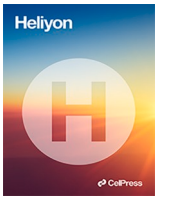
Heliyon 10 (2024) e39934

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

The impact of site-specific DNA methylation in *KCNJ11* promoter on type 2 diabetes

Mengmeng Zhu [a,b,1], Qiaoliang Huang [c,1], Heng Li [a], Yujie Zhao [a], Heming Guo [b], Tao Wang [b], Xiaodan Liu [b], Yun Huang [b], Ji Hu [b], Chen Fang [b,**], Jian Huang [a,*]

[a] *School of Basic Medical Sciences, Suzhou medical college of Soochow University, Suzhou, Jiangsu, 215123, China*
[b] *Department of Endocrinology, The Second Affiliated Hospital of Soochow University, Suzhou, Jiangsu, 215004, China*
[c] *Suzhou Center for Disease Control and Prevention, Suzhou, Jiangsu, 215000, China*

ARTICLE INFO

Keywords:
Type 2 diabetes
DNA methylation
KCNJ11 gene
TCF12 transcription factor

ABSTRACT

Aims: This study explores the correlation between site-specific methylation levels of the *KCNJ11* promoter and type 2 diabetes mellitus (T2DM), analyzing potential molecular mechanisms.
Methods: Thirty patients newly diagnosed with T2DM and 30 healthy controls were selected to determine the CpG methylation levels in the promoter region of the *KCNJ11* gene using the bisulfite assay. The online software JASPAR was used to predict transcription factors binding to differentially methylated sites. Key transcription factors were further validated through quantitative PCR (q-PCR) and chromatin immunoprecipitation followed by PCR (ChIP-PCR).
Results: Methylation at multiple CpG sites within the *KCNJ11* gene promoter was generally reduced in newly diagnosed T2DM patients compared with healthy individuals. The methylation status of CpG-471, a site crucial for the binding of the transcription factor TCF12, emerged as potentially influential in T2DM pathogenesis. This reduction in methylation at CpG-471 may enhance TCF12 binding, thereby altering *KCNJ11* expression.
Conclusion: Hypomethylation of specific CpG sites in the promoter region of the *KCNJ11* gene in patients with incipient T2DM potentially contributes to the disease's pathogenesis. This hypomethylation may influence TCF12 binding, with potential regulatory effects on KCNJ11 expression and pancreatic beta-cell function, though further studies are needed to confirm the exact mechanisms involved.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a complex polygenic disease [1], primarily driven by insulin resistance and inadequate insulin production from β cells [2]. According to the updated version of the International Diabetes Federation (IDF) Diabetes Atlas, more than 500 million patients worldwide have diabetes, and this number is expected to increase by 20 % by 2030 and 46 % by 2045 [3]. China has the highest prevalence of diabetes and prediabetes, with an estimated 10.9 % of Chinese adults diagnosed with diabetes and 35.7 % with prediabetes [4]. Due to the significant increase in the incidence of T2DM and its concomitant acute and chronic complications, it

* Corresponding author.
** Corresponding author.
E-mail addresses: afa9911@sina.com (C. Fang), huangjian79@suda.edu.cn (J. Huang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.heliyon.2024.e39934
Received 12 July 2024; Received in revised form 26 October 2024; Accepted 28 October 2024
Available online 29 October 2024

is an important cause of patient death and increased healthcare costs, which brings a huge economic burden to the society, and there is an urgent need to actively intervene in the treatment of T2DM from the etiologic and early prevention perspectives [5,6]. The etiology of T2DM is complex, resulting from a combination of genetic and environmental factors [7]. An important area of current research is the search for reliable, sensitive and easily accessible biomarkers for T2DM. In this context, a growing number of studies [8–13]suggest that genetic and epigenetic mechanisms together play an important role in the pathogenesis of T2DM patients.

Differential variability in DNA methylation, often indicative of epigenetic dysregulation, is associated with various phenotypes, pathologies, and adverse environments [9]. Initial epigenetic studies in pancreatic islets and skeletal muscle of T2DM patients identified altered methylation patterns [14].Recent genome-wide DNA methylation studies have confirmed these findings, revealing altered methylation levels in diabetes-associated genes like TCF7L2 [15], PDX-1 [16], CDKN1A [17], FTO [18], and PPARGC1A [19], with changes in their promoter regions correlating with diabetes risk and severity. Additionally, data suggest that the *KCNJ11* gene is closely related to the development of T2DM, and methylation of its promoter region may play an important role in it [20]. *KCNJ11* is a gene encoding Kir6.2, which constitutes the pore-forming subunit of the ATP-sensitive K+ channel (KATP channel) [21], and this channel plays a critical role in ATP-dependent insulin secretion in pancreatic β-cells [22]. Previous studies have confirmed that inactivating mutations in the *KCNJ11* gene can lead to congenital hyperinsulinemia [23]; heterozygous mutations in the *KCNJ11* gene cause neonatal diabetes mellitus [24,25]; a common single nucleotide polymorphism, E23K, in Kir6.2 increases susceptibility to T2DM [26,27]; and along with genes such as ADCY5 and FTO, the *KCNJ11* gene is a well-characterized type 2 diabetes susceptibility gene [28]. These findings highlight the complex interplay of genetic and epigenetic factors in T2DM, emphasizing the potential of methylation studies in understanding and addressing this widespread disease.

While genome-wide association studies have extensively documented changes in methylation patterns, few have focused on methylation at specific sites. Therefore, our study aims to investigate the correlation between specific methylation sites in the *KCNJ11* gene promoter and the onset of T2DM, as well as how these modifications affect gene expression through transcription factor regulation. Supporting literature suggests that age-related methylation changes detectable in blood could serve as biomarkers for T2DM [29–31]. To this end, we selected 30 patients newly diagnosed with T2DM (20 men and 10 women), average age 57.0 ± 13.1 years. We also recruited 30 matched controls—18 males and 12 females, average age 56.7 ± 10.1 years, all meeting specific criteria for glucose tolerance. Exclusion criteria were a prior diabetes diagnosis, serious concurrent conditions, pregnancy, and dropout from follow-up. Comparisons between baseline data of the two groups showed no significant differences ($P > 0.05$), confirming their comparability. Preliminary results indicate an overall decrease in methylation at several sites within the *KCNJ11* promoter in T2DM patients compared with controls, and the CpG-471 site was found to be critical for TCF12 binding—highlighting its potential role in T2DM pathogenesis.

2. Materials and methods

2.1. Sample collection

After receiving approval from the Institutional Ethics Committee, blood samples were collected from 30 newly diagnosed patients with T2DM at the Department of Endocrinology, Second Affiliated Hospital of Soochow University, and 30 healthy volunteers between November 2022 and November 2023. Samples were drawn from the patients' arm veins using disposable sterile syringes and stored in EDTA-containing tubes to prevent coagulation. Collection occurred after an overnight fast, adhering to the WHO 1999 diagnostic criteria. Patients were informed and voluntarily participated in the study as advised by their physicians.

2.2. DNA extraction

DNA extraction was performed using the TIANamp Genomic DNA kit (DP304; TIANGEN, Germany) according to the manufacturer's standard procedure. Genomic DNA was isolated from 200 μl of the collected peripheral blood samples. The purity of the DNA samples was assessed by calculating the absorbance ratio at 260 nm–280 nm using an Invitrogen Nanodrop spectrophotometer. The isolated DNA samples were stored at −80 °C for further analysis.

2.3. McrBC-PCR analysis

McrBC is a GTP-dependent specific nucleic acid endonuclease that acts on methylcytosine-containing DNA and does not act on unmethylated DNA [32]. The DNA methylation level of the region upstream of the *KCNJ11* transcription start site was detected by McrBC-PCR. McrBC digestion was performed with at least 200 ng of genomic DNA using the McrBC kit (M0272S; NEB) according to the manufacturer's instructions. The same amount of DNA without McrBC digestion was used as a negative control. Primers were designed using Primer3, sequences of primers used for McrBC PCR are listed in Supplementary Table 1, 50ul of the PCR amplification system was used and the products were analyzed by electrophoresis on a 1.5 % agarose gel.

2.4. Bisulfite sequencing PCR(BSP)

DNA Bisulfite Conversion Kit (E3318S; NEB) was applied to DNA bisulfite modification according to the instructions. The total PCR system was 50 μL, to which 6 μL of bisulfate-modified DNA template, 25 μL of 2 × PCR buffer, 2 μL each of upstream and downstream primers were added, and the final volume was adjusted to 50 μL with double-distilled water. The PCR conditions and setting reaction

procedures were as follows: pre-denaturation at 94 °C for 3 min, denaturation at 94 °C for 30s, annealing at 55 °C for 30s, and extension at 72 °C for 16s for 35 cycles, and the reaction ended with a final extension at 72 °C for 7 min. The PCR products were electrophoresed in a 2 % agarose gel, after which the target fragments were recovered. The pMD18-T vector (6011, TaKaRa) was used for TA clonal ligation, followed by transformation of DH5α receptor cells. Positive clones were selected and sent to AZENTA for sequencing, and the methylation rate was calculated as the number of methylated cytosine (mC) clones/10 × 100 %. The sequencing results were analyzed online according to Web-based Kismeth software (http://katahdin.Mssm.edu/ismet/revpage.pl) [33].

2.5. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was used to validate the binding of the transcription factor TCF12 to the promoter of the *KCNJ11* gene. 293T cells were fixed with formaldehyde, and glycine was added for cross-linking. The samples were then placed on ice and sonicated to isolate DNA. Cell lysates were incubated overnight with antibodies (anti-TCF12 antibody, sc-28364, Santa Cruz Biotechnology or control IgG) at a dilution of 1:50 and then with protein G-agarose beads (#3956088, Merck KgaA, Germany) at 4 °C overnight. Mouse control IgG (#3452481, Merck KgaA, Germany) was used as a negative control for the reaction. The bound DNA-protein mixture was eluted and the cross-linking was reversed after several washes. The DNA fragments were then purified and subjected to PCR with the *KCNJ11* primer. The PCR products (172 bp) were separated on a 2 % agarose gel and visualized on a UV transilluminator.

2.6. Bioinformatic analysis

We began by searching for BioProjects on the National Library of Medicine using the keyword "T2DM patients" and discovered 89 projects in total. We then filtered using the keywords "human" and "SRA" and found 24 projects. We then filtered the projects involving microbes and other mixed diseases. Because this was a blood study, we ultimately chose PRJNA649863 (SRP274496), which included blood transcriptome data from six healthy people and six diabetic patients.

Short reads downloaded from the SRA database with accession SRP274496 were used to produce quality control reports through FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and subjected to low-quality filtering and adapter trimming using EtoKi (https://pubmed.ncbi.nlm.nih.gov/31809257/). Clean data were aligned to the reference genome using Hisat2 (https://pubmed.ncbi.nlm.nih.gov/31375807/), and the mapped results were sorted using Samtools (https://pubmed.ncbi.nlm.nih.gov/19505943/). Transcriptome assembly and counting were carried out using StringTie (https://pubmed.ncbi.nlm.nih.gov/25690850/). Bioinformatic analysis was performed using the R command line, and differential expression analysis was conducted using edgeR (https://pubmed.ncbi.nlm.nih.gov/19910308/). R packages pheatmap (https://cran.r-project.org/web/packages/pheatmap/index.html) were used for visualization.

2.7. In vitro siRNA transfection and insulin secretion assay

The sequences of the small interfering RNAs (mus TCF12 siRNAs) used were as follows (5′–3′): si-1: CAGGCUGUCAGUCUAGUCUTT; si-2: GCAGCAACUUCACGAGCAUTT; and si-3: GGUCCUGUCUAGUACUGUUTT, which were constructed by GenePharma (Shanghai, China), and used to inhibit the expression of TCF12. MIN6 cells were cultured in six-well plates to 60–70 % confluence and were transfected with 75 pM negative control (NC) or TCF12 siRNA using siRNA-mate plus transfection reagents (GenePharma) according to the manufacturer's instructions. After 48 h, half of the cell lysates were obtained to analyze the expression of the genes of interest [34].

The remaining half of the post-transfected MIN6 cells were incubated in 24-well plates and was grown to approximately 80–90 % confluence, MIN6 cells were washed with Krebs–Ringer–bicarbonate HEPES (KRBH) buffer containing 2.8 mm glucose (low glucose) twice and then preincubated for 30 min in KRBH containing low glucose. After preincubation, the cells were stimulated with KRBH containing 30 mm KCl for 1 h. Following incubation, the supernatants were collected for measurement of the amount of released insulin [35]. The cell lysates were collected to determine the total amount of protein [36]. The Insulin release content was measured via a highly sensitive mouse insulin immunoassay kit (EZassay, China, MS200). The protein concentration was measured via a BCA protein assay kit (Solarbio, China, #PC0020).

2.8. Western blot

The same transfection method described in 2.7. was performed on MIN6 cells, and after 48 h, half of the cell lysates were also obtained to analyze the expression of the genes of interest; the remaining half of the post-transfected MIN6 cells were collected to analyze the expression of the protein of interest.

For protein extraction, the cells were lysed with RIPA lysis buffer. The protein concentrations were determined via BCA protein assay kits (Solarbio, China, #PC0020). Protein samples were mixed with SDS sample buffer and heated to 100 °C for 10 min. Subsequently, equal amounts of protein were separated by 8%–10 % SDS–polyacrylamide gel electrophoresis, electroblotted onto PVDF membranes, blocked with bovine serum albumin (BSA) for 2 h, and then incubated first with primary antibodies against KCNJ11 (1:600) and α-Tubulin(1:6000) overnight at 4 °C and, after washing, with the corresponding secondary antibodies (1:6000). The immunoreactive bands were developed using chemiluminescence.

2.9. Quantitative real time polymerase chain reaction assay

Peripheral blood leukocyte RNA was extracted with TRIzol ™ Reagent (15596026CN, Thermo Fisher Scientific), and reverse transcription was performed according to the instructions of the HiScript II Q RT SuperMix for qPCR(+gDNA wiper)(R223,Vazyme) kit. The total volume of the QPCR amplification mixture was 10 μL, and 1 μL of cDNA template, 5 μL of 2*ChamQ SYBR qPCR Master Mix (Q341,Vazyme), and 0.2 μL of upstream and downstream primers were added. The volume was adjusted to a final volume of 10 μL with double-distilled water. The PCR conditions and the setup of the reaction program were as follows: pre-denaturation at 95 °C for 180s, denaturation at 95 °C for 15s, and annealing and extension at 60 °C for 60s, and the reaction was run for 39 cycles.

2.10. Statistical analysis

The data are presented as the mean ± SD. Differences in mean values between two groups were analyzed using the two-tailed *t*-test. Statistical analyses were performed using the GraphPad Prism version 6.0 (GraphPad Software, USA). Correction for multiple testing was performed using FDRs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ or ****$p < 0.0001$ were considered statistically significant.

Clinically included data were categorized into two types of continuous and categorical variables. Continuous variables are expressed as the means ± standard deviations and were subjected to a *t*-test (normal distribution) or the Kruskal-Wallis rank sum test (nonnormal distribution) depending on the normality of the distribution. Correction for multiple testing was performed using FDRs or Bonferroni, respectively. And categorical variables are expressed as percentages and were subjected to the $\chi 2$ test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical characteristics of the study population

To maintain a consistent genetic background and characterize the course of the disease, several potential confounders were considered in this study based on the literature and clinical judgment. Covariates included age, sex, body mass index, smoking status, alcohol consumption status, family history, physical activity, creatinine, total cholesterol, triglyceride, and low-density lipoprotein levels of the included subjects. The data included in this study were categorized into two types of continuous and categorical variables. Continuous variables are expressed as the mean ± standard deviation and were subjected to *t*-test (normal distribution) or Kruskal-Wallis rank sum test (non-normal distribution) depending on the normality of the distribution. Correction for multiple testing was performed using FDRs or Bonferroni, respectively. And categorical variables were expressed as percentages and were subjected to the $\chi 2$ test (Table 1). The subjects were divided into a healthy control group and a first-onset type 2 diabetes group according to the WHO 1999 diabetes diagnostic criteria. After the application of strict inclusion criteria, Table 1 shows that the differences between the two groups were statistically significant only for fasting blood glucose, 2-h postprandial blood glucose, and glycosylated hemoglobin($P < 0.01$).

Table 1
Clinical Characteristics of the subjects.

Parameters	Healthy controls	T2DM	P value
n (male/female)	30(20/10)	30(18/12)	
Age (year)	56.7 ± 10.1	57.0 ± 13.1	0.895
BMI (kg/m2)	25.9 ± 3.6	28.5 ± 4.7	0.287
HbA1c(%)	5.7 ± 0.8	7.3 ± 1.2	0.0024
FBG(mmol/L)	4.9 ± 0.5	8.3 ± 1.1	0.0024
PBG (mmol/L)	6.8 ± 0.6	12.4 ± 0.5	0.0024
Family history	4(13.3 %)	8(26.67 %)	0.36
Smoking	5(16.67 %)	7(23.33 %)	0.747
Alcohol	5(16.67 %)	9(30 %)	0.36
Physical activity	12(40 %)	10(33.33 %)	0.789
Scr (umol/L)	95.51 ± 0.4	96.35 ± 0.6	0.895
TG(mmol/l)	1.54 ± 0.3	1.59 ± 0.5	0.895
TC(mmol/l)	4.34 ± 0.2	4.39 ± 0.4	0.895
LDL-C(mmol/L)	2.83 ± 0.25	2.86 ± 0.34	0.895

Data are presented as mean ± standard deviation or as the numbers (%) of participants. Continuous variables are subjected to a *t*-test (normal distribution) or the Kruskal-Wallis rank sum test (nonnormal distribution) depending on the normality of the distribution, and P-values are shown after applying the FDR or Bonferroni multiple tests, respectively; categorical variables are subjected to the $\chi 2$ test. Abbreviations: BMI – body mass index; HbA1c – hemoglobin A1c; FBG – fasting blood glucose; PBG – postprandial blood glucose; Scr – serum creatinine; TG – triglyceride; TC – serum total cholesterol; LDL-C – low-density lipoprotein cholesterol; p – statistical significance.

3.2. McrBC-PCR analysis

Agarose gel electrophoresis of the PCR products of diabetic and healthy control samples with the same amount of DNA before and after 1h of McrBC digestion and analysis of gray values are shown in (Fig. 1). The results demonstrated that DNA methylation modification exists in the promoter region of the *KCNJ11* gene in patients with first-onset T2DM and healthy controls, and that the degree of methylation modification in healthy control samples is likely to be greater than that in patients with first-onset T2DM.

3.3. DNA methylation patterns in the KCNJ11 promoter of healthy vs. T2DM groups

We analyzed DNA methylation at selected locations in this region in the healthy and T2DM groups via bisulfite sequencing method and selected the sequencing results of one of the most representative samples from each group to be displayed in Fig. 2B. As shown in Fig. 2B, there are 30 cytosines in the 151 bp bases sequenced from the *KCNJ11* promoter region in this study, of which 9 are CG, 9 are CHG, and 12 are CHH. The red solid dots represent the CG sites, and the blue solid dots represent CHG, implying that the two bases immediately following the methylated cytosine are H (H for either A, C, or T) and G, and the green solid dots represent CHH, which means that the methylated cytosine is immediately followed by H (H for either A, C, or T), with no guanine involved. DNA methylation (DNAm) in mammals is mostly examined within the context of CpG dinucleotides [37,38].

After bisulfite treatment and sequencing of positive clones, it was demonstrated that there were nine methylation sites, including CpG-449, CpG-454, CpG-471, CpG-499, CpG-504, CpG-510, CpG-513, CpG-520, and CpG-539.The methylation ratios of the above loci were lower overall in patients in the type 2 diabetes group, whereas they were relatively hypermethylated in the healthy normal control group (Table S2). Moreover, at the CpG-471 locus, the methylation rate was significantly lower and statistically different in the T2DM group compared with that in the healthy control group (Fig. 2C).

3.4. TCF12 directly bind to KCNJ11 promoter

DNA methylation has been extensively studied over the past few decades, and the attachment of methyl groups to the promoter region of a gene might inhibit transcription factor binding, and hence usually reduce transcription; the opposite phenomenon also holds true [39,40]. The results analyzed by JASPAR and AlphaFold online software showed that the TCF12 transcription factor binds to the differentially methylated site CpG-471 in the promoter region of the *KCNJ11* gene, which may explain the hypomethylation of the *KCNJ11* promoter region in patients with T2DM, which can affect protein expression by enhancing the activity of the transcription factor TCF12 and thus predispose them to developing T2DM (Fig. 3A). CHIP-PCR gel electrophoresis revealed target bands in the input group and anti-TCF12 group but no bands in the IgG or blank group (Fig. 3B), which also confirmed that the transcription factor TCF12 binds to the promoter region of the *KCNJ11* gene and may be involved in the regulation of KCNJ11 expression. possibly influencing the regulatory pathways involved in pancreatic beta-cell function.

3.5. TCF12 knockdown effects on KCNJ11 level and insulin secretion in MIN6 cells

To explore the relationship between TCF12 activity and phenotypic characteristics of T2DM, we designed three pairs of small

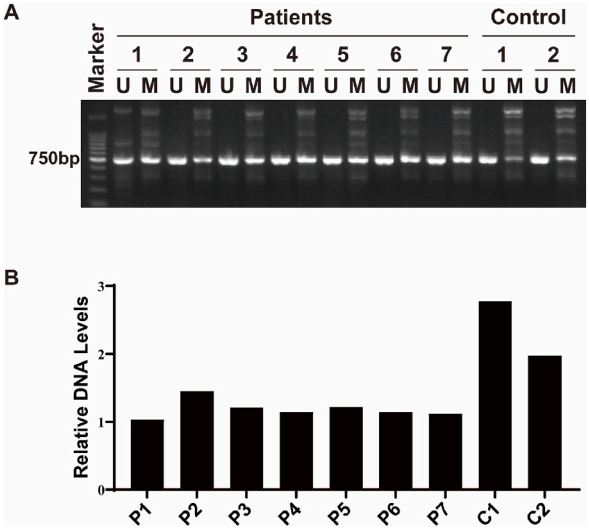


Fig. 1. Gel electrophoresis of different diabetic samples and healthy subjects after 1h digestion by McrBC. The same amount of DNA from the same sample on the left side is the uncut control, and on the right side is the digestion for 1h (A); The below panel shows the bar graph of gray value analysis (B). Abbreviations: U – undigested; M – McrBC digested; P – patient; C – healthy control.

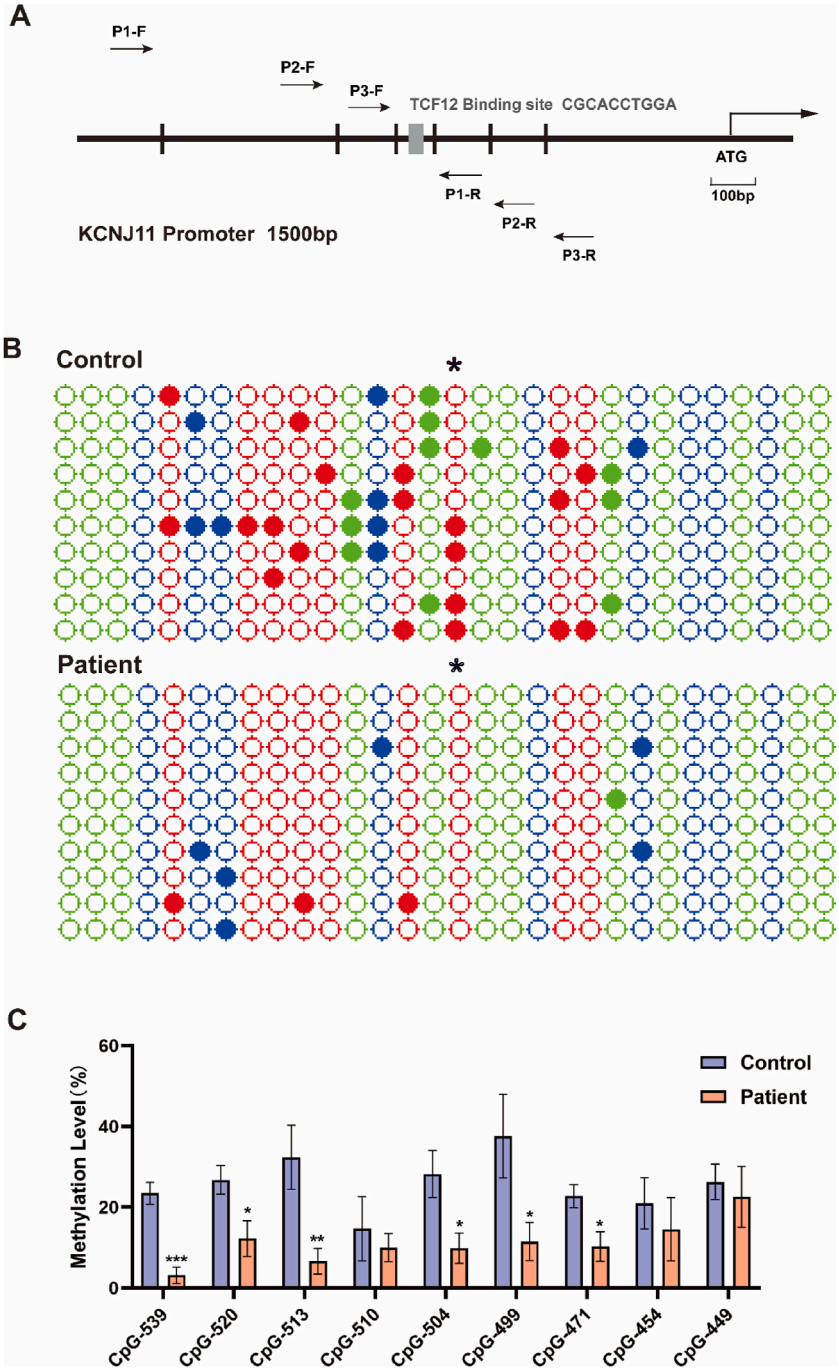


Fig. 2. Schematic illustration of the amplification positions of each primer pair in the promoter region of *KCNJ11* and the binding site of the TCF12 transcription factor.P1 represents the McrBC primer (668bp), P2 represents the BSP primer (151bp), and P3 represents the CHIP primer (172bp)(A). Healthy control and T2DM genomic DNA were sequenced for different clones after treatment with sodium bisulfite and one representative sample of each group was selected for presentation, scatters represent cytosines at the positions indicated in the *KCNJ11* 5′ region, the red solid dots represent the CG sites, the blue solid dots represent CHG, and the green solid dots represent CHH,* labeled at the CpG-471 site (B).Histogram of the methylation rate of the *KCNJ11* promoter region in the first-onset T2DM group (red bars) and healthy control group(blue bars) (C). Abbreviations: P – primer; F – forward; R – reverse.

interfering RNAs (siRNAs) to knock down *TCF12* expression in the MIN6 cell line. Among these, siRNA-2 effectively reduced *TCF12* levels by 43.7 % (Fig. 3C). Although the relative mRNA levels of *KCNJ11* did not reach statistical significance (P = 0.09) (Fig. 3D), a trend towards down-regulation was observed. Furthermore, Western blot analysis demonstrated a significant decrease in KCNJ11

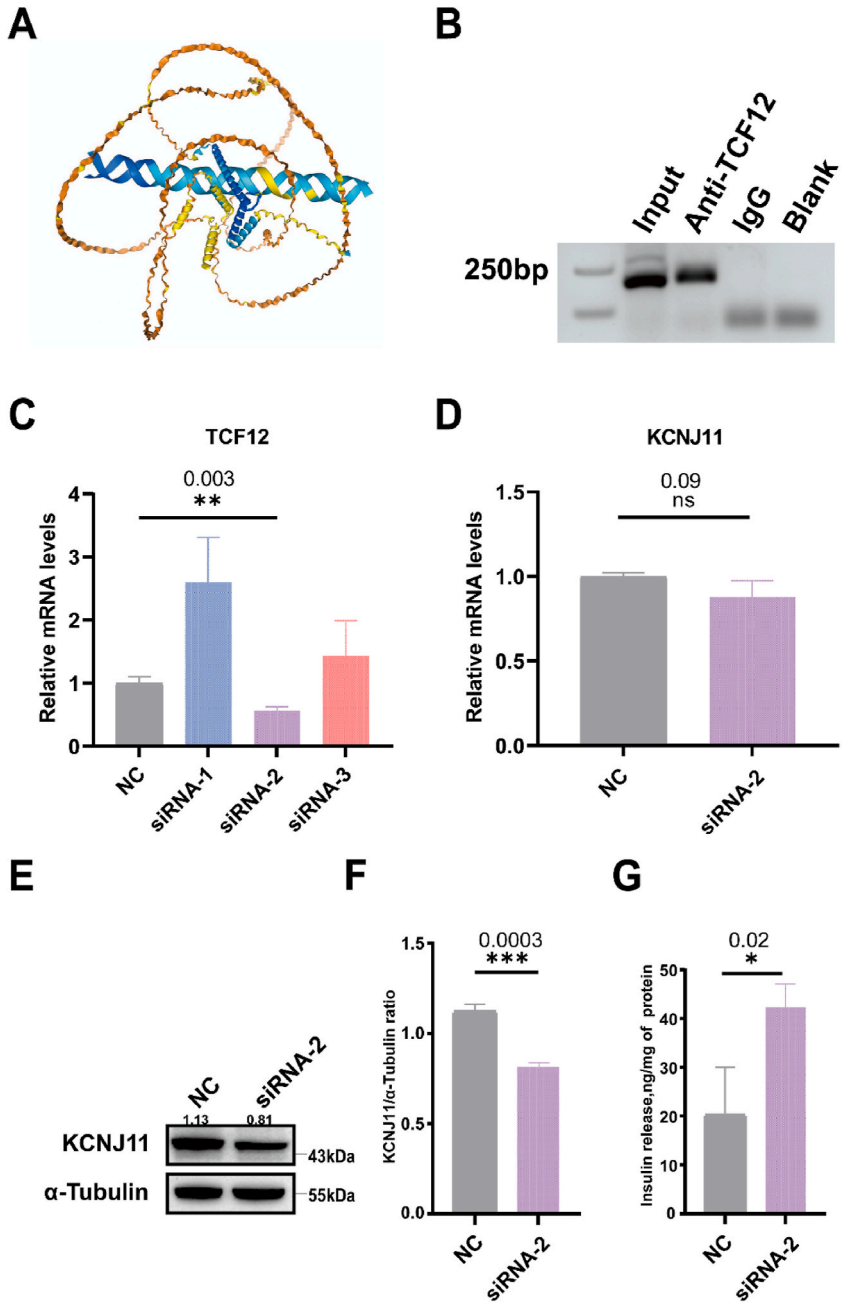


Fig. 3. Structural pattern of the binding of the transcription factor TCF12 to the *KCNJ11* promoter region demonstrated by AlphaFold software(A). The PCR products of the 293T cells from the ChIP assay were visualized on a UV transilluminator (B). Verification of the interference efficiency of TCF12 by real-time quantitative PCR (C). Quantitation of the *KCNJ11* relative mRNA level relative to β-actin. The mean value of the relative quantification of *KCNJ11* gene expression in the siRNA-2 group was 0.877, and the P value was 0.09 (D). Western blot showing a significant decrease in KCNJ11 protein levels in the siRNA-2 knockdown group. Densitometric analysis was performed by comparing the intensity of KCNJ11 bands to the corresponding α-Tubulin bands, with α-Tubulin serving as the loading control (set to a value of 1). The relative values are displayed above each respective band(E). The expressions and quantitative analysis of KCNJ11 protein(mean ± SD, n = 3)(F). Knock down TCF12 expression by siRNA, and detected insulin release was detected via an enzyme-linked immunosorbent assay (G).

protein levels following the knockdown of *TCF12* using siRNA-2 (Fig. 3E and F). Importantly, insulin secretion was significantly increased in MIN6 cells transfected with *TCF12* siRNA-2 (Fig. 3F).

3.6. *KCNJ11* expression in human peripheral blood

All 12 people involved are of Han ethnicity in China. The patient group had been diagnosed with T2DM for at least 3 months, while the healthy group had Fasting Plasma Glucose (FPG) < 5.6 mmol/L. There was no clear smoking or drinking habits documented for them. In the T2DM patients' group, there were three males and three females, with an age of 53.50 ± 9.44, a BMI of 26.08 ± 5.48 kg/m^2, and an FPG of 8.15 ± 1.68 mmol/L. In the Healthy control group, there were six females, with an age of 43.5 ± 6.02, a BMI of 23.00 ± 1.38 kg/m^2, and an FPG of 5.02 ± 0.39 mmol/L.

The RNA-seq data from 12 blood samples obtained from the SRA database, including 6 from healthy individuals and 6 from type 2 diabetes patients, were included in the analysis. Differential gene expression analysis revealed that the *KCNJ11* gene was upregulated in the type 2 diabetes group (logFC = 1.478151, p = 0.09869249, FDR = 0.3574878) (Fig. 4A). We then extracted RNA from fresh peripheral blood of enrolled healthy controls and T2DM patients for quantitative analysis of the *KCNJ11* gene, and the results demonstrated that *KCNJ11* mRNA expression was increased in peripheral blood from patients with T2DM compared with that in peripheral blood from healthy controls (p < 0.001) (Fig. 4B).

4. Discussion

In this study, BSP-sequencing was utilized to examine methylation levels at specific sites within the *KCNJ11* gene promoter in blood samples from individuals at the early stages of Type 2 Diabetes Mellitus (T2DM). Employing bisulfite conversion, a well-established technique for DNA cytosine methylation analysis [41], our results demonstrated distinct differences in methylation patterns at several critical sites of the *KCNJ11* gene promoter in the peripheral blood between newly diagnosed T2DM patients and healthy controls.

The current literature establishes a clear link between DNA methylation at gene promoters, CpG islands, and proximal gene body sites with changes in transcriptional activity and gene expression [42,43]. Our findings reinforce this paradigm, revealing overall hypomethylation at pivotal sites within the *KCNJ11* promoter in the peripheral blood of T2DM patients. Research indicates that methylation patterns of *KCNJ11* vary by geography and ethnicity, with significant differences observed between Indian and European T2DM populations. In India, whole blood samples from type 2 diabetic individuals in the Lucknow region exhibited lower *KCNJ11* methylation compared to those from the Hyderabad region. Additionally, the European group showed significantly lower *KCNJ11* methylation levels compared to the urban Indian population [44]. Another genome-wide methylation analysis also demonstrated that the *KCNJ11* gene was also differentially methylated at sites in European T2DM populations compared with healthy control pancreatic islets and that T2DM patients were hypomethylated [45]. Both studies revealed reduced methylation of the *KCNJ11* promoter region in T2DM patients in either peripheral blood or islets, which is consistent with our findings. This epigenetic alteration may facilitate TCF12 binding, though its exact role in regulating KCNJ11 expression and T2DM susceptibility requires further investigation.

Our siRNA experiments achieved a knockdown efficiency of 43.7 % for TCF12, which may account for the lack of a statistically significant change in KCNJ11 mRNA levels. The reduction in KCNJ11 protein levels, despite the modest mRNA changes, suggests that TCF12 may also affect KCNJ11 post-transcriptionally, potentially influencing protein stability or translation. Future experiments with higher knockdown efficiency or genetic deletion like CRISPR-based deletion of TCF12 could provide more insight into its role in regulating KCNJ11 transcription.

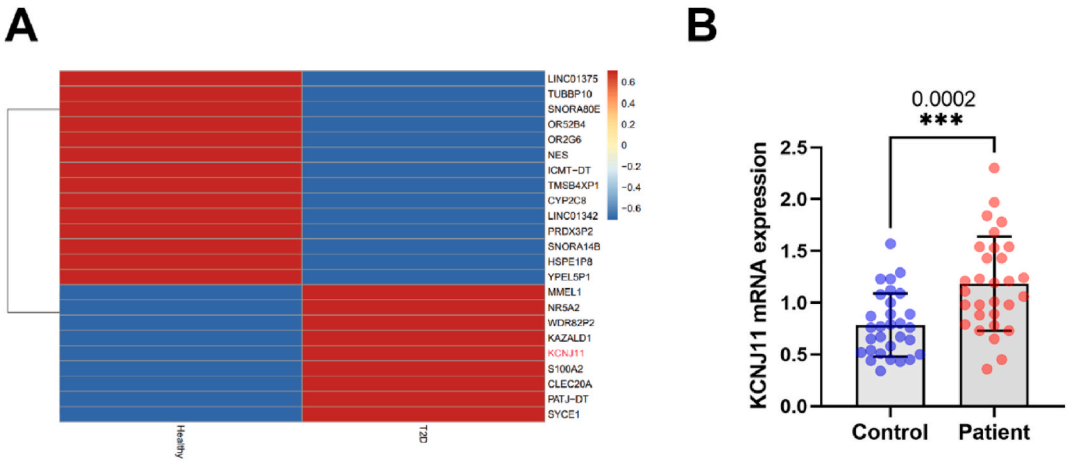


Fig. 4. RNA-seq data shows that the *KCNJ11* gene is upregulated in the type 2 diabetes group (logFC = 1.478151, p = 0.09869249, FDR = 0.3574878) (A). *KCNJ11* mRNA expression levels in human peripheral blood from healthy controls and patients with T2DM. Gene expression was analyzed using quantitative RT-PCR. The results are expressed as the mean ± SD (***p < 0.001, n = 30) (B).

Research within the diabetes field is predominantly focused on two approaches: comprehensive scans for differential DNA methylation across the genome [46] and targeted investigations into known diabetes-associated genes using specific primers to pinpoint differential methylation [15,16,47,48]. Our study's focus on the *KCNJ11* promoter methylation in peripheral blood contributes significantly to the broader epigenetic narrative of T2DM pathogenesis, paving the way for novel insights into disease mechanisms and therapeutic innovation [49]. The role of methylation in other gene loci related to diabetes progression remains an open question warranting further exploration.

With diabetes prevalence increasing globally, the imperative for innovative therapeutic approaches is more pressing than ever. Future treatment modalities might include epigenetic editing of specific genes [50], a strategy supported by emerging research advocating for targeting the enzymes that modify epigenetic landscapes in tissues affected by diabetes [51,52]. Identifying epigenetic biomarkers, readily detectable in blood and derivative from target tissues, could profoundly impact clinical practices by facilitating easier analysis in diabetic and at-risk populations. Epigenetic biomarkers that are translated from target tissue to blood may subsequently be of great clinical relevance [9].However, the discovery of epigenetic markers with superior predictive strength over existing diabetes indicators remains a critical research need.

It is notable that KCNJ11 has not been consistently identified as differentially expressed in previous studies of pancreatic islets in T2DM. There are several potential reasons for this discrepancy. First, KCNJ11 regulation may be tissue-specific, with differential expression more prominent in peripheral blood than in pancreatic islets due to different local microenvironments and signaling pathways. Second, the timing of disease progression could be a factor, as the differential expression may be more evident in early-stage T2DM, which may not have been the focus of previous islet studies.

From a technical standpoint, previous studies may have had limited sample sizes, reducing statistical power, or used experimental methods such as microarray that may not capture subtle expression changes [17,45,46]. these biological and technical differences suggest that the role of KCNJ11 in T2DM may be more complex and context-dependent than previously appreciated.

In summary, our study has identified significant epigenetic modifications in the promoter of the *KCNJ11* gene in the peripheral blood of T2DM patients, highlighting a robust link between epigenetics and the pathophysiology of diabetes in humans. These epigenetic markers offer promising avenues as biomarkers for predicting T2DM, gauging the risk of vascular complications, and assessing the efficacy of therapeutic and lifestyle interventions, thus heralding a new era of precision medicine in diabetes care.

CRediT authorship contribution statement

Mengmeng Zhu: Writing – review & editing, Writing – original draft, Project administration, Formal analysis. **Qiaoliang Huang:** Investigation, Conceptualization. **Heng Li:** Investigation, Data curation. **Yujie Zhao:** Methodology, Data curation. **Heming Guo:** Resources. **Tao Wang:** Resources. **Xiaodan Liu:** Validation, Data curation. **Ji Hu:** Supervision, Ye Lu, Validation. **Chen Fang:** Supervision, Conceptualization. **Jian Huang:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

We confirm that this manuscript has not been published previously and is not under consideration for publication elsewhere. We declare no conflicts of interest.

Acknowledgements

This research was supported by grants from the National Natural Science Foundation of China (82371544,82070814); Suzhou Healthcare Science and Technology Innovation Program, China, (SKYD2022030); Open project of Jiangsu Health Development Research Center , China,(JSHD2022035); A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, China.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39934.

References

[1] A.L. Gloyn, M.I. McCarthy, The genetics of type 2 diabetes, Best Pract Res Clin Endocrinol Metab 15 (2001) 293–308, https://doi.org/10.1053/beem.2001.0147.
[2] R. Taylor, Type 2 diabetes: etiology and reversibility, Diabetes Care 36 (2013) 1047–1055, https://doi.org/10.2337/dc12-1805.
[3] H. Sun, P. Saeedi, S. Karuranga, M. Pinkepank, K. Ogurtsova, B.B. Duncan, et al., IDF Diabetes Atlas: global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045, Diabetes Res. Clin. Pract. 183 (2022) 109119, https://doi.org/10.1016/j.diabres.2021.109119.
[4] L. Wang, P. Gao, M. Zhang, Z. Huang, D. Zhang, Q. Deng, et al., Prevalence and ethnic pattern of diabetes and prediabetes in China in 2013, JAMA 317 (2017) 2515–2523, https://doi.org/10.1001/jama.2017.7596.

[5] Y. Zheng, S.H. Ley, F.B. Hu, Global aetiology and epidemiology of type 2 diabetes mellitus and its complications, Nat. Rev. Endocrinol. 14 (2018) 88–98, https://doi.org/10.1038/nrendo.2017.151.

[6] X. Zhuo, P. Zhang, T.J. Hoerger, Lifetime direct medical costs of treating type 2 diabetes and diabetic complications, Am. J. Prev. Med. 45 (2013) 253–261, https://doi.org/10.1016/j.amepre.2013.04.017.

[7] A.T. Kharroubi, H.M. Darwish, Diabetes mellitus: the epidemic of the century, World J. Diabetes 6 (2015) 850–867, https://doi.org/10.4239/wjd.v6.i6.850.

[8] D. Bernstein, M.L. Golson, K.H. Kaestner, Epigenetic control of beta-cell function and failure, Diabetes Res. Clin. Pract. 123 (2017) 24–36, https://doi.org/10.1016/j.diabres.2016.11.009.

[9] C. Ling, T. Rönn, Epigenetics in human obesity and type 2 diabetes, Cell Metab. 29 (2019) 1028–1044, https://doi.org/10.1016/j.cmet.2019.03.009.

[10] J.D. Wren, H.R. Garner, Data-mining analysis suggests an epigenetic pathogenesis for type 2 diabetes, J. Biomed. Biotechnol. 2005 (2005) 104–112, https://doi.org/10.1155/JBB.2005.104.

[11] M.I. McCarthy, Genomics, type 2 diabetes, and obesity, N. Engl. J. Med. 363 (2010) 2339–2350, https://doi.org/10.1056/NEJMra0906948.

[12] C. Ling, L. Groop, Epigenetics: a molecular link between environmental factors and type 2 diabetes, Diabetes 58 (2009) 2718–2725, https://doi.org/10.2337/db09-1003.

[13] T. Ronn, C. Ling, DNA methylation as a diagnostic and therapeutic target in the battle against Type 2 diabetes, Epigenomics 7 (2015) 451–460, https://doi.org/10.2217/epi.15.7.

[14] R. Barres, M.E. Osler, J. Yan, A. Rune, T. Fritz, K. Caidahl, et al., Non-CpG methylation of the PGC-1alpha promoter through DNMT3B controls mitochondrial density, Cell Metab. 10 (2009) 189–198, https://doi.org/10.1016/j.cmet.2009.07.011.

[15] H.O. Smail, D.A. Mohamad, Identification of DNA methylation change in TCF7L2 gene in the blood of type 2 diabetes mellitus as a predictive biomarker in Iraq Kurdistan region by using methylation-specific PCR, Endocr. Regul. 57 (2023) 53–60, https://doi.org/10.2478/enr-2023-0007.

[16] B.T. Yang, T.A. Dayeh, P.A. Volkov, C.L. Kirkpatrick, S. Malmgren, X. Jing, et al., Increased DNA methylation and decreased expression of PDX-1 in pancreatic islets from patients with type 2 diabetes, Mol. Endocrinol. 26 (2012) 1203–1212, https://doi.org/10.1210/me.2012-1004.

[17] T. Dayeh, P. Volkov, S. Salo, E. Hall, E. Nilsson, A.H. Olsson, et al., Genome-wide DNA methylation analysis of human pancreatic islets from type 2 diabetic and non-diabetic donors identifies candidate genes that influence insulin secretion, PLoS Genet. 10 (2014) e1004160, https://doi.org/10.1371/journal.pgen.1004160.

[18] G. Toperoff, D. Aran, J.D. Kark, M. Rosenberg, T. Dubnikov, B. Nissan, et al., Genome-wide survey reveals predisposing diabetes type 2-related DNA methylation variations in human peripheral blood, Hum. Mol. Genet. 21 (2012) 371–383, https://doi.org/10.1093/hmg/ddr472.

[19] C. Ling, S. Del Guerra, R. Lupi, T. Ronn, C. Granhall, H. Luthman, et al., Epigenetic regulation of PPARGC1A in human type 2 diabetic islets and effect on insulin secretion, Diabetologia 51 (2008) 615–622, https://doi.org/10.1007/s00125-007-0916-5.

[20] T.R. Dreszer, D. Karolchik, A.S. Zweig, A.S. Hinrichs, B.J. Raney, R.M. Kuhn, et al., The UCSC Genome Browser database: extensions and updates 2011, Nucleic Acids Res. 40 (2012) D918–D923, https://doi.org/10.1093/nar/gkr1055.

[21] F.M. Ashcroft, ATP-sensitive potassium channelopathies: focus on insulin secretion, J. Clin. Invest. 115 (2005) 2047–2058, https://doi.org/10.1172/JCI25495.

[22] N. Inagaki, T. Gonoi, JPt Clement, N. Namba, J. Inazawa, G. Gonzalez, et al., Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor, Science 270 (1995) 1166–1170, https://doi.org/10.1126/science.270.5239.1166.

[23] I. Wieland, I. Schanze, I.M. Felgendreher, W. Barthlen, S. Vogelgesang, K. Mohnike, et al., Integration of genomic analysis and transcript expression of ABCC8 and *KCNJ11* in focal form of congenital hyperinsulinism, Front. Endocrinol. 13 (2022) 1015244, https://doi.org/10.3389/fendo.2022.1015244.

[24] A.I. Tarasov, H.J. Welters, S. Senkel, G.U. Ryffel, A.T. Hattersley, N.G. Morgan, et al., A Kir6.2 mutation causing neonatal diabetes impairs electrical activity and insulin secretion from INS-1 beta-cells, Diabetes 55 (2006) 3075–3082, https://doi.org/10.2337/db06-0637.

[25] A.L. Gloyn, E.R. Pearson, J.F. Antcliff, P. Proks, G.J. Bruining, A.S. Slingerland, et al., Activating mutations in the gene encoding the ATP-sensitive potassium-channel subunit Kir6.2 and permanent neonatal diabetes, N. Engl. J. Med. 350 (2004) 1838–1849, https://doi.org/10.1056/NEJMoa032922.

[26] C. Schwanstecher, U. Meyer, M. Schwanstecher, K(IR)6.2 polymorphism predisposes to type 2 diabetes by inducing overactivity of pancreatic beta-cell ATP-sensitive K(+) channels, Diabetes 51 (2002) 875–879, https://doi.org/10.2337/diabetes.51.3.875.

[27] C. Schwanstecher, B. Neugebauer, M. Schulz, M. Schwanstecher, The common single nucleotide polymorphism E23K in K(IR)6.2 sensitizes pancreatic beta-cell ATP-sensitive potassium channels toward activation through nucleoside diphosphates, Diabetes 51 (Suppl 3) (2002) S363–S367, https://doi.org/10.2337/diabetes.51.2007.s363.

[28] P.A. Fujita, B. Rhead, A.S. Zweig, A.S. Hinrichs, D. Karolchik, M.S. Cline, et al., The UCSC Genome Browser database: update 2011, Nucleic Acids Res. 39 (2011) D876–D882, https://doi.org/10.1093/nar/gkq963.

[29] K. Bacos, L. Gillberg, P. Volkov, A.H. Olsson, T. Hansen, O. Pedersen, et al., Blood-based biomarkers of age-associated epigenetic changes in human islets associate with insulin secretion and diabetes, Nat. Commun. 7 (2016) 11089, https://doi.org/10.1038/ncomms11089.

[30] M. Bysani, A. Perfilyev, V.D. de Mello, T. Ronn, E. Nilsson, J. Pihlajamaki, et al., Epigenetic alterations in blood mirror age-associated DNA methylation and gene expression changes in human liver, Epigenomics 9 (2017) 105–122, https://doi.org/10.2217/epi-2016-0087.

[31] T. Ronn, P. Volkov, L. Gillberg, M. Kokosar, A. Perfilyev, A.L. Jacobsen, et al., Impact of age, BMI and HbA1c levels on the genome-wide DNA methylation and mRNA expression patterns in human adipose tissue and identification of epigenetic biomarkers in blood, Hum. Mol. Genet. 24 (2015) 3792–3813, https://doi.org/10.1093/hmg/ddv124.

[32] E. Sutherland, L. Coe, E.A. Raleigh, McrBC: a multisubunit GTP-dependent restriction endonuclease, J. Mol. Biol. 225 (1992) 327–348, https://doi.org/10.1016/0022-2836(92)90925-a.

[33] E. Gruntman, Y. Qi, R.K. Slotkin, T. Roeder, R.A. Martienssen, R. Sachidanandam, Kismeth: analyzer of plant methylation states through bisulfite sequencing, BMC Bioinf. 9 (2008) 371, https://doi.org/10.1186/1471-2105-9-371.

[34] S. Chen, L. Lei, Z. Li, F. Chen, Y. Huang, G. Jiang, et al., Grem1 accelerates nucleus pulposus cell apoptosis and intervertebral disc degeneration by inhibiting TGF-beta-mediated Smad2/3 phosphorylation, Exp. Mol. Med. 54 (2022) 518–530, https://doi.org/10.1038/s12276-022-00753-9.

[35] L. Zhao, C.M. Carmean, M. Landeche, B. Chellan, R.M. Sargis, Selenomethionine modulates insulin secretion in the MIN6-K8 mouse insulinoma cell line, FEBS Lett. 595 (2021) 3042–3055, https://doi.org/10.1002/1873-3468.14232.

[36] Y. Yang, Z. Cai, Z. Pan, F. Liu, D. Li, Y. Ji, et al., Rheb1 promotes glucose-stimulated insulin secretion in human and mouse beta-cells by upregulating GLUT expression, Metabolism 123 (2021) 154863, https://doi.org/10.1016/j.metabol.2021.154863.

[37] P. Titcombe, R. Murray, M. Hewitt, E. Antoun, C. Cooper, H.M. Inskip, et al., Human non-CpG methylation patterns display both tissue-specific and inter-individual differences suggestive of underlying function, Epigenetics 17 (2022) 653–664, https://doi.org/10.1080/15592294.2021.1950990.

[38] R. Lister, M. Pelizzola, R.H. Dowen, R.D. Hawkins, G. Hon, J. Tonti-Filippini, et al., Human DNA methylomes at base resolution show widespread epigenomic differences, Nature 462 (2009) 315–322, https://doi.org/10.1038/nature08514.

[39] P. Volkov, K. Bacos, J.K. Ofori, J.L. Esguerra, L. Eliasson, T. Ronn, et al., Whole-genome bisulfite sequencing of human pancreatic islets reveals novel differentially methylated regions in type 2 diabetes pathogenesis, Diabetes 66 (2017) 1074–1085, https://doi.org/10.2337/db16-0996.

[40] P.A. Jones, Functions of DNA methylation: islands, start sites, gene bodies and beyond, Nat. Rev. Genet. 13 (2012) 484–492, https://doi.org/10.1038/nrg3230.

[41] Q. Gouil, A. Keniry, Latest techniques to study DNA methylation, Essays Biochem. 63 (2019) 639–648, https://doi.org/10.1042/EBC20190027.

[42] X. Yang, H. Han, D.D. De Carvalho, F.D. Lay, P.A. Jones, G. Liang, Gene body methylation can alter gene expression and is a therapeutic target in cancer, Cancer Cell 26 (2014) 577–590, https://doi.org/10.1016/j.ccr.2014.07.028.

[43] R.A. Irizarry, C. Ladd-Acosta, B. Wen, Z. Wu, C. Montano, P. Onyango, et al., The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores, Nat. Genet. 41 (2009) 178–186, https://doi.org/10.1038/ng.298.

[44] H.R. Elliott, G.K. Walia, A. Duggirala, A. Groom, S.U. Reddy, G.R. Chandak, et al., Migration and DNA methylation: a comparison of methylation patterns in type 2 diabetes susceptibility genes between indians and europeans, J Diabetes Res Clin Metab 2 (2013) 6, https://doi.org/10.7243/2050-0866-2-6.

[45] T. Ronn, J.K. Ofori, A. Perfilyev, A. Hamilton, K. Pircs, F. Eichelmann, et al., Genes with epigenetic alterations in human pancreatic islets impact mitochondrial function, insulin secretion, and type 2 diabetes, Nat. Commun. 14 (2023) 8040, https://doi.org/10.1038/s41467-023-43719-9.

[46] M. Volkmar, S. Dedeurwaerder, D.A. Cunha, M.N. Ndlovu, M. Defrance, R. Deplus, et al., DNA methylation profiling identifies epigenetic dysregulation in pancreatic islets from type 2 diabetic patients, EMBO J. 31 (2012) 1405–1426, https://doi.org/10.1038/emboj.2011.503.
[47] N. Fatima, J.F. Schooley Jr., W.C. Claycomb, T.P. Flagg, Promoter DNA methylation regulates murine SUR1 (Abcc8) and SUR2 (Abcc9) expression in HL-1 cardiomyocytes, PLoS One 7 (2012) e41533, https://doi.org/10.1371/journal.pone.0041533.
[48] B.T. Yang, T.A. Dayeh, C.L. Kirkpatrick, J. Taneera, R. Kumar, L. Groop, et al., Insulin promoter DNA methylation correlates negatively with insulin gene expression and positively with HbA(1c) levels in human pancreatic islets, Diabetologia 54 (2011) 360–367, https://doi.org/10.1007/s00125-010-1967-6.
[49] S.K. Kota, L.K. Meher, S. Jammula, S.K. Kota, K.D. Modi, Genetics of type 2 diabetes mellitus and other specific types of diabetes; its role in treatment modalities, Diabetes Metab Syndr 6 (2012) 54–58, https://doi.org/10.1016/j.dsx.2012.05.014.
[50] H. Chen, H.G. Kazemier, M.L. de Groote, M.H. Ruiters, G.L. Xu, M.G. Rots, Induced DNA demethylation by targeting Ten-Eleven Translocation 2 to the human ICAM-1 promoter, Nucleic Acids Res. 42 (2014) 1563–1574, https://doi.org/10.1093/nar/gkt1019.
[51] D.P. Christensen, M. Dahllof, M. Lundh, D.N. Rasmussen, M.D. Nielsen, N. Billestrup, et al., Histone deacetylase (HDAC) inhibition as a novel treatment for diabetes mellitus, Mol Med 17 (2011) 378–390, https://doi.org/10.2119/molmed.2011.00021.
[52] D.P. Christensen, C. Gysemans, M. Lundh, M.S. Dahllof, D. Noesgaard, S.F. Schmidt, et al., Lysine deacetylase inhibition prevents diabetes by chromatin-independent immunoregulation and beta-cell protection, Proc Natl Acad Sci U S A. 111 (2014) 1055–1059, https://doi.org/10.1073/pnas.1320850111.