


Whole genome bisulfite sequencing of human spermatozoa reveals differentially methylated patterns from type 2 diabetic patients

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ABSTRACT

Aims/Introduction: The incidence of type 2 diabetes mellitus is increasing worldwide, and it might partly cause metabolic disorder and type 2 diabetes mellitus susceptibility in patients' offspring through epigenetic modification. However, the underlying mechanisms remain largely unclear. Recent studies have shown a potential link between deoxyribonucleic acid methylation in paternal sperm and susceptibility to type 2 diabetes mellitus in offspring, so this article focuses on whether the whole-genome methylation profiles of spermatozoa in type 2 diabetes mellitus patients have changed.

Materials and Methods: We investigated the genome-wide deoxyribonucleic acid methylation profiles in spermatozoa by comparing eight individuals with type 2 diabetes mellitus and nine non-diabetic controls using whole-genome bisulfite sequencing method.

Results: First, we found that the proportion of methylated cytosine in the whole genome of the type 2 diabetes mellitus group was slightly lower than that of the control group. Interestingly, the proportion of methylated cytosines in the CG context decreased, and the proportion of methylated cytosines in the CHG context (H = A, T or C) increased in the type 2 diabetes mellitus group, but the proportion of methylated cytosines in the CHH context (H = A, T or C) barely changed. The methylated cytosines in the CG context were mainly distributed at the high methylated level, whereas methylated cytosines in the CHG context and methylated cytosines in the CHH context were mainly distributed at the low and middle methylated level in both groups. Second, functional enrichment analysis showed that differentially methylated genes played a significant role in nervous system development and cell metabolism. Finally, we identified 10 top type 2 diabetes mellitus-related differentially methylated genes, including *IRS1*, *PRKCE*, *FTO*, *PPARGC1A*, *KCNQ1*, *ATP10A*, *GHR*, *CREB1*, *PRKAR1A* and *HNF1B*.

Conclusions: Our study provides the first evidence for deoxyribonucleic acid methylation reprogramming in spermatozoa of type 2 diabetes mellitus patients, and provides a new basis for explaining the complex mechanism of type 2 diabetes mellitus susceptibility in offspring.

INTRODUCTION

The global incidence of type 2 diabetes mellitus is increasing in modern societies¹. According to several human epidemiological

studies, the susceptibility to diabetes and related chronic diseases is increasing in offspring among type 2 diabetes mellitus patients^{2,3}. However, the molecular mechanisms of paternal type 2 diabetes mellitus that mediate these impacts remain

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largely unclear, particularly the role of epigenetics factors, such as deoxyribonucleic acid (DNA) methylation.

DNA methylation is a common pattern of epigenetic modification that mainly occurs at the CpG sites, adding a methyl to the fifth position of cytosine. DNA methylation plays an important role in regulating gene activities through modulating DNA transcription^{4,5}, and protecting genome integrity by restricting transposal element activity in the germ line and perhaps in somatic cells^{6,7}.

To our knowledge, fathers contribute to the developing offspring mostly just by sperm, and studies have found that sperm DNA methylation is crucial in transmitting epigenetic information from father to offspring. Furthermore, although sperm DNA methylation is erased and established anew during germ cell and pre-implantation development, the DNA methyl group can be inherited in approximately 20% of each generation^{8–10}.

Several independent research groups have shown that abnormal sperm DNA methylation usually increases the offspring's susceptibility to disorders and affects their phenotypes^{11–16}. For example, a study of C57B/6J mice showed that offspring of fathers who received 12 weeks of long exercise training were more likely to be adversely affected by a high-fat diet, characterized by weight and fat gain, impaired glucose tolerance, and elevated insulin levels. Interestingly, several differentially methylated metabolic genes are also expressed in the skeletal muscle of offspring, including *Oga*, *Ogt*, *H19*, *Pdk4*, *Glut4* and *Ptpn1*¹³. In another study, the connection of abnormal DNA methylation in fathers' sperm with the risk of autism in offspring was tested, and suggested that several differentially methylated regions (DMRs) in fathers' sperm were associated with autism risk in offspring¹⁴. In addition, sperm DNA methylation was altered with age, and possibly increased susceptibility to disease in the next generation¹⁵.

Although accumulating evidence shows that many paternal factors might contribute to differential DNA methylation in sperm and influence the health of the next generation, there are very limited studies focused on the relationship between sperm DNA methylation in type 2 diabetes mellitus fathers and the susceptibility to type 2 diabetes mellitus in their offspring. Wei *et al.* found that male mice with prediabetes altered overall DNA methylation patterns in sperm that matched changed methylation in their pancreatic islets in offspring, and increased their susceptibility to diabetes¹⁷. However, whether there are observed differences in sperm DNA methylation in humans is still unknown.

In the present study, our aim was to assess the distribution of genome-wide DNA methylation in sperm isolated from type 2 diabetes mellitus patients and non-diabetic individuals through whole-genome bisulfite sequencing (WGBS) technology. Here, we found that sperm DNA methylation profiles are altered in type 2 diabetes mellitus patients, which is of great significance to explain the susceptibility of type 2 diabetes mellitus and the overall health in offspring.

METHODS

Study cohorts

The study protocol was approved by the ethics committee of Fujian Province Hospital (K2016-09-014). Semen samples were collected from age-matched volunteers aged 20–45 years. There were eight type 2 diabetes mellitus patients (fasting plasma glucose >6.1 mmol/L), and nine control volunteers without diabetes (fasting plasma glucose 3.9–6.1 mmol/L). More detailed information was provided in Table S1.

Isolation of motile spermatozoa

The routine operation of sperm treatment was mainly referred to the 5th edition of the World Health Organization laboratory manual for human semen examination and processing¹⁸. Motile spermatozoa were isolated and purified by the pellet swim-up procedure¹⁹. The corresponding volume of preheated HTF solution was added to a round-bottom tube containing 1–5 mL of liquefied semen, then incubated at a 45° angle for 30 min in a 37°C incubator. The upper components were collected and placed in centrifuge tubes and centrifuged at 340 g for 15 min. The supernatant was discarded, and 1–2 mL of HTF solution was slowly added for suspension. The spermatozoa were then counted by microscopy and the presence of somatic cells was checked. The purified spermatozoa were frozen and stored in the refrigerator at –80°C for subsequent DNA methylation analysis.

Library construction and WGBS

DNA from semen samples was extracted by tissue/cell genome DNA isolation kit (Aidlab Biotechnologies, Beijing, China). The WGBS library was constructed according to the manufacturer's instructions. Genomic DNA was fragmented to an average size of approximately 250 bp by sonication using a bioruptor (Diagenode, Liege, Belgium). The fragments were then followed by end-repaired, dA addition to 3'-end and adaptor ligation. According to the manufacturer's instructions, ligated DNA was bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA). Then, 200–250 bp fragments were excised from the same lane of a 2% TAE agarose gel electrophoresis. The selected fragments were purified by using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and amplified by polymerase chain reaction. At last, the qualified WGBS library was sequenced using the Illumina HiSeq 2000 (BGI, Shenzhen, China).

WGBS data procession and analysis pipeline

The reads from WGBS were aligned to hg19 reference genome converted by bisulfite using Bismark v0.19.0 software²⁰. We used the Bismark methylation extractor script in Bismark to extract the methylated status. Only CpGs covered by at least three reads were used for downstream analysis. Differentially methylated CpGs (DMCs) were identified by methylKit v1.10.0²¹ and RRBSseq²². DMRs were selected as regions that

contain at least five DMCs in the 250 bp window. Promoters were defined as the regions containing 2 kb upstream and downstream of the transcription start site of the reference genome. Promoter methylation for each gene was calculated based on the mean of methylation levels from all CpGs covered in the promoter. We only selected DMRs with >10% methylation change and with a *Q*-value <0.01 , $pval_perm_1000 \leq 0.01$ were included for further analysis.

Functional enrichment analysis of differentially methylated genes (DMGs) was realized through the OmicsBean proteomics kit (www.omicsbean.com:88). Gene Ontology (GO) analysis was carried out by using Fisher's exact test and the GO database (<http://www.ebi.ac.uk/QuickGO/>)²³. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was carried out by KEGG Mapper v2.8 software (<http://www.kegg.jp/kegg/mapper.html>)²⁴. Protein–protein interaction analysis mainly used Cytoscape Web to generate a protein–protein interaction network model²⁵.

RESULTS

DNA methylation mapping

To identify the characteristics of the methylome in human spermatozoa, we collected WGBS data from nine controls and eight type 2 diabetes mellitus donors. After filtering low-quality reads, N reads and adapter sequences, each sample generated an average of 93.036 GB clean bases. Clean reads were then mapped to the human reference genome (hg19), with genome mapping rates ranging from 87.33% to 90.70% across all genomic CpG sites. Approximately 6.56% and 6.71% of all genomic C loci were methylated in the type 2

diabetes mellitus group and control group, respectively (Table 1).

General characteristics of methylated cytosines in the CG context, methylated cytosines in the CHG context and methylated cytosines in the CHH context

The methylated cytosine in DNA sequences can be divided into three types: CG, CHG and CHH (H = A, G or T), and these types showed a similar proportion in both groups. We also found that the whole-genome methylated cytosine levels in the type 2 diabetes mellitus group were 89.20% CGs, 2.35% CHGs and 8.45% CHHs, whereas those in the control group were 89.97% CGs, 2.13% CHGs and 8.45% CHHs (Figure 1a). Furthermore, compared with the control group, the proportion of methylated cytosines in the CG context (mCG) decreased slightly, whereas methylated cytosines in the CHG context (mCHG) showed an upward trend in the type 2 diabetes mellitus group. By comparing the methylation levels of different types of cytosine, we found that the methylated cytosines in the CPC context (mCpG) sites were highly methylated, whereas mCHG and mCHH sites were at low levels of methylation with wide sections (Figure 1b).

To show the relationship between sequence context and human methylation preference, we calculated the methylation percentage of all cytosine 9-mer sequences in which methylated cytosine located in the fourth position. We found that there was no significant difference in methylation preference in both groups. Furthermore, the sequence motifs of mCHG and mCHH have almost the same frequency in both groups (Figure 1c).

Table 1 | Whole-genome deoxyribonucleic acid bisulfite sequencing data

Groups	Sample ID	Clean reads	Mapping rate (%)	Bisulfite conversion rate (%)	Total mC (%)
T2DM	T2DM 1	633333338	88.62	99.53	7.04
	T2DM 2	609888844	88.61	99.53	6.83
	T2DM 3	633333336	90.60	99.55	6.77
	T2DM 4	564932690	89.18	99.58	7.12
	T2DM 5	633333336	90.66	99.47	6.27
	T2DM 6	633333342	90.70	99.53	5.98
	T2DM 7	633333342	89.84	99.49	6.17
	T2DM 8	608786878	90.12	99.56	6.30
Control	Control 1	633333342	90.16	99.53	6.86
	Control 2	633333338	89.37	99.56	6.96
	Control 3	633333346	90.49	99.52	6.76
	Control 4	633333338	90.18	99.53	6.70
	Control 5	587013322	89.35	99.55	6.86
	Control 6	633333336	88.00	99.54	7.04
	Control 7	627205050	89.31	99.51	6.02
	Control 8	633333338	87.33	99.47	6.92
	Control 9	579568080	88.04	99.52	6.26

We randomly selected 633333336–46 reads when the sequenced data were more than 95G. mC, methylated cytosines; T2DM, type 2 diabetes mellitus.

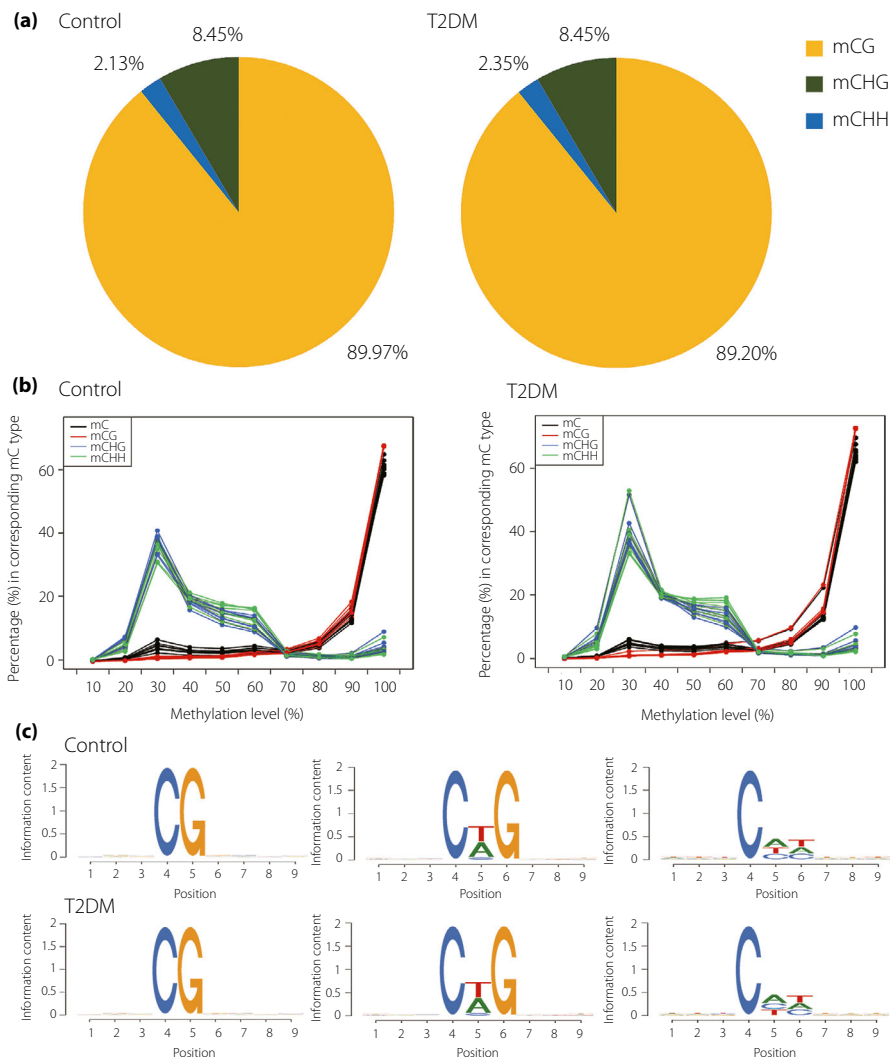


Figure 1 | (a) The average ratio of deoxyribonucleic acid methylation types in the whole genome in type 2 diabetes mellitus (T2DM) patients and control groups (H = A, C or T), the methylated cytosines in the CG context (mCG), methylated cytosines in the CHG context (mCHG) and methylated cytosines in the CHH context (mCHH) are denoted by yellow, green and blue colors respectively. (b) Distribution of methylation level of methylated cytosines (mC) in different sequence contexts. The x-axis is defined as the percentage of mC readings shown at the reference cytosine site, and the y-axis represents the score of the total mC calculated in 10% of the bins. (c) Sequence preferences for methylation in CG, CHG and CHH contexts. The horizontal axis represents the base position and methylated cytosine is in the fourth position, whereas the vertical axis showed the entropy of the base.

DMRs analysis

DMRs were defined as regions containing at least five DMCs (CG, CHH or CHG) within a 250bp window. A total of 9,025 DMRs were significantly differentially methylated between diabetes patients and controls (methylation difference $\geq 10\%$, $Q < 0.01$, $p_{\text{permutation}_1000} \leq 0.01$). Of these, 5,756 DMRs were hypomethylated and 3269 DMRs were hypermethylated (Table S2).

Functional annotation of DMGs

DMGs were defined as a class of genes whose promoter, intron or exon regions overlap with DMRs. Apart from

the overlap, we found approximately 2,019 DMGs in all (Table S3–S5). To probe gene functions of DMGs in human sperm exposed to type 2 diabetes mellitus, GO and KEGG pathway analyses were carried out to characterize the DMGs (Table S6–S7). The GO analysis showed that DMGs were significantly enriched in multiple biological processes, such as multicellular organism development, system development, cell differentiation, neuron system development, neurogenesis, regulation of cell communication, signal transduction and so on (Figure 2a). The KEGG analysis showed that DMGs were significantly enriched in the phosphoinositide-3-kinase–protein kinase B signaling pathway, cyclic

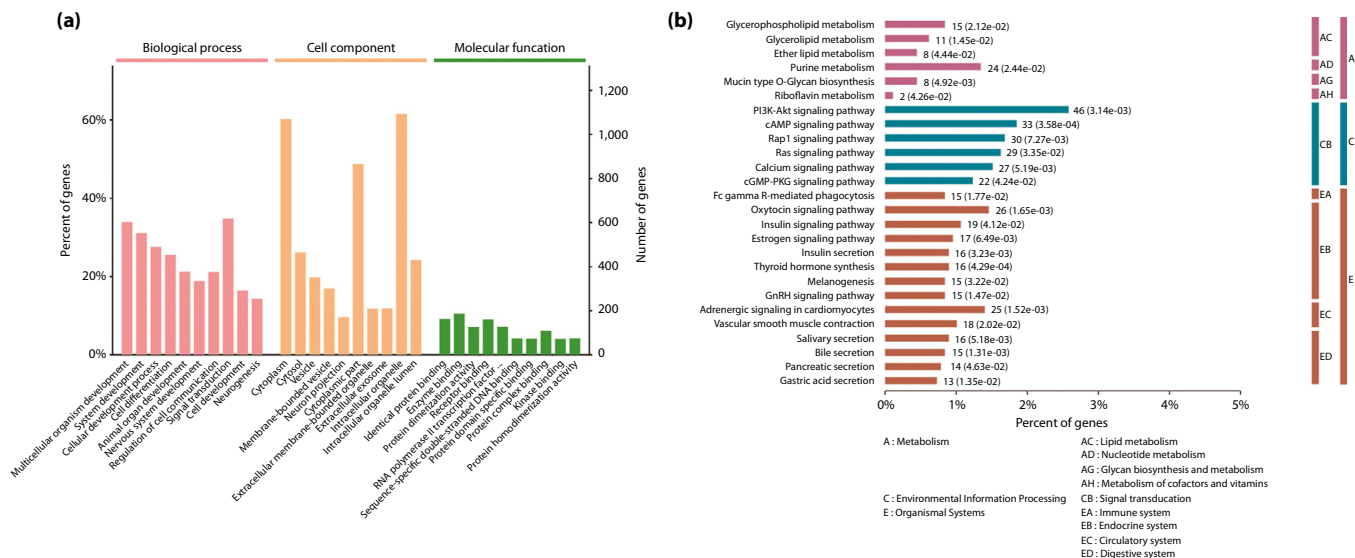


Figure 2 | (a) Gene Ontology analysis of differentially methylated genes. A total of 10 significantly enriched terms of biological processes, cell components and molecular functions are shown, respectively. The *P*-value was set to 0.05, and terms of the same category were sorted by *P*-value. (b) Kyoto Encyclopedia of Genes and Genomes pathway analysis of differentially methylated genes. Kyoto Encyclopedia of Genes and Genomes pathways were divided into the following subcategories, containing metabolism, environmental information processing and organismal systems, and the *P*-value was set to 0.05.

adenosine monophosphate signaling pathway, calcium signaling pathway, insulin signaling pathway and so on (Figure 2b). More importantly, we found that some DMGs were involved in the endocrine and digestive system, including insulin secretion, thyroid hormone synthesis and pancreatic secretion. These results suggested that these DMGs could affect insulin secretion and energy metabolism, subsequently contributing to metabolism dysregulation and the pathogenesis of type 2 diabetes mellitus in offspring.

Identification of the type 2 diabetes mellitus-related DMGs

To further explore the relationship between aberrant sperm DNA methylation and type 2 diabetes mellitus, we searched for type 2 diabetes mellitus-related differential DNA methylation genes. First, a protein–protein interaction network model of DMGs was built by choosing type 2 diabetes mellitus-related biological processes and pathways, such as insulin receptor signaling pathway (*P*-value = 2.17E-07), insulin signaling pathway (*P*-value = 0.0412), insulin secretion (*P*-value = 2.63E-10), calcium signaling pathway (*P*-value = 0.00519) and so on (Figure 3a). The genes-matched confidence score (>400) was shown in the network. Second, we compared 2,019 DMGs and 832 top relevant score genes (>8) searched by the term “diabetes” in the GeneCards website, and found that 77 DMGs overlap with type 2 diabetes mellitus candidate genes identified by the GeneCards (Figure 3b). Finally, we identified 10 top type 2 diabetes mellitus-related DMGs, including *IRS1*, *PRKCE*, *FTO*, *PPARGCIA*, *KCNQ1*, *ATP10A*, *GHR*, *CREB1*, *PRKARIA* and *HNF1B* (Figure 3c).

DISCUSSION

Does type 2 diabetes mellitus change DNA methylation profiles in human spermatozoa? To answer this question, we carried out the present study and uniquely showed DNA methylome alterations in the spermatozoa of type 2 diabetes mellitus patients. As the main type of epigenetic regulation mechanism, DNA methylation plays a key role in regulating gene expression and genomic imprinting. Previous studies have shown that DNA methylation was closely related to the pathogenesis of type 2 diabetes mellitus^{26–28}, and an abnormal DNA methylation spectrum from type 2 diabetes mellitus patients was found in different somatic cells, such as pancreatic islets^{29–32}, liver^{33,34}, skeletal muscle^{35,36} and adipose tissue^{37–39}. However, there is no report about the DNA methylation pattern in sperm from type 2 diabetes mellitus patients at the genome-wide level. In the mouse model, Wei *et al.* first found that overall DNA methylation profiles of spermatozoa in male mice with prediabetes were changed, with most of DMGs overlapping with that of pancreatic islets in offspring, and increasing the susceptibility to diabetes in offspring¹⁷. To extend this previous study, we explored the epigenetic changes of DNA methylation in type 2 diabetes mellitus spermatozoa by WGBS, which allowed us to comprehensively study the DNA methylation of the whole genome at single-nucleotide resolution⁴⁰.

In the present study, we found that the proportion of methylated cytosine in the whole genome in the type 2 diabetes mellitus group was slightly lower than that in the control group, similar to the previous study⁴¹. Interestingly, the proportion of mCG decreased, whereas mCHG showed an upward trend in the type 2 diabetes mellitus group. Unlike mCHH, there is a certain

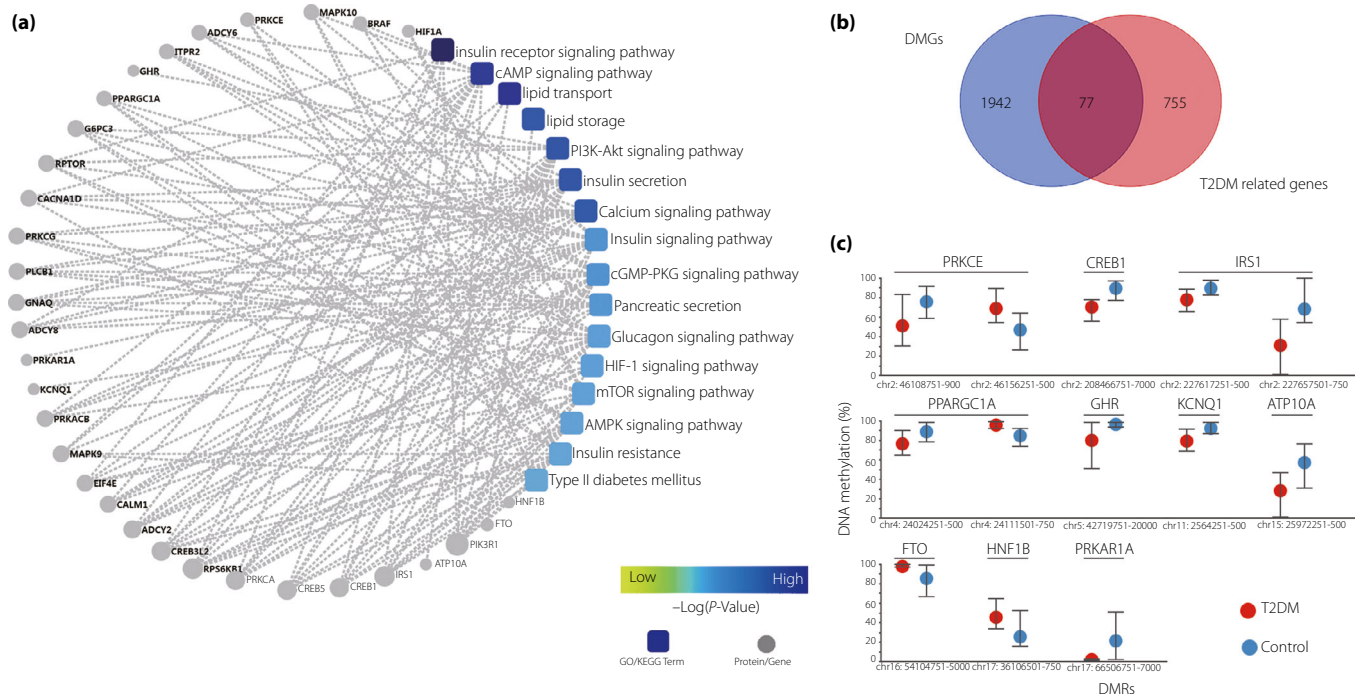


Figure 3 | (a) A protein-protein interaction network model was generated using the Cytoscape Web application. The circle nodes represent genes/proteins, and the rectangle represents the Kyoto Encyclopedia of Genes and Genomes pathway or biological process. The pathway color changes from yellow to blue, yellow represents a smaller P -value, and blue represents a larger P -value. (b) Venn diagram comparing the 2019 differentially methylated genes (DMGs) and 832 top relevance score ($RS > 8$) genes that were searched by the term “diabetes”. (c) Differentially methylated regions (DMRs) of the 10 top type 2 diabetes mellitus (T2DM)-related DMGs are shown. The circles represent the median values of methylation levels, and the whiskers represent the minimum and maximum levels of methylation.

symmetry in mCG and mCHG, especially as mCG methylation occurs predominantly at the symmetrical dinucleotide CpG, and as a result of that, DNA methyltransferase 1 prefers a hemimethylated substrate to maintain specific patterns of methylation in the genome⁴². This provided a means to explain how differentially methylated patterns could be maintained by populations of cells. Furthermore, mCGs were mainly distributed at the high methylated level, whereas the mCHGs and mCHHs were at the low and middle methylated level in both groups. In addition, sequence preferences in mCGs, mCHGs and mCHHs were obtained in the present study, and CTG and CAG were the most common sequence motifs in mCHG sites, and the sequence motif between the two groups had no obvious difference.

We also compared the whole-genome methylation sites between type 2 diabetes mellitus and control groups. Overall, we identified 9,025 DMRs. Recent studies have shown that both promoter and gene body regions could regulate gene expression, and DNA methylation in the gene body regions was related to the regulation of alternative splicing^{4,43}.

The GO analysis showed that DMGs were significantly enriched in terms related to nervous system development and cell metabolism. Previous epigenetic studies have shown that genes associated with energy metabolism and neurological disorders escaped demethylation in human primordial germ cells,

suggesting that certain genomic regions, especially those located between the central nervous system and metabolic regulation, are hot spots of epigenetic variation of gametes^{44,45}. Interestingly, DMGs were significantly enriched in the KEGG signaling pathway “insulin secretion” ($P = 0.00323$), which was closely associated with the occurrence and development of type 2 diabetes mellitus. Furthermore, many pathways involved in energy metabolism were also enriched, including calcium signaling pathway, adenosine monophosphate protein kinase signaling pathway, cyclic guanosine monophosphate-dependent protein kinase signaling pathway, cyclic adenosine monophosphate signaling pathway, glycerophospholipid metabolism, lipid metabolism and glycerolipid metabolism. These results suggested that aberrant DNA methylation in spermatozoa might affect cell metabolic function and increase the risk of diabetes in offspring.

Abnormal insulin secretion and insulin resistance are the main characteristics of type 2 diabetes mellitus. In the DMGs list, we found 16 genes associated with insulin secretion and 14 genes associated with insulin resistance. To find the key genes associated with type 2 diabetes mellitus, the 10 top DMGs were identified by the Genecards database and protein-protein interaction analysis, including *IRS1*, *PRKCE*, *FTO*, *PPARGC1A*, *KCNQ1*, *ATP10A*, *GHR*, *CREB1*, *PRKAR1A* and *HNF1B*, and some of which have been validated in previous studies. The

IRS1 and *FTO* genes were reported to have significant differences in CpG sites in human pancreatic islets from individuals with type 2 diabetes versus non-diabetic donors^{46,47}. Another genome-wide methylome study of the liver found that the activating transcription factor-motif of the *PRKCE* gene showed decreased DNA methylation from obese men with type 2 diabetes mellitus compared with non-obese controls³⁴. Type 2 diabetes mellitus is associated with differential DNA methylation in human tissues. A total of 23 DMGs have been reported in the liver, adipose tissue and islets²⁶. Importantly, nine DMGs were also found in the present study, including *IRS1*, *ATP10A*, *PPARGCIA*, *KCNQ1*, *FTO*, *PRKCE*, *PDE7B*, *GRB10* and *MOGAT1*. Interestingly, we also found 10 paternal methylation imprinting sites, including *KCNQ1*, *CALCR*, *RNU5D-1*, *INPP5F*, *PHACTR2*, *RB1*, *GRB10*, *DSCAM*, *DLGAP2* and *SNHG14*. The *KCNQ1* and *CALCR* genes are closely related to the occurrence and development of type 2 diabetes mellitus. These results suggested that sperm DNA methylation profiles associated with type 2 diabetes mellitus might be established and passed on to offspring at the early stage of development, and participate in insulin secretion or insulin resistance in offspring.

In conclusion, the present study uniquely showed that global cytosine methylation profiles were altered in spermatozoa from men with type 2 diabetes mellitus. Although we identified several candidate DMGs in type 2 diabetes mellitus men that might affect islet β -cell function and insulin resistance, the extent to which these DMGs influence energy metabolism of their offspring is not clear. Thus, we will validate those DMGs in different tissues of the offspring from type 2 diabetes mellitus patients in further studies by flow sorting technology or single-cell sequencing technology. We believe this work not only provides some new clues for deciphering the epigenetic mechanisms of the susceptibility to diabetes in offspring, but also inspires new ideas for the diagnosis and treatment of type 2 diabetes mellitus susceptibility.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 | Clinical information on sperm donors.

Table S2 | Differentially methylated regions were significantly differentially methylated between type 2 diabetes mellitus patients and controls (methylation difference $\geq 10\%$, $Q < 0.01$, $pval_perm_1000 \leq 0.01$).

Table S3 | Differentially methylated genes associated with promoters were significantly differentially methylated between type 2 diabetes mellitus patients and controls (methylation difference $\geq 10\%$, $Q < 0.01$, $pval_perm_1000 \leq 0.01$).

Table S4 | Differentially methylated genes associated with exons were significantly differentially methylated between type 2 diabetes mellitus patients and controls (methylation difference $\geq 10\%$, $Q < 0.01$, $pval_perm_1000 \leq 0.01$).

Table S5 | Differentially methylated genes associated with introns were significantly differentially methylated between type 2 diabetes mellitus patients and controls (methylation difference $\geq 10\%$, $Q < 0.001$, $pval_perm_1000 \leq 0.01$).

Table S6 | Gene Ontology analysis of differentially methylated genes.

Table S7 | Kyoto Encyclopedia of Genes and Genomes pathway analysis of differentially methylated genes.