worldwide by 2045 [1]. T2DM is characterized not only by hyperglycemia and insulin resistance (IR), but also by substantial alterations in epigenetic regulation. Among these, DNA methylation has emerged as an important mechanism linking environmental stressors to changes in gene expression in patients with T2DM [2, 3]. DNA methylation has been proposed as one of the epigenetic mechanisms that may help explain the missing contribution of genetic variants to T2DM, as large genome-wide association studies have accounted for only 20% or less of the estimated heritability of T2DM [4, 5].

DNA methylation involves the addition of a methyl group (CH_3) to the fifth carbon atom of a cytosine residue to form 5-methylcytosine (5mC), primarily at cytosine-phosphate-guanine (CpG) sites [6]. Accordingly, alterations in this process may influence gene expression and consequently affect gene function [7]. DNA methylation dynamics are mediated by a family of enzymes known as DNA methyltransferases (DNMTs), with S-adenosylmethionine serving as the methyl-group donor [8]. The major DNMTs include DNMT1, DNMT3A, and DNMT3B [7]. DNMT1 is primarily responsible for maintaining methylation patterns during DNA replication, whereas DNMT3A and DNMT3B are responsible for establishing new methylation patterns [9]. DNMT3A establishes de novo DNA methylation patterns and therefore plays a role in normal development, including the development of hematopoietic stem cells and neural progenitors. It has also been implicated in several disorders, including T2DM and cancer [10, 11].

Poor glycemic control is considered a hallmark of T2DM and contributes substantially to oxidative stress [12]. Oxidative stress broadly refers to the detrimental effects on cells,

tissues, and organs resulting from an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses. Kowluru et al. reported that ROS may modify DNA methylation through direct DNA damage or interactions with DNMTs [13]. It has also been proposed that ROS may contribute to epigenetic regulation involving 5mC through nucleophilic substitution reactions [14].

Despite growing evidence indicating that epigenetic mechanisms are involved in the pathogenesis and progression of T2DM, available data remain limited. Accordingly, the present study investigated the interplay among genetic, epigenetic, oxidative stress, and glycemic factors in T2DM. Specifically, we evaluated whether the investigated DNMT3A single-nucleotide polymorphisms are associated with T2DM susceptibility and whether they are associated with alterations in global DNA methylation, oxidative stress, and glycemic control in patients with T2DM.

Materials and Methods

Study Participants and Setting

The present case-control study included 100 patients diagnosed with T2DM who were recruited from the Diabetes and Endocrine Care Center of Marjan Teaching Hospital, Hilla City, Iraq. The mean age of the patient group was 58.37 ± 11.23 years, and the mean body mass index (BMI) was 32.46 ± 5.80 kg/m^2. For comparison, 100 healthy individuals without a history of diabetes or other chronic diseases were included as controls. The control group was age-matched to the patient group, with a mean age of 55.76 ± 8.12 years and a mean BMI of 30.94 ± 5.18 kg/m^2.

All participants were non-smoking men to minimize the potential influence of sex-related hormonal differences. Patients were selected based on laboratory findings, including HbA1c and fasting blood glucose (FBG), as well as clinical diagnosis by specialist physicians at the center. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. The study protocol, participant information, and consent forms were reviewed and approved by the local Ethics Committee of the Department of Biology, College of Science for Women, University of Babylon (Document No. 90, May 13, 2026). Written informed consent was obtained from all participants before blood sample collection. Disease duration was included in the present analysis. Complete information on medication use, diet, and comorbidities was not available for all participants and therefore could not be included as adjustment variables.

Assay of Studied Biomarkers

HbA1c levels (%) were measured using an automated Epithod® 616 Analyzer (DxGen, Korea). Fasting blood glucose (FBG; mg/dL) was determined using an enzymatic colorimetric method with a Linear kit (Spain; references 1980005, HUMANS MULTISERA NORMAL/Borderline glucose levels, and 1985005, HUMANS MULTISERA ABNORMAL/raised glucose levels). Serum insulin levels (µIU/mL) were measured using an ELISA kit (Calbiotech, USA; Catalog No. IS130D). Insulin resistance (IR) and insulin sensitivity (IS) were estimated using the homeostasis model assessment and quantitative insulin sensitivity check index, respectively, according to the equations referenced in [15]. Total antioxidant status (TAS) was assessed using laboratory-prepared reagents according to the published method of Apak et al [16].; no commercial assay kit was used. Total oxidant status (TOS) was assessed using laboratory-prepared reagents according to the method described by Erel [17]. The oxidative stress index (OSI) was calculated based on the relationship between TAS and TOS according to the equation described previously [18]:

$$OSI = \frac{ROSs}{TAS} \times 100$$

Extraction of DNA

Genomic DNA was extracted using the ReliaPrep™ Blood gDNA Miniprep System (Promega, USA; Catalog No. A5081) according to the manufacturer's instructions. DNA concentration and purity were assessed using a BioDrop µLITE spectrophotometer (BioDrop, England). Agarose gel electrophoresis was performed to assess the integrity of the extracted genomic DNA and PCR products. Although DNA samples were initially extracted from 100 T2DM patients and 100 healthy controls, only samples from 90 patients

and 76 controls were included in the genetic analyses. Samples that did not meet the required DNA quality and purity criteria for reliable downstream analysis were excluded.

Assessment of Global DNA Methylation

Global DNA methylation levels were assessed using the MethylFlash™ Methylated DNA 5-mC Quantification Kit (Epigentek, USA; Catalog No. P-1034) according to the manufacturer's instructions. This assay provides an estimate of the overall abundance of 5-methylcytosine (5mC) in genomic DNA and therefore reflects global DNA methylation.

Amplification and Genotyping of DNMT3A Polymorphisms

Primer sets supplied by Macrogen (Korea) were used to amplify the investigated SNPs. For rs11683424, tetra-primer amplification refractory mutation system PCR (tetra-ARMS-PCR) was performed using the following specific primers: F1, 5′-CTGTGCCTACTCCAAACATCATCATT-3′; R1, 5′-AGTTCAACACCCTTTCCCTGGT-3′; F2, 5′-CAAAAATAACATCACCCTTGAAGGAG-3′; and R2, 5′-CTCCTCTGACTTTACAACCCTGC-3′. PCR products corresponded to the control fragment (724 bp), C allele (407 bp), and T allele (368 bp). The annealing temperature was 58°C for 40 s.

For rs1550117, amplification was performed using the forward primer 5′-AGTGAGTTCCCCGTACCTTG-3′ and reverse primer 5′-CACCACCAACTCCAGCAATC-3′, with an annealing temperature of 58°C for 30 s. Positive PCR products of approximately 420 bp were sent to Macrogen (Korea) for DNA sequencing using a 3500 Genetic Analyzer.

PCR was performed using Green Master Mix (Promega). PCR products were visualized by electrophoresis in 1% agarose using 0.5× TBE buffer at 70 V and 20 mA for 30 min, followed by staining with ethidium bromide.

Statistical Analysis

Data are presented as mean ± standard deviation (SD). Data distribution was assessed using the Shapiro–Wilk test. Normally distributed continuous variables were analyzed using the independent-samples t-test or one-way analysis of variance (ANOVA), as appropriate. Categorical variables were compared using the chi-square test. Multiple linear regression analysis was performed to identify potential predictors, with statistical significance defined as $p<0.05$.

Genetic analyses were performed using MEGA11. Associations were evaluated using odds ratios (ORs) with 95% confidence intervals (CIs). Hardy–Weinberg equilibrium and single-locus association analyses were performed according to Hu et al [19].. Haplotype analysis was performed according to [20], while linkage disequilibrium (LD) was evaluated according to [21, 22] using the SNPStats platform. Statistical significance was defined as $p<0.05$. Fisher's exact test was used where appropriate. No formal correction for multiple testing was applied. Therefore, analyses involving multiple biomarker correlations, genetic variants, haplotypes, and genotype–phenotype associations were considered exploratory. Nominal p-values are reported, and the findings should be interpreted cautiously because of the potential for type I error arising from multiple comparisons.

Results

Comparison of Diabetes-Related Parameters, Oxidative Stress Parameters, and Global DNA Methylation Between T2DM Patients and Healthy Controls

Table 1 summarizes the clinical and diabetes-related parameters, oxidative stress parameters, and global DNA methylation levels in T2DM patients and healthy controls. T2DM patients had significantly higher HbA1c levels (9.10 ± 1.74% vs. 5.19 ± 0.49%; $p=0.001$), fasting blood glucose (FBG) levels (206.35 ± 72.24 vs. 86.58 ± 17.12 mg/dL; $p=0.001$), insulin levels (22.11 ± 9.82 vs. 6.11 ± 1.48 μIU/mL; $p=0.001$), and insulin resistance (IR) values (12.06 ± 8.69 vs. 1.30 ± 0.36; $p=0.001$) compared with healthy controls. Conversely, insulin sensitivity (IS) was significantly lower in T2DM patients than in healthy controls (0.280 ± 0.022 vs. 0.371 ± 0.017; $p=0.001$).

Table 1. Comparison of studied biomarkers between T2DMpatients and healthy individuals

Variable	Controls (N=100)	T2DMPatients (N=100)	p-value
	Mean ± SD		
HbA1c (%)	5.19 ± 0.49	9.10 ± 1.74	0.001*
FBG (mg/dL)	86.58 ± 17.12	206.35 ± 72.24	0.001*
Insulin (µIU/mL)	6.11 ± 1.48	22.11 ± 9.82	0.001*
IS	0.371 ± 0.017	0.280 ± 0.022	0.001*
IR	1.30 ± 0.36	12.06 ± 8.69	0.001*
TAS (mmol/l)	867.55 ± 214.71	1011.84 ± 212.99	0.001*
TOS (µmol/l)	15.09 ± 6.15	30.55 ± 19.48	0.001*
OSI (%)	1.81 ± 0.74	3.13 ± 2.11	0.001*
DNA methylation level (%)	0.367 ± 0.245	0.826 ± 0.257	0.001*
Disease duration	-	8.13 ± 6.19	-

SD: standard deviation, * Highly significant difference at the 0.05 level,

Regarding oxidative stress parameters, total antioxidant status (TAS) was significantly higher in T2DM patients than in healthy controls (1011.84 ± 212.99 vs. 867.55 ± 214.71 mmol/L; p=0.001). Total oxidant status (TOS) levels were also significantly higher in patients (30.55 ± 19.48 vs. 15.09 ± 6.15 µmol/L; p=0.001). Accordingly, the oxidative stress index (OSI) was significantly increased in T2DM patients compared with healthy controls (3.13 ± 2.11 vs. 1.81 ± 0.74%; p=0.001).

Global DNA methylation levels were significantly higher in T2DM patients than in healthy controls (0.826 ± 0.257 vs. 0.367 ± 0.245%; p=0.001). The mean duration of diabetes among patients was 8.13 ± 6.19 years; no corresponding value was applicable to the healthy control group (Table 1).

Correlation of Global DNA Methylation with the Studied Biomarkers

Table 2 presents the correlations between global DNA methylation levels and the investigated metabolic and oxidative stress parameters in T2DM patients and healthy controls.

In T2DM patients, a weak but statistically significant inverse correlation was observed between global DNA methylation and BMI (r=−0.241, p=0.016). Global DNA methylation also showed weak inverse correlations with TAS (r=−0.180, p=0.074) and TOS (r=−0.182, p=0.070); however, neither association reached statistical significance. No significant associations were observed between global DNA methylation and OSI, HbA1c, FBG, fasting insulin, IS, IR, or disease duration in T2DM patients (all p>0.05). In healthy controls, none of the investigated parameters showed a statistically significant correlation with global DNA methylation (Table 2).

Multiple Linear Regression Analysis of Factors Associated with Global DNA Methylation

Multiple linear regression analyses were performed separately for T2DM patients and healthy controls to identify factors independently associated with global DNA methylation (Table 3).

In T2DM patients, the overall regression model was not statistically significant ($F(5, 94)=0.852$, $p=0.516$) and explained 4.3% of the variance in global DNA methylation ($R^2=0.043$). The adjusted R^2 was −0.008. None of the assessed variables was a significant independent predictor of global DNA methylation. BMI showed the largest inverse association (B=−0.009, β=−0.199), although this association did not reach statistical significance (p=0.059). OSI, HbA1c, IR, and disease duration were also not significantly associated with global DNA methylation.

Similarly, the regression model for healthy controls was not statistically significant ($F(4, 95)=0.592$, $p=0.669$) and explained 2.4% of the variance in global DNA methylation ($R^2=0.024$; adjusted $R^2=-0.017$). None of the investigated predictors was significantly associated with global DNA methylation. BMI showed the largest standardized coefficient (B=0.008, β=0.138), but the association was not statistically significant (p=0.199) (Table 3).

Table 2. Correlation analysis of global DNA methylation with other studied biomarkers in T2DMpatients and healthy individuals

Variable	Controls		T2DMPatients	
	r	p-values	r	p-values
BMI	0.111	0.273	−0.241	0.016*
HbA1c (%)	0.021	0.838	0.050	0.624
FBG (mg/dL)	0.006	0.951	−0.043	0.667
Insulin (µIU/mL)	0.137	0.175	0.025	0.809
IS	0.004	0.970	−0.044	0.662
IR	0.044	0.662	−0.004	0.970
TAS (mmol/l)	−0.125	0.215	−0.180	0.074
TOS (µmol/l)	−0.033	0.742	−0.182	0.070
OSI (%)	0.045	0.658	−0.078	0.440
Duration		-	0.152	0.131

Table 3. Multiple linear regression analysis of factors associated with global DNA methylation in T2DMpatients and healthy individuals

Predictor	T2DMPatients				Controls			
	B	β	p-values	VIF	B	β	p-values	VIF
BMI	−0.009	−0.199	0.059	1.066	0.008	0.138	0.199	1.118
OSI	0.004	0.030	0.773	1.039	0.009	0.028	0.788	1.081
HbA1c	0.012	0.082	0.516	1.567	−0.013	−0.026	0.812	1.193
IR	−0.001	−0.038	0.768	1.593	0.034	0.050	0.638	1.079
Duration	0.002	0.040	0.698	1.044	—	—	—	—

Genetic Association Analysis of DNMT3A rs11683424 and rs1550117

Genotype and allele distributions of the two investigated DNMT3A polymorphisms, rs11683424 and rs1550117, are presented in Table 4, while representative electrophoresis and sequencing results are shown in Fig. 1.

For rs11683424, genotype distribution differed significantly between T2DM patients and healthy controls (χ^2=7.902, p=0.005). Among T2DM patients, the CC genotype was detected in 84.4% and the CT genotype in 15.6%, whereas 97.4% of controls carried the CC genotype and 2.6% carried the CT genotype. The CT genotype was associated with increased odds of T2DM compared with the CC genotype (OR=6.81, 95% CI=1.50–31.25).

Table 4. .Genotype distribution and association of rs11683424 and rs1550117 polymorphisms with patient status

Polymorphism	Analysis	Genotype/ allele	Patients (N=90) n (%)	Controls (N=76) n (%)	OR (95% CI)	χ^2	p-value
rs11683424	Genotype	CC	76 (84.4)	74 (97.4)	1.00 (reference)	7.902	0.005*
		CT	14 (15.6)	2 (2.6)	6.81 (1.50–31.25)		
	Allele	C	166 (92.2)	150 (98.7)	1.00 (reference)	7.502	0.006*
		T	14 (7.8)	2 (1.3)	6.33 (1.41–28.29)		
rs1550117	Genotype	GG	78 (86.7)	70 (92.1)	1.00 (reference)	1.261	0.262 NS
		GA	12 (13.3)	6 (7.9)	1.79 (0.64–5.04)		
	Allele	G	168 (93.3)	146 (96.1)	1.00 (reference)	1.188	0.276 NS
		A	12 (6.7)	6 (3.9)	1.74 (0.64–4.75)		

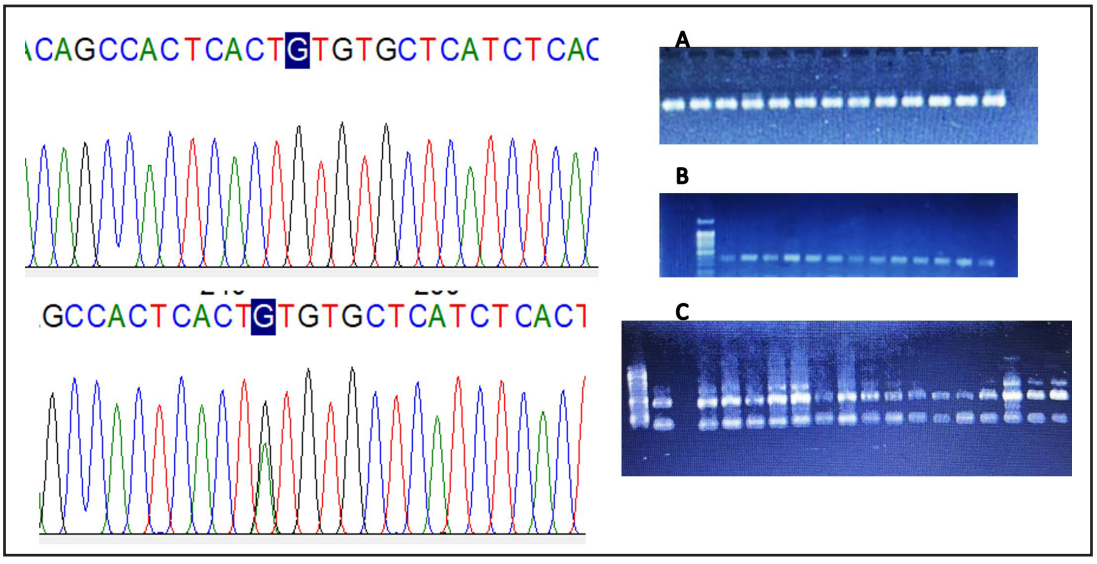

Fig. 1. Electrophoresis patterns and sequencing chromatograms of rs11683424 and rs1550117 amplification products. (A) Genomic DNA extracted from whole blood. (B) Amplification product of rs1550117. (C) Amplification product of rs11683424. Sequencing chromatograms of rs1550117 demonstrate the GG and GA genotypes.

Allele distributions also differed significantly between the groups (χ^2=7.502, p=0.006). The C allele was present at frequencies of 92.2% in T2DM patients and 98.7% in controls, whereas the T allele was present at frequencies of 7.8% and 1.3%, respectively. The T allele was associated with increased odds of T2DM (OR=6.33, 95% CI=1.41–28.29) (Table 4).

In contrast, rs1550117 was not significantly associated with T2DM. The GA genotype was detected in 13.3% of patients and 7.9% of controls, whereas the GG genotype was present in 86.7% and 92.1%, respectively. Genotype distribution did not differ significantly between the groups (χ^2=1.261, p=0.262; OR for GA vs. GG=1.79, 95% CI=0.64–5.04). Similarly, allele frequencies did not differ significantly between patients and controls (χ^2=1.188, p=0.276; OR for A vs. G=1.74, 95% CI=0.64–4.75) (Table 4).

Hardy–Weinberg Equilibrium Analysis

Observed and expected genotype distributions for rs11683424 and rs1550117 are presented in Table 5. For rs11683424, the observed genotype counts among T2DM patients were 76 CC, 14 CT, and 0 TT, compared with expected counts of 76.54, 12.91, and 0.54, respectively. Among controls, the observed counts were 74 CC, 2 CT, and 0 TT, compared with expected counts of 74.01, 1.97, and 0.01, respectively.

For rs1550117, the observed genotype counts among patients were 78 GG, 12 GA, and 0 AA, compared with expected counts of 78.40, 11.20, and 0.40, respectively. Among controls, the observed counts were 70 GG, 6 GA, and 0 AA, compared with expected counts of 70.12, 5.76, and 0.12, respectively (Table 5). Hardy–Weinberg equilibrium analysis showed no significant deviation for rs11683424 in either patients (χ^2=0.640, p=0.424) or controls (χ^2=0.014, p=0.907). Similarly, rs1550117 genotype distributions did not deviate significantly from Hardy–Weinberg equilibrium in patients (χ^2=0.459, p=0.498) or controls (χ^2=0.128, p=0.720). Exact HWE tests were also non-significant for all comparisons (exact p=1.000) (Table 6).

Linkage Disequilibrium Analysis

Linkage disequilibrium (LD) analysis demonstrated statistically significant non-random associations between rs11683424 and rs1550117 in T2DM patients, healthy controls, and the combined study population (Table 7). Among patients, D was 0.0263, D′ was 0.4281, and r was 0.3940 (p<0.001). Among controls, D was 0.0058, D′ was 0.4615, and r was 0.2628

Table 5. Expected genotype distribution of rs11683424 and rs1550117 under Hardy–Weinberg equilibrium

Group	Genotype	Observed, n (%)	Expected frequency	Expected count
rs11683424				
	CC	76 (84.4)	p2=0.8505	76.54
Patients (n = 90)	CT	14 (15.6)	2pq=0.14352	12.91
	TT	0 (0.0)	q2=0.0060	0.54
	CC	74 (97.4)	p2=0.9739	74.01
Controls (n = 76)	CT	2 (2.6)	2pq=0.02602	1.97
	TT	0 (0.0)	q2=0.0002	0.01
rs1550117				
	GG	78 (86.7)	p2=0.8711	78.40
Patients (n = 90)	GA	12 (13.3)	2pq=0.12442	11.20
	AA	0 (0.0)	q2=0.0044	0.40
	GG	70 (92.1)	p2=0.9226	70.12
Controls (n = 76)	GA	6 (7.9)	2pq=0.07592	5.76
	AA	0 (0.0)	q2=0.0016	0.12

(p=0.0012). In the combined study population, D was 0.0174, D′ was 0.3823, and r was 0.3593 (p<0.001). D′ values of approximately 0.38–0.46 indicated incomplete or moderate LD rather than complete co-inheritance of the two SNPs. Although D′ was slightly higher in controls, the allelic correlation coefficient r was higher in patients than in controls (Table 7).

Haplotype Analysis

Haplotype frequencies and their associations with T2DM status are presented in Table 8. The CG haplotype was the most common haplotype in both groups and was more frequent in controls than in patients (95.37% vs. 88.71%). The CA haplotype showed no significant association with T2DM (OR=1.18, 95% CI=0.34–4.06; p=0.790). The TG and TA haplotypes were more frequent among T2DM patients and showed increased odds of disease. For the TG haplotype, the OR was 7.89 (95% CI=0.96–64.74; p=0.056), whereas for the TA haplotype the OR was 5.80 (95% CI=0.63–53.44; p=0.120). Neither association reached statistical significance. Accordingly, the observed differences in T-containing haplotypes should be regarded as exploratory (Table 8).

Genotype–Phenotype Association Analysis

Genotype–phenotype associations between the investigated DNMT3A variants and global DNA methylation are presented in Table 9. For rs11683424, T2DM patients carrying the CC genotype had a mean global DNA methylation level of 0.816 ± 0.266, whereas

Table 6. The statistical analysis outcomes of HWE

Group	C frequency	T frequency	HWE χ^2	Chi-square p-value	Exact HWE p-value
rs11683424					
Patients	0.922	0.078	0.640	0.424	1.000
Controls	0.987	0.013	0.014	0.907	1.000
rs1550117					
Patients	0.933	0.067	0.459	0.498	1.000
Controls	0.961	0.039	0.128	0.720	1.000

Table 7. Comparison of linkage disequilibrium and estimated haplotype frequencies between patients and controls

SNP pair	Group	n	D	D′	r	p-value
rs11683424- rs1550117	Patients	90	0.0263	0.4281	0.3940	<0.001
rs11683424- rs1550117	Controls	76	0.0058	0.4615	0.2628	0.0012
	Study population	166	0.0174	0.3823	0.3593	<0.001

Table 8. rs11683424- rs1550117 haplotype frequencies and association with disease status

Haplotype	Total frequency (%)	Controls (%)	Patients (%)	OR (95% CI)	p-value
CG	91.76	95.37	88.71	1.00 (reference)	—
CA	3.42	3.31	3.52	1.18 (0.34–4.06)	0.790
TG	2.82	0.68	4.63	7.89 (0.96–64.74)	0.056
TA	2.00	0.63	3.15	5.80 (0.63–53.44)	0.120

Table 9. DNA Methylation level according to rs11683424 and rs1550117 genotypes among patients and controls

Study group	Polymorphism	Genotype	Methylation level, mean ± SD	p-value
Patients	rs11683424	CC	0.816 ± 0.266	0.176
		CT	0.876 ± 0.118	
	rs1550117	GG	0.806 ± 0.223	0.477
		GA	0.947 ± 0.367	
Controls	rs11683424	CC	0.366 ± 0.260	0.811
		CT	0.385 ± 0.021	
	rs1550117	GG	0.367 ± 0.261	0.992
		GA	0.365 ± 0.213	

patients carrying the CT genotype had a mean level of 0.876 ± 0.118. The difference was not statistically significant (p=0.176). Among healthy controls, mean global DNA methylation levels were 0.366 ± 0.260 for the CC genotype and 0.385 ± 0.021 for the CT genotype, with no significant difference (p=0.811). For rs1550117, T2DM patients carrying the GA genotype had a mean global DNA methylation level of 0.947 ± 0.367 compared with 0.806 ± 0.223 among GG genotype carriers; this difference was not statistically significant (p=0.477). Among controls, global DNA methylation levels were 0.367 ± 0.261 for GG carriers and 0.365 ± 0.213 for GA carriers, with no significant difference between genotypes (p=0.992) (Table 9).

Discussion

The comparison of clinical, glycemic, and metabolic parameters between T2DM patients and healthy controls revealed the characteristic biochemical profile of diabetes and was consistent with previous findings in different populations [12, 23–25]. Elevated HbA1c levels reflect long-term glucose dysregulation and are also associated with an increased risk of microvascular and macrovascular complications [26]. The significantly elevated insulin concentrations in T2DM patients, together with reduced insulin sensitivity (IS) and increased insulin resistance (IR), further reflect the metabolic dysfunction characteristic of these patients. This pattern of hyperinsulinemia and impaired insulin sensitivity has been widely reported, with mechanistic studies indicating that insulin resistance is associated with obesity, chronic inflammation, and ectopic fat accumulation, ultimately contributing to β-cell stress and progressive metabolic dysfunction [27–29].

Oxidative stress parameters also differed significantly between the study groups. TOS and OSI were markedly higher in T2DM patients, whereas TAS was also elevated. The increased TAS may reflect a compensatory upregulation of antioxidant defenses in response to excessive free-radical production. Nevertheless, the increased OSI indicates that these defenses were insufficient to restore oxidative balance, consistent with previous studies showing that persistent oxidative stress is a characteristic feature of diabetes and contributes to vascular complications [2, 30]. Our findings are also consistent with reports indicating that oxidative stress and associated inflammation may result from increased TOS accumulation and/or impaired antioxidant defenses in several chronic diseases, including diabetes mellitus, cardiovascular disease, and respiratory disease [31, 32].

The present study also examined global DNA methylation in the context of T2DM, with particular attention to the relationship between metabolic dysregulation and epigenetic regulation. The significantly increased global DNA methylation observed in T2DM patients

compared with healthy controls adds an epigenetic dimension to the pathophysiological differences between the groups. Several mechanisms have been proposed to explain altered DNA methylation in diabetes, including the interplay between hyperglycemia and oxidative stress [10].

Chronic hyperglycemia may influence epigenetic regulation through metabolic intermediates such as α-ketoglutarate and succinate. α-Ketoglutarate is an important cofactor for ten-eleven translocation (TET) enzymes involved in DNA demethylation, whereas succinate and other tricarboxylic acid-cycle intermediates may inhibit TET activity and thereby favor DNA hypermethylation [33]. Oxidative stress may additionally influence DNA methylation by inducing DNA damage and modifying DNMT activity. Experimental studies have indicated that oxidative stress can increase DNMT expression and activity, potentially promoting hypermethylation of metabolic and inflammatory genes [34]. Ling and Rönn further described interactions between hyperglycemia, oxidative stress, and persistent epigenetic modifications, which may contribute to the concept of "metabolic memory" in T2DM [35].

The increased global DNA methylation observed in the present T2DM cohort is consistent with genome-wide analyses of pancreatic islets showing altered DNA methylation at genes involved in insulin secretion and glucose uptake, potentially contributing to impaired β-cell function and glycemic control [36]. Our findings are also in line with the meta-analysis by Juvinao-Quintero et al., which included four European populations and identified associations between DNA methylation patterns and T2DM [37]. Similarly, Smail reported increased methylation of three metabolic genes in patients with T2DM, particularly in individuals diagnosed at a younger age [38].

DNA methylation has attracted increasing interest in diabetes research because epigenetic modifications are dynamic and potentially reversible, raising the possibility that epigenetic approaches may eventually complement conventional strategies for glycemic control and prevention of complications [35]. However, findings across studies are not uniform. Rohde et al. reported no significant methylation differences between T2DM patients and controls [39], whereas Vujcic et al. reported decreased global DNA methylation in T2DM patients and proposed that such changes may reflect interactions among oxidative stress, inflammation, and epigenetic regulation in T2DM [40].

According to the correlation analysis presented in Table 2, BMI was the only investigated variable significantly associated with global DNA methylation, and this association was observed exclusively in T2DM patients. The correlation was weak and inverse ($r=-0.241$, $p=0.016$), indicating that BMI accounted for only a limited proportion of the variability in methylation within this cohort. This finding is consistent with epigenome-wide association studies linking adiposity to subtle but reproducible epigenetic modifications. Wahl et al. reported that BMI explains only a relatively small proportion of epigenetic variation across populations [41]. Meta-analytical studies have likewise shown that DNA methylation signatures associated with BMI are detectable but heterogeneous and often context-dependent, reflecting interactions among genetic background, metabolic status, and environmental exposures [42]. A study in Arab populations identified associations between BMI and DNA methylation at loci including SOCS3, SREBF1, and CPT1A, while also emphasizing ethnic variation and the need for caution when interpreting findings across different populations [43].

The multiple linear regression analyses presented in Table 3 showed no evidence of problematic multicollinearity, with all variance inflation factor (VIF) values below 2.0. BMI, OSI, HbA1c, IR, and diabetes duration were not independently associated with global DNA methylation in the patient model. The low explanatory power of both regression models suggests that variation in global DNA methylation may be influenced by additional genetic, epigenetic, environmental, or clinical determinants that were not included in the present analysis.

The absence of significant independent associations between the investigated variables and global DNA methylation may partly reflect the sample size of the present study. More broadly, this finding is consistent with the complexity of epigenetic regulation in T2DM. The meta-analysis by Juvinao-Quintero et al. showed that DNA methylation alterations in blood cells were associated with T2DM, although individual associations were generally modest and frequently required large sample sizes to achieve statistical significance [37].

The concept of "metabolic memory" may also provide a possible explanation, as epigenetic marks established during earlier stages of disease may persist independently of current metabolic status [35]. Such persistence could help explain why current measures of glycemic control and oxidative stress were not significantly associated with global DNA methylation in the present analysis. In this context, DNA methylation may reflect cumulative long-term metabolic exposure rather than only concurrent biochemical parameters.

Our findings are also consistent with those of Chambers et al., who reported that DNA methylation signatures predicted future T2DM risk independently of conventional metabolic markers, emphasizing the potential relevance of epigenetic variation to diabetes susceptibility [44]. Additional factors may contribute to variation in glycemic control and may consequently influence methylation patterns [45–47].

Aberrant DNA methylation has been proposed as an important early event in the development of T2DM [48, 49]. DNMTs are key enzymes involved in DNA methylation in eukaryotic cells, with DNMT3A functioning as a de novo DNA methyltransferase that contributes to dynamic methylation processes during development and disease. Genetic variation in DNMT3A may potentially influence gene expression and enzymatic function. However, evidence specifically addressing rs11683424 remains limited compared with more extensively investigated DNMT3A variants.

To the best of our knowledge, relatively little is known about the involvement of DNMT3A variants in genetic susceptibility to T2DM. The present case-control study therefore investigated the rs11683424 and rs1550117 polymorphisms in relation to T2DM susceptibility.

The significant association observed between rs11683424 and T2DM susceptibility suggests that variation in this epigenetic regulator may be relevant to disease susceptibility. The heterozygous CT genotype and T allele were significantly more frequent among T2DM patients, with an OR of 6.81 for the CT genotype. In contrast, rs1550117 was not significantly associated with T2DM in the studied population. Given the relatively small number of rs11683424 variant carriers and the wide confidence interval, however, this association should be interpreted cautiously and requires validation in larger, independent, and multi-ethnic populations.

Importantly, the present data do not demonstrate that rs11683424 directly affects global DNA methylation. The genotype–phenotype analysis did not reveal a statistically significant difference in methylation according to rs11683424 genotype. Thus, the genetic association and the observed alteration in global DNA methylation should be regarded as related observations within the study rather than evidence of a demonstrated functional pathway.

The observed genotype distributions were compatible with those expected from the corresponding allele frequencies and provided no evidence of substantial deviation from Hardy–Weinberg equilibrium.

Linkage disequilibrium analysis indicated D′ values of approximately 0.38–0.46, consistent with incomplete or moderate LD rather than complete co-inheritance of rs11683424 and rs1550117. Although D′ was slightly higher in controls, the allelic correlation coefficient r was higher in patients than in controls (Table 7).

Haplotype analysis may provide additional information beyond single-SNP analyses by considering the combined genetic structure of linked variants. Haplotype-based approaches using SNP markers have therefore received increasing attention in candidate-gene studies across different clinical conditions [50].

In the present study, the CG haplotype was the most frequent haplotype in both groups, whereas the TG and TA haplotypes were more frequent among patients. However, neither the TG nor the TA association reached statistical significance. Consequently, the potential biological significance of these haplotypes remains exploratory.

Previous studies have suggested that chronic hyperglycemia may dysregulate DNMT activity and contribute to persistent epigenetic alterations associated with "metabolic memory" [51]. Other studies have reported that DNMT3A variants may alter methyltransferase activity and downstream gene expression [52, 53]. These observations provide a biological context in which DNMT3A haplotypes may warrant further investigation; however, the present study does not establish a functional effect of the observed haplotypes on DNMT3A activity or metabolic memory. More generally, LD- and haplotype-based approaches may complement single-SNP analyses when investigating complex genetic and epigenetic traits. Persistent epigenetic modifications despite improved glycemic control have been discussed in the context of metabolic memory by Mishra and Kowluru [54].

Finally, genotype–phenotype analysis integrated global DNA methylation measurements with the genotype data for rs11683424 and rs1550117 (Table 9). Only limited differences in global DNA methylation were observed among the investigated genotypes, and none of the comparisons reached statistical significance. For rs11683424, the absence of a significant difference between genotypes in T2DM patients may partly reflect the small number of CT genotype carriers, which limits the statistical power of this comparison.

The present study has several limitations. The number of variant carriers was relatively small, and complete information on potential confounders, including medication use, diet, and comorbidities, was not available for all participants. In addition, no formal adjustment for multiple testing was applied; consequently, the genetic, haplotype, biomarker-correlation, and genotype–phenotype analyses should be regarded as exploratory. These findings therefore require confirmation in larger, independent cohorts.

Conclusion

The present study demonstrated significantly altered global DNA methylation levels in patients with T2DM compared with healthy controls and identified an association between the DNMT3A rs11683424 polymorphism and T2DM susceptibility, with the CT genotype and T allele occurring more frequently among patients. In contrast, rs1550117 was not significantly associated with T2DM.

Although oxidative stress parameters were also significantly altered in T2DM patients, the present findings do not establish a causal relationship between oxidative stress, DNMT3A genetic variation, and global DNA methylation. Furthermore, the genotype–phenotype analyses did not demonstrate significant differences in global DNA methylation according to the investigated genotypes.

Accordingly, these findings should be regarded as associative and exploratory and require confirmation in larger, independent, and ethnically diverse populations.

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Author contributions

Ali Majeed Allami, Hawraa Sabah Al-Musawi, and Hiba Abdulameer Mohammed: Study design and manuscript writing.

Ahmed M. Amshawee, Maryam A. Hussain, and Thura Alyasiri: Study design and manuscript writing.

Mohammed Al-Daraji, Islam K. Alazzawi, Mona N. Al-Terehi, and Ahmed Flayyih Hasan: Study design, methodology, molecular analyses, and manuscript writing; all authors contributed to funding acquisition.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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