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Insulin-like growth factor binding protein 1 DNA methylation in type 2 diabetes

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Abstract

Background: Type 2 diabetes (T2D) is a complex trait in humans. Several environmental and hereditary factors contribute to the overall pathogenesis of this disease. The association between genes, environment, and T2D was unknown for decades until epigenetics was discovered. Epigenetics affects gene transcription, which, in turn, influences organ function. One of the epigenetic regulatory mechanisms is DNA methylation. This mechanism permits modification of gene function without changes in the DNA sequence.

There are several risk factors for type 2 diabetes such as harmful intrauterine environment, obesity, poor physical activity, increasing age, a family history of the disease, and an unhealthy diet. All these factors have been proven to influence the DNA methylation sequence in target tissues for insulin resistance in humans. We aimed to evaluate insulin-like growth factor binding protein-1 (IGFBP1) gene methylation levels in T2D. In all, 100 Egyptian individuals were included in this study: 50 patients with T2D versus 50 healthy controls. Genomic DNA was extracted from peripheral blood and IGFBP1 methylation levels were analyzed using pyrosequencing.

Results: DNA methylation levels in the IGFBP1 gene at each of the six CpG sites were significantly higher in the T2D patients than in the controls at P values of 0.001, 0.002, 0.010, 0.007, 0.014, and 0.001, respectively.

Conclusion: According to this study, T2D is due to interactions between genetics, epigenetics, and lifestyle. This study also revealed that DNA methylation levels of the IGFBP-1 gene are higher in T2D patients than in healthy control.

Keywords: Epigenetics, IGFBP1, DNA methylation, Type 2 diabetes mellitus

Background

Type 1 diabetes (T1D) is an autoimmune disorder in which insulin is not produced. Therefore, insulin treatment is needed for patients with this disease to maintain life. Unlike type 1 diabetes, type 2 diabetes (T2D) is characterized by decreased production of insulin or insulin resistance as the body does respond to the released insulin. Genetic and environmental factors have a great influence on T2D.

Several T2D risk factors, such as age, sex, ethnicity, smoking, obesity, and central obesity, an unhealthy diet

containing low percentages of fibers and high percentages of saturated fat, poor physical activity, family history of T2D, history of gestational diabetes mellitus, history of the non-diabetic elevation of fasting or 2-h glucose, and different drug treatments have been associated with modification of epigenetic marks [1].

Genome-wide association studies (GWAS) have detected some genetic susceptibility variants for type 2 diabetes. These findings can explain approximately 10% of the overall risk of T2D [2].

Several studies suggest that genes and environmental interactions through epigenetic modifications influence a person's susceptibility to diabetes because environmental factors can stimulate intracellular signaling pathways, leading to alterations in gene expression through

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epigenetic modifications such as DNA methylation, which integrates genetic susceptibility and environmental factors by adjusting transcriptional regulation with no change in the sequence of underlying DNA [3].

Epigenetic factors may be the reason for the missing information on inheritance. Due to the limited information provided on epigenetic factors associated with T2D until now, further epigenetic studies have been required, to help in better understand T2D pathogenesis [4].

Insulin-like growth factor-binding protein-1 (*IGFBP-1*) is a protein secreted by the liver that has an inhibitory influence on insulin-like growth factor (IGF)-I and IGF-II actions in peripheral tissues. Low circulating levels of *IGFBP-1* are associated with insulin resistance and metabolic syndrome. Moreover, they predict T2D development [5].

DNA methylation variation is hypothesized to affect a person's susceptibility to T2D and its levels are detected at clusters of CpG methylation sites in the genes that are used to indicate epigenetic effects [6].

Methods
Study population
This study was performed at the Molecular Biology Unit, Clinical Pathology Department. The study included 50 subjects with T2D diagnosed according to the American Diabetes Association Diagnosis and classification of diabetes mellitus, 2014 [7].

There were 21 males and 29 females aged between 38 and 69 years. Fifty apparently healthy volunteers with normal fasting plasma glucose and HBA1c levels were included in the study as controls. There were 25 males and 25 females and their age ranged between 34 and 58 years. All participants were subjected to detailed medical and family history and clinical examination including weight and height measurements to calculate body mass index (BMI) according to the standard formula BMI = weight (kg)/[height (m)]^2.

A family history of diabetes (FHD) was defined as having at least one first-degree relative or two second-degree relatives with diabetes.

The exclusion criteria were type 1 diabetes, secondary diabetes, major organ failure, metabolic disorders, endocrine diseases other than T2D, autoimmune diseases, malignancy, and pregnancy (the demographic, clinical and laboratory data of T2D, and control subjects are summarized in Table 1).

Informed consent was obtained from all participants. The study was approved by the Ethical Committee of the National Research Centre.

Methodology
Morning venous blood samples were drawn from all subjects after an 8-h fasting. Fasting plasma glucose

Table 1 Demographic, clinical, and laboratory data of the studied groups

	T2D (no.=50)	Controls (no.=50)
N (male/female)	50 (21/29)	50 (25/25)
Age (years)	48 ± 9	39± 10
BMI (kg/m^2)	32 ± 4	29± 8
FPG (mg/dl)	202± 83	82± 9.5
PP (mg/dl)	226±69	94± 11
HBA1c (%)	8.9± 2	5.2± 0.7
Fasting insulin (uIU/ml)	9 (3.7-20.5)	3.4 (2.8-4.6)

BMI body mass index, *FPG* fasting plasma glucose, *PP* postprandial plasma glucose, *HBA1c* glycated hemoglobin. Age (years), BMI (kg/m^2), FPG (mg/dl), PP (mg/dl), and HBA1c (%) are represented as the mean ± SD, sex is represented as number, and fasting insulin is represented as the median and interquartile range (IQR)

(FPG) level was assayed on an Automated Clinical Chemistry analyzer Olympus AU 400. HbA1c level was assayed by a Nycocard Reader II analyzer using kits supplied by Alere Technologies (Alere Technologies AS Kjelsasveien 161 P.O. Box 6863 Rodelokka N-0504 Olso, Norway).

Fasting serum insulin levels were determined using an ELISA kit supplied by immunospec (Immunospec Corporation, 7018 Owesmounth Ave. Suit 103 Canoga Park, CA91303, USA, catalog no. E29-072) [8].

Analysis of *IGFBP-1* DNA methylation
DNA extraction and bisulfite treatment
Genomic DNA was extracted using a QIAamp DNA extraction kit (DNA Blood Mini Kits -50-catalog no. 51104) supplied by QIAGEN (QIAGEN GmbH, D-40724 Hilden Germany). The concentration and purity of DNA were determined using a NanoDrop 2000C (Thermo Fisher Scientific -168 Third Avenue Walthman, MA USA 02451). DNA integrity was checked by agarose gel electrophoresis. Samples were migrated at 100 volts for 30 min and viewed on an E-Gel Imager (Thermo Fisher-USA). The stock solution of DNA samples was stored at −80 °C until use. For epigenetic analysis, the extracted DNA samples were bisulfite-treated using the EpiTect Fast DNA Bisulfite Kit, -50-catalog no. 59824 supplied by QIAGEN, giving complete conversion of unmethylated cytosine to uracil and subsequent purification in less than 6 h. This highly sensitive method utilizes an innovative protection against DNA degradation and ensures high conversion rates of over 99%. DNA extraction and bisulfite treatment were conducted according to the manufacturer's instructions.

DNA amplification
DNA amplification was performed by PCR using a Pyro-Mark PCR Kit, (catalog no. 978703) and an Applied Biosystems Veriti Thermal Cycler from (Thermo Fisher-USA)

.The amplification products were separated on a 2% agarose gel stained with ethidium bromide. Bands were visualized by UV transillumination.

Preparation of single-stranded DNA template followed by pyrosequencing methylation analysis of CPG sites

IGFBP1 DNA methylation analyses were performed by PyroMark Gold Q24 reagents (catalog no. 970802) and PyroMark Q24 CpG assay (catalog no.978746) using the PyroMark Q24 pyrosequencing system (catalog no. 9001514) supplied by QIAGEN.

Data analysis

Pyrogram outputs were analyzed by PyroMark Q24 software (cat. no. 9019062) used for methylation data analysis. The relative peak height of T versus C at that CpG is measured to give percent methylation. Quality control consists of using the non-CpG dispensions (C) as reference peaks.

Statistical analysis

Data were analyzed using SPSS© Statistics version 17 ((SPSS Inc., Chicago, IL)). Parametric numerical variables are presented as the mean ± SD, and intergroup differences were compared using the unpaired t test. Nonparametric numerical variables are presented as medians and interquartile ranges. Categorical variables are presented as numbers and percentages and intergroup differences were compared using the chi squared test. Normality was checked for all data before analysis. Comparisons between different groups were tested using two-sample t tests, ANOVA with Duncan's post hoc test, and the Mann-Whitney U test. Linear regression was used to determine factors affecting methylation. Correlations among numerical variables were tested parametrically using the Pearson product-moment correlation or nonparametrically using the Spearman rank correlation, as appropriate. A P value <0.05 was considered statistically significant.

Results

Analysis of *IGFBP1* DNA methylation levels in the studied groups

DNA methylation of six CpG sites in the human *IGFBP1* gene was analyzed by bisulfite pyrosequencing (referred to as CpG1 to CpG6). The T2D group showed significantly higher *IGFBP1* DNA methylation levels at each of the six CpG sites (CpG %) (CpG1, 33.2%; CpG 2, 29.8%; CpG 3, 25%; CpG 4, 24.8. %; CpG 5, 26.2 %; CpG 6, 44.7) than the control group (CpG 1, 26.7%; CpG 2, 24.1%; CpG 3, 19.1%; CpG 4, 18.6%; CpG 5, 20.8%; CpG 6, 28%) with P values of 0.001, 0.002, 0.010, 0.007, 0.014, and 0.001, respectively. Moreover, when all six CpG sites were combined (CpG mean), the mean values of *IGFBP1*

DNA methylation levels were significantly higher in the T2D group than in the control group (30.6% versus 22.8% P=0.008) as shown in Fig. 1.

Further analysis of *IGFBP-1* DNA methylation in the T2D group according to the type of treatment was performed with 28 T2D patients on insulin treatment and 22 patients on oral anti-diabetic drugs (OAD). *IGFBP1* DNA methylation levels at CpG 1, 2, and 6, and the CpG mean were significantly lower in the control group than in the insulin and oral anti-diabetic drug (OAD) groups with no significant difference between insulin and OAD groups. CpGs 3, 4, and 5 were significantly higher in the OAD group than in the control group with no significant difference between the insulin group and each of the OAD and control groups as shown in Table 2.

Association of *IGFBP1* DNA methylation level and family history

The relation between *IGFBP1* DNA methylation and family history of diabetes (FHD) was assessed and there was a significant difference concerning *IGFBP1* DNA methylation levels among T2D patients between those with a positive FHD and those with a negative FHD (Table 3).

Correlation of *IGFBP1* DNA methylation level to duration of treatment of T2D

After assessing of the correlation *between IGFBP1* DNA methylation and the duration of treatment, a statistically significant correlation was found between the duration of treatment and *IGFBP1* DNA methylation levels (P= 0.001, r= −0. 479).

Association of *IGFBP1* DNA methylation with BMI

To investigate the influence of obesity on *IGFBP1* DNA methylation levels, T2D patients and controls were divided according to BMI into two subgroups based on a BMI cut-off of 30 kg/m^2.

The results of this study demonstrated no statistically significant differences in *IGFBP1* DNA methylation levels at any of the six CpG sites in T2D patients with a BMI ≥30kg/m^2 in comparison with those with a BMI < 30kg/m^2 (P value = 0.5, 0.8, 0.8, 0.9, 0.8, and 0.7, respectively). Moreover, when all six CpG sites were combined (CpG mean), the mean of *IGFBP1* DNA methylation levels were also not significantly different (P=0.06) (Fig. 2).

Correlation between *IGFBP1* DNA methylation levels and laboratory parameters in T2D

In the current study, statistically significant correlations between *IGFBP1 DNA* methylation levels and FPG (r = −0.244, P = 0.01), 2-h PP (r = −0.315, P = 0.001), and

Fig. 1 Comparison of the *IGFBP-1* DNA methylation level in six CPG sites between the studied groups
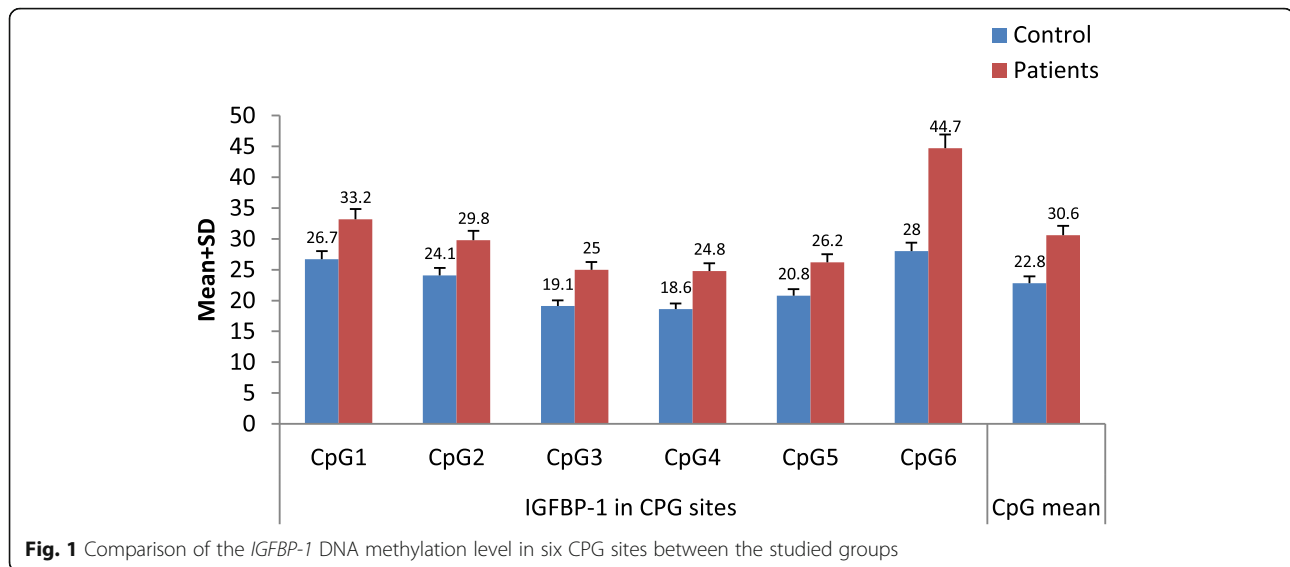
HBA1C ($r = -0.350$, $P = 0.001$) were detected in T2D patients at the CpG mean.

The influencing factors of *IGFBP1* DNA methylation

The influencing factors of *IGFBP1* DNA methylation were then confirmed using multiple stepwise regression analysis. The results showed that a positive family history was the only significant factor that affected (increased) different methylations among cases, while age was the only significant factor that affected (increased) different methylations except P2 (Tables 4 and 5).

Discussion

Several studies suggest that genes and environmental interactions through epigenetic modifications influence a person's susceptibility to diabetes because environmental factors can stimulate intracellular signaling pathways, leading to alterations in gene expression through epigenetic

modifications, such as DNA methylation, which integrates genetic susceptibility and environmental factors by adjusting transcriptional regulation with no change in the sequence of underlying DNA [3].

CpG methylation can prevent adjacent gene expression partially or completely. Accordingly, the genes containing the 5′-CpG region transcription rate are frequently inversely proportional to their methylation rates [9].

It has been demonstrated that DNA methylation is affected by age, weight, sex, lifestyle, and genetic background [10]. In the present study, age- and sex-matched T2D and nondiabetic subjects were selected to avoid the effect of age and sex.

This paper introduces an epigenetic study of the *IGFBP1 gene* that was conducted on an Egyptian cohort. The results indicated an increase in *IGFBP1* DNA methylation levels in patients with T2D compared to nondiabetic controls.

A previous study has shown that the region of high CpG density at the 5′-UTR recruits a methyl-CpG binding protein to the promoter region [11].

Some researchers found that fasting *IGFBP-1* is a good predictor of abnormal glucose homeostasis, with low values anticipating impaired fasting glucose development, impaired glucose tolerance and T2D in 8–17 years [12].

Type 1 diabetes is associated with a decline in *IGFBP-1* DNA methylation levels; this implies the possibility of *IGFBP-1* conferring different epigenetic effects in T1D and T2D. Therefore, different *IGFBP-1* DNA methylation levels in T1D and T2D may be related to insulin activity [11].

Both types of diabetes are characterized by hyperglycemia; however, the underlying pathogenic mechanism of T1D is different from that of T2D. The development of T1D is due to autoimmune destruction of pancreatic

Table 2 Comparison of *IGFBP-1* DNA methylation of the six combined CPG sites in the studied groups

	Insulin (N=28)	Oral (N=22)	Control (N=50)	^P value
CpG 1	33.0±7.2 a	33.4±7.3 a	26.7±9.5 b	**0.001****
CpG 2	29.8±8.1 a	29.9±8.2 a	24.1±10.0 b	**0.009****
CpG 3	24.5±10.6 ab	25.5±10.6 a	19.1±11.6 b	**0.034***
CpG 4	24.3±10.6 ab	25.4±10.3 a	18.6±11.7 b	**0.024***
CpG 5	25.6±10.7 ab	27.0±10.0 a	20.8±11.2 b	**0.045***
CpG 6	43.4±24.6 a	46.5±28.6 a	28.0±24.3 b	**0.005****
CpG mean	30.1±11.6 a	31.3±12.2 a	22.9±12.6 b	**0.008****

^ANOVA test with post hoc Duncan's test, homogeneous groups had the same symbol (a, b)
*Significant
**Highly significant

Table 3 Relation of *IGFBP1* DNA methylation levels to the presence of family history of diabetes among T2D

IGFBP-1 in CPG sites T2D patients	Positive FHD (*N*=27)	Negative FHD (*N*=23)	*P* value
AverageIGFBP1methylation value	36.9±12.0	23.0±10.8	<0.001*

FHD family history of diabetes; *T2D* type 2 diabetes
Independent *t* test
**p* significant

beta cells, leading to insulin deficiency, while T2D hyperglycemia is due to a combination of impaired insulin secretion and insulin resistance. Nevertheless, *IGFBP-1* secretion is inhibited by insulin, which means that increased insulin levels in T2D due to insulin resistance are associated with increased methylation of the *IGFBP-1* gene [13].

The present work found no significant differences between T2D patients treated with insulin and those treated with OADs, signifying no effect of diabetes treatment on *IGFBP1* methylation levels. This might be due to the small numbers of individuals in the subgroups, which goes hand by hand with Gu and his colleagues (2013) [11].

Although nonsignificant, stratification of the subjects according to BMI revealed that type 2 diabetes patients with a BMI >30 kg/m^2 had higher DNA methylation levels than those with a BMI <30 kg/m^2. The difference in the *IGFBP1* DNA methylation levels for the obese group may explain how obesity could contribute to T2D development. The countless metabolic changes taking place during T2D pathogenesis support the presence of a role for epigenetics in chronic disease development. A new data mining methodology was used to

search for a possible association between epigenetics and the pathogenesis of T2D [14]. This methodology was based on a search of Medline records where they discovered the presence of methylation and chromatin among the top-five ranked items associated with T2D, based on common shared relationships. The shared relationships were obesity, maternal influence, fatty acids, methylene tetrahydrofolate reductase, cytokines, homocysteine, variable severity, and late-onset of the disease. Based on these findings, a model for T2D pathogenesis was introduced, suggesting that an increase in short-chain fatty acid production as by-products of long-chain fatty acids leads to modifications in DNA methylation that result in dysregulation of pro-inflammatory cytokines [14].

Both genetic and environmental factors have a role in type 2 diabetes development. Another important risk factor for T2D is obesity, which mainly contributes to the rapid increase in T2D patients worldwide [15].

In addition, *IGFBP1* DNA methylation levels were discovered to be influenced by family history (FH), which is in agreement with previous work [11] that found that patients with FHD had higher *IGFBP1* DNA methylation than those without family history.
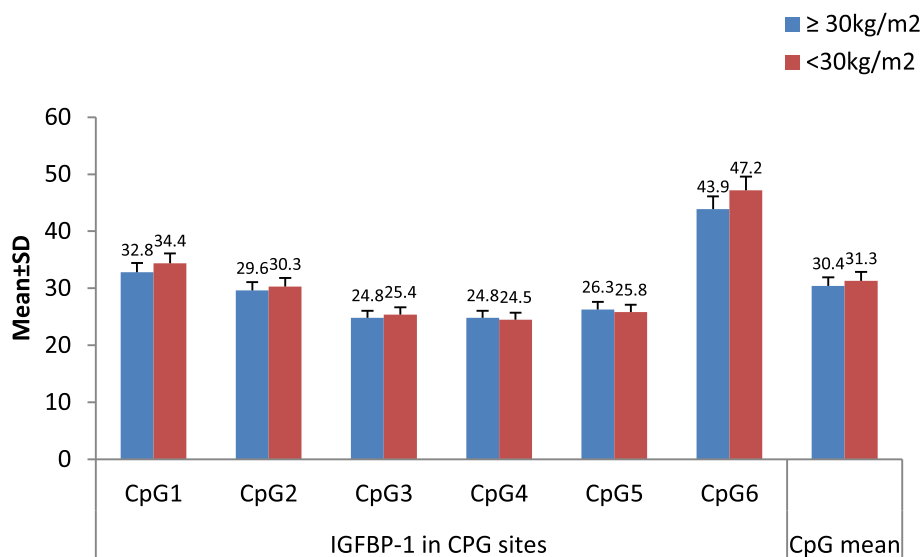

Fig. 2 Comparison of *IGFBP1* DNA methylation levels in six CPG sites in T2D patients with a BMI ≥ 30 kg/m^2 and a BMI < 30 kg/m^2. T2D, type 2 diabetes; BMI, body mass index

Table 4 Linear regression for factors affecting methylation in the T2D patients

Marker	Factor	β	SE	*P* value	95% CI	R^2
CpG 1	**Constant**	24.06	10.32	**0.024**[a]	3.25–44.87	0.373
	Age	0.11	0.16	0.516	−0.22–0.43	
	BMI	−0.05	0.12	0.655	−0.30–0.19	
	Duration	0.28	0.27	0.307	−0.27–0.83	
	Family history	8.49	1.79	**<0.001**[a]	4.88–12.11	
	Male	−0.18	1.75	0.919	−3.71–3.36	
	Insulin treatment	−2.81	2.56	0.277	−7.97–2.34	
CpG 2	**Constant**	27.11	11.68	**0.025**[a]	3.56–50.67	0.371
	Age	0.05	0.18	0.780	−0.32–0.42	
	BMI	−0.17	0.14	0.231	−0.44–0.11	
	Duration	0.30	0.31	0.339	−0.32–0.92	
	Family history	8.75	2.03	**<0.001**[a]	4.66–12.84	
	Male	−1.85	1.98	0.356	−5.86–2.15	
	Insulin treatment	−2.81	2.89	0.336	−8.65–3.02	
CpG 3	**Constant**	12.36	15.43	0.427	−18.75–43.47	0.345
	Age	0.18	0.24	0.460	−0.31–0.67	
	BMI	−0.12	0.18	0.513	−0.48–0.24	
	Duration	0.51	0.41	0.219	−0.31–1.33	
	Family history	11.41	2.68	**<0.001**[a]	6.01–16.81	
	Male	−1.42	2.62	0.591	−6.70–3.87	
	Insulin treatment	−5.23	3.82	0.178	−12.94–2.48	
CpG 4	**Constant**	17.83	14.69	0.231	−11.79–47.44	0.394
	Age	0.05	0.23	0.813	−0.41–0.52	
	BMI	−0.12	0.17	0.470	−0.47–0.22	
	Duration	0.45	0.39	0.255	−0.34–1.23	
	Family history	12.43	2.55	**<0.001**[a]	7.29–17.57	
	Male	−0.43	2.50	0.864	−5.46–4.60	
	Insulin treatment	−5.02	3.64	0.175	−12.36–2.31	
CpG 5	**Constant**	15.66	14.89	0.299	−14.36–45.69	0.374
	Age	0.15	0.23	0.521	−0.32–0.62	
	BMI	−0.16	0.17	0.368	−0.51–0.19	
	Duration	0.52	0.39	0.190	−0.27–1.32	
	Family history	11.65	2.59	**<0.001**[a]	6.44–16.86	
	Male	−0.53	2.53	0.835	−5.63–4.57	
	Insulin treatment	−5.68	3.69	0.131	−13.11–1.76	
CpG 6	**Constant**	29.91	37.99	0.435	−46.70–106.52	0.366
	Age	0.17	0.60	0.778	−1.03–1.37	
	BMI	−0.39	0.44	0.389	−1.28–0.51	
	Duration	1.10	1.00	0.278	−0.92–3.13	
	Family history	29.53	6.60	**<0.001**[a]	16.22–42.83	
	Male	−2.67	6.45	0.681	−15.69–10.35	
	Insulin treatment	−12.78	9.41	0.181	−31.76–6.19	
CpG mean	**Constant**	21.16	16.80	0.215	−12.73–55.04	0.387
	Age	0.12	0.26	0.654	−0.41–0.65	

Table 4 Linear regression for factors affecting methylation in the T2D patients *(Continued)*

Marker	Factor	β	SE	P value	95% CI	R^2
	BMI	−0.17	0.20	0.397	−0.56–0.23	
	Duration	0.53	0.44	0.242	−0.37–1.42	
	Family history	13.71	2.92	**<0.001[a]**	7.83–19.59	
	Male	−1.18	2.85	0.681	−6.94–4.58	
	Insulin treatment	−5.72	4.16	0.176	−14.12–2.67	

β regression coefficient, *SE* standard error, *CI* confidence interval, R^2 coefficient of determination
[a]Significant

Table 5 Linear regression for factors affecting methylation in the controls

Marker	Factor	β	SE	P value	95% CI	R^2
CpG 1	**Constant**	18.52	14.85	0.219	−11.37–48.41	0.176
	Age	0.40	0.18	**0.030[a]**	0.04–0.75	
	BMI	−0.37	0.33	0.272	−1.04–0.30	
	Male	0.63	2.90	0.829	−5.21–6.47	
CpG 2	**Constant**	31.12	15.79	0.055	−0.66–62.90	0.150
	Age	0.29	0.19	0.133	−0.09–0.67	
	BMI	−0.67	0.36	0.067	−1.38–0.05	
	Male	0.03	3.09	0.993	−6.18–6.24	
CpG 3	**Constant**	7.26	18.25	0.693	−29.49–44.00	0.164
	Age	0.52	0.22	**0.021[a]**	0.08–0.96	
	BMI	−0.43	0.41	0.302	−1.26–0.40	
	Male	−1.00	3.57	0.780	−8.19–6.18	
CpG 4	**Constant**	18.94	17.82	0.293	−16.93–54.81	0.215
	Age	0.51	0.21	**0.021[a]**	0.08–0.93	
	BMI	−0.78	0.40	0.057	−1.59–0.02	
	Male	−1.14	3.48	0.745	−8.15–5.87	
CpG 5	**Constant**	8.94	17.17	0.605	−25.63–43.50	0.208
	Age	0.57	0.20	**0.008[a]**	0.15–0.98	
	BMI	−0.49	0.39	0.207	−1.27–0.28	
	Male	−1.38	3.36	0.683	−8.14–5.38	
CpG 6	**Constant**	29.33	37.22	0.435	−45.59–104.26	0.204
	Age	0.95	0.44	**0.038[a]**	0.06–1.84	
	BMI	−1.53	0.84	0.074	−3.22–0.15	
	Male	0.91	7.27	0.901	−13.74–15.55	
CpG mean	**Constant**	19.02	19.25	0.328	−19.74–57.78	0.201
	Age	0.54	0.23	**0.023[a]**	0.08–1.00	
	BMI	−0.71	0.43	0.107	−1.58–0.16	
	Male	−0.33	3.76	0.931	−7.90–7.25	

β regression coefficient, *SE* standard error, *CI* confidence interval, R^2 coefficient of determination
[a]Significant

The limitations of the present study include the small number of included subjects, and the performance of DNA methylation analyses using whole blood; we did not evaluate the cell-specific pattern for the gene region beforehand.

Conclusion

This work concluded that type 2 diabetes occurrence is due to interactions between genetics, epigenetics, and lifestyle. It also provides evidence that type 2 diabetes patients had higher DNA methylation levels of the *IGFBP*-1 gene.

Abbreviations
IGFBP1: Insulin-like growth factor binding protein-1; T2D: Type 2 diabetes

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Informed Written consent was obtained from all participants. The study has been approved by the Ethical Committee of National Research Centre. Registration Number: 15003. Written consent was obtained from study participants.

Consent for publication
"Not applicable"

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