Original Article
Genome-wide DNA methylation analysis of oligospermia and asthenozoospermia in a Chinese population

Li Qin[1,2], Xiao-Qiong Luo[2], Jing-Xi Wei[2], Yu-Xia Wei[2], Jun-Li Wang[2], Lian-Ling Zhang[2], Hong-Bao Huang[2], Xiang-Cai Wei[1,3]

[1]Jinan University, Guangzhou, Guangdong, China; [2]Center for Reproductive Medicine, The Affiliated Hospital of Youjiang Medical University for Nationalities, Baise, Guangxi, China; [3]Guangdong Women and Children Hospital, Guangzhou, Guangdong, China

Abstract: Aberrant DNA methylation in sperm has been associated with infertility in male patients demonstrating low semen quality or defective spermatogenesis. The aim of this study was to analyze methylation levels of DNA in different types of disorders related to spermatogenesis, including oligospermia and asthenozoospermia. Illumina Infinium HD Human Methylation 450K arrays were used to identify genomic regions demonstrating variations in sperm DNA methylation patterns between 36 fertile and 36 infertile individuals. Based on genome-wide methylation chips, this study screened out a batch of differentially methylated genes related to the process of spermatogenesis. There were 776 genes showing significant methylation differences between the oligospermia group and fertile control group (Diff Score > 50, P < 0.001), while 2,142 genes with aberrant methylation were found in the asthenozoospermia group (Diff Score > 50, P < 0.001). About 189 genes showed differential methylation patterns in fertile individuals with a history of smoking or alcohol use (Diff Score > 30, P < 0.05). This study identified nine genes, predominantly expressed in the testis, and three genes that have not been commonly studied in relation to male infertility: PEG3, DYDC1, and MMEL1. Cluster analysis revealed that abnormally methylated genes in oligospermia were mainly associated with apoptosis and cell cycle proteins, while abnormally methylated genes in asthenozoospermia were mainly involved in ion channels, cytoskeleton, and flagellum motility. Thus, these genetic variations may lead to male infertility. Overall, present data suggests that aberrant sperm DNA methylation, along with certain risk factors, might contribute to different types of spermatogenic disorders. The current study provides a promising basis for future exploration of the molecular mechanisms underlying male infertility.

Keywords: DNA methylation, male infertility, sperm, genomic chips, epigenetic

Introduction

Human infertility is a disorder affecting 13-15% of couples, worldwide [1]. The proportion of infertility caused by male factors is about 26% to 30%. Other combined factors are nearly 40%, while 25% to 28% are unexplained. In the majority of cases, male factor infertility is closely related to infection, varicocele, testicular dysfunction, anatomic variances, injury, toxin exposures, chromosomal abnormalities, systemic diseases, and sperm antibodies [1]. Additional risk factors may include smoking, alcohol use, obesity, and older age [2]. Semen analysis is the cornerstone of male infertility evaluation. Based on information obtained by traditional semen analysis, patient symptoms can be classified as oligospermia, asthenozoospermia, teratozoospermia, and oligoasthenoteratozoo-

spermia (OTA). However, male infertility evaluation must go far beyond simple semen analysis. It has to be complemented with a comprehensive clinical history, proper physical examination, and relevant endocrine, genetic, and other investigations [3].

Recently, epigenetics has emerged as one of the promising research areas in the understanding of male infertility. Many studies have indicated that epigenetic modifications, especially DNA methylation in imprinted and developmental genes, may have a role in male infertility [4, 5]. Epigenetics has been recognized as a study of heritable changes affecting gene expression that are not caused by any change in DNA sequence. The best-known epigenetic marker is DNA methylation [6], a dynamic process that occurs throughout the development

of multicellular organisms and ensures the maintenance of normal expression patterns. Recent evidence has suggested that DNA methylation changes are associated with developmental processes [7], different human pathologies [8], and aging [9]. Changes at the hereditary level may occur well before the disease is diagnosed.

In addition, DNA methylation plays a critically important role during spermatogenesis in mammals [10, 11]. DNA methylation of germ cells is required for many processes, including genomic imprinting [12], sex chromosome inactivation [13], silence or activation of transposable elements [14], meiosis-related events [11], and DNA compaction [15, 16]. In males, widespread erasure of DNA methylation occurs in primordial germ cells and subsequent *de novo* DNA methylation takes place during the maturation of germ cells and spermatogenesis before meiosis, accounting for the unique pattern of sperm DNA methylation [17]. As many researchers have suggested, correct sperm DNA methylation is essential for both fertilization and early embryo viability [18-22]. Therefore, enhancing the knowledge of epigenetics in the sperm is not only necessary to understand these processes but may also provide some clues to the potential causes of male infertility.

Several exploratory studies have established that altered sperm DNA methylation in single or multiple genes is associated with male infertility [23, 24]. Early studies of sperm DNA methylation analysis were specifically performed in imprinted genes, suggesting that children conceived through assisted reproductive technologies (ART) might have an increased risk of congenital imprinting diseases. Some imprinted genes with aberrant methylation patterns may result in poor quality sperm, such as *IGF2/H19* [25], *H19,* and *MEST/PEG1* [26]. Likewise, altered sperm DNA methylation patterns have also been found in non-imprinted genes associated with spermatogenic disorders, such as *DNMT* [27], *MTHFR* [28], *CREM* [29], and *DAZL* [30].

Since evidence for defective sperm DNA methylation patterns being associated with alterations in semen quality has emerged, more and more studies have used genome-wide approaches for their investigations [31]. Analyzing DNA methylation alterations at the whole genome level has become a reality with the ad-

vent of new technologies. Using modern technologies, abnormal sperm DNA methylations in imprinted and non-imprinted genes have been identified in sterile individuals with poor semen quality. Pacheco's study [32] identified CpG methylation profiles and mRNA alterations associated with low sperm motility. Heyn's research [33] revealed that nearly 600 genes were differentially methylated in humans with spermatogenic disorders, of which two are noteworthy, Piwi-like RNA-mediated gene silencing 2 (*PIWIL2*) and Tudor domain containing 1 (*TDRD1*). However, most of these studies have evaluated just a few genes, focusing only on a single symptom or including very small cohorts.

The current project aimed to evaluate genome-wide sperm DNA methylation patterns in a large cohort of fertile and infertile men. For the first time, this study sought to investigate the influences of some risk factors (smoking or alcohol use) on sperm DNA methylation at the whole genome level. To address this issue, high-throughput 450K methylation arrays were used, covering the whole genome. The aim was to determine whether epigenetic changes in sperm could explain different types of spermatogenic disorders and whether male infertility could be dependent on certain risk factors.

Material and methods

Subjects

Clinical assessment of fertile and infertile individuals was conducted at the Center for Reproductive Medicine, a Clinical Department in the Affiliated Hospital of Youjiang Medical University for Nationalities. Assessment included full personal and family medical histories to rule out heritable conditions, physical examinations with special emphasis on sexual characters, gonads, and genitalia, and a minimum of two semen analyses performed in accordance with World Health Organization (WHO) guidelines [34]. Semen data included volume, pH, sperm concentration, motility assessment, vitality, and morphology. Motility and sperm counts were done in duplicate aliquots of ≥ 200 cells. Measures were adopted to adjust for acceptable differences between duplicates. Sperm concentrations were performed on diluted and immobilized samples using hemocytometer chambers. Computer-assisted sperm analysis (CASA) was performed on fresh ejaculates

to obtain objective measurements of sperm kinematics.

To analyze differential methylation patterns, this study divided the samples into different groups based on semen data. One group had 36 fertile individuals with normal seminal quality (sperm count ≥ 15 × 10⁶/mL, progressive motility ≥ 32%, total motility ≥ 40%), including 24 volunteers of proven fertility and 12 fertile people with risk factors (smoking or alcohol use). The other group contained 36 infertile patients consulting for couple infertility with defects of semen quality values, including 15 oligospermia patients (sperm count < 15 × 10⁶/mL, normal motility), 20 asthenozoospermia patients (progressive motility < 32%, normal sperm count), and 11 oligoasthenozoospermia patients (sperm count < 15 × 10⁶/ml and progressive motility < 32%). The oligospermia group was subdivided into three groups on the basis of the semen sperm concentration standards: severe oligospermia or azoospermia (n = 5, sperm count < 5 × 10⁶/mL), moderate oligospermia (n = 5, 5 × 10⁶/mL ≤ sperm count < 10 × 10⁶/ml), and mild oligospermia (n = 5, 10 × 10⁶/mL ≤ sperm count < 15 × 10⁶/mL). The asthenozoospermia group was also divided into three subgroups, according to semen sperm motility standards: severe asthenozoospermia or necrospermia (n = 6, progressive motility < 10%), moderate asthenozoospermia (n = 7, 10% ≤ progressive motility < 20%), and mild asthenozoospermia (n = 7, 20% ≤ progressive motility < 32%). Semen samples from 24 fertile volunteers with normal seminal quality were studied as methylation controls of fertile spermatozoa. This study ruled out teratozoospermia cases. The female partners presented no known risk factors. Reference values of fertility were those most recently defined by the WHO [34]. The average age of research subjects was 33.75 ± 7.16 years and there was no statistical difference between the groups. Consent was obtained from all participants of the study in accordance with the Ethics Committee.

Isolation of sperm from semen samples

Semen samples were liquefied and homogenized with a mechanical mixer at 37°C (30-60 min). They were subsequently processed using a differential centrifugation technique employing density gradients (65-90% Puresperm, Nidacon International AB, Molndal, Sweden) to remove somatic contaminants and to enrich samples with spermatozoa. Isolated germ cells were normalized to a concentration of 1 × 10⁶/mL.

Sperm DNA extraction

Isolated germ cells were processed to obtain sperm DNA [QIAampR DNA Micro Kit (50) 56304, QIAGEN, Germany], following manufacturer instructions for this specific cell type. DNA extraction included RNAse A treatment of DNA samples. Eluted DNA was subjected to DNA quantification, purity detection, integrity detection, and storage at -80°C before experimental use.

Genome-wide DNA methylation analysis with high-density arrays

Microarray-based DNA methylation profiling was performed with the Illumina Infinium Human Methylation 450 BeadChip (Illumina Inc., USA) [35]. Bisulfite conversion of DNA was carried out using the EZ DNA Methylation Kit (Zymo Research, Orange, CA), following manufacturer instructions, but with certain modifications described in the Infinium Assay Methylation Protocol Guide. Processed DNA samples were then hybridized to the BeadChip (Illumina) following the Illumina Infinium HD Methylation Protocol. The chip was transferred to the iScan Reader for scanning. Data from the microarrays were further processed using the Genome Studio (GS) software methylation module to eliminate the influence of system variation on gene expression levels, ensuring that normalized data truly reflected biological significance.

Detection of differentially methylated probes and statistical analyses

To adjust for different probe-design types present in the 450k architecture, red and green signals from the files were corrected. Probes with detection *P*-values over 0.01, in at least two samples, were filtered out. Cluster analysis and significance tests were used to analyze methylation differences of differentially expressed genes in different types of spermatogenic disorders. The dmCpGs were defined as hypermethylated or hypomethylated when methylation values were, respectively, higher or lower in infertile samples, compared with fertile controls. In accordance with GS software, both Delta Data values and Diff Score values were

computed and applied to the data analyzed. The following terms used for analysis are defined below:

Average Beta: The average of the CpG sites in which the representative gene is detected, indicating the average value of the methylation ratio in each group.

Delta Data: This indicates the change in the degree of methylation. The methylation level in the sample is determined by calculating the ratio of the methylated CpG sites density to the entire CpG sites density. The Beta value is 0-1. Compared with references, a positive number indicates an increase in methylation and a negative number indicates a decrease. Delta Data > 0.2 (more than 20%) is an increase in methylation and < -0.2 (less than 20%) is a decrease in methylation.

Diff Score Significant Difference Test: Compares the differential methylation of each gene in different semen groups. A positive value of Diff Score indicates that the experimental group had an increased degree of methylation relative to the control group and a negative value denotes the opposite. Absolute value of Diff Score greater than 30 means *P* value < 0.05. Diff Score greater than 50 or less than -50 means *P* value < E-5 or P < 0.00001, with differences considered significant.

Results

Quality control analysis of methylation chips

The hybridization process of the methylation chips was strictly analyzed in the test to ensure the reliability of results. Analysis indicators included: (1) Staining Control: Chip dye quality control, red, and green signals were subjected to DNP (deoxyribonucleoprotein) and biotin monitoring at all levels. Therefore, sensitivity and efficiency had to be high in both the green and red channels (values greater than 5000, as shown in **Figure 1A**); (2) Extension Controls: The single base extension step was controlled by a green signal representing the GC base and a red signal representing the AT base. Thus, both the green and red channel values had to be high (values greater than 5000, as shown in **Figure 1B**); (3) Hybridization Controls: Hybridization control was applied, using three concentrations of biotin-labeled probes (green signal) to detect hybridization effects of the chip. Three high-concentration signals could be seen in the green channel (values greater than

10,000, as shown in **Figure 1C**); (4) Target Removal Controls: Residual base impurity quality control indicated by low signals in both green and red channels (value less than 5000, as shown in **Figure 1D**).

Global DNA methylation patterns: alterations of sperm DNA methylation were found in infertile individuals

To identify genomic regions showing differences in sperm DNA methylation patterns between fertile and infertile individuals, methylation arrays of 72 sperm samples were performed, analyzing the site-specific methylation status of 485577 CpG sites across the human genome [35, 36]. Statistical analysis showed 2,918 dmCpGs between fertile and infertile men. Of these, 2,201 CpG sites were hypermethylated, while 717 were hypomethylated in infertile patients. Given that testis is a critical organ in spermatogenesis, this study compared these dmCpGs with the genes that showed restricted expression toward testis or predominantly expressed in the testis. Nine genes were found, of which four genes had restricted expression in the testis and showed differential methylation patterns in oligospermia, asthenozoospermia, and oligoasthenozoospermia groups. Some imprinted genes were also found to be aberrantly methylated in infertile patients. Differences in DNA methylation in semen samples are shown in the deviation plot (**Figure 2**). It can be observed from this plot that greater differences in color indicate more significant methylation differences. Hierarchical clustering of DNA methylation data for the most variable CpG sites highlighted differences between the fertile controls and infertile patients (**Figure 3**). A histogram of four genes with restricted expression in the testis shows methylation differences among the different groups (**Figure 4**). Cluster analysis of the candidate genes showed that abnormally methylated genes in the infertile group participated in cell adhesion, cell morphogenesis, differentiation, apoptosis, muscle contraction, protein signal transduction, regulation of transcription, fertilization, immune response, and so forth (**Table 1**). These genes have the potential to cause male infertility.

*Comparative methylation analysis between the oligospermia group and fertile control group*

There were 776 genes showing different methylation levels in the oligospermia group and fertile control group (Diff Score > 50, P < 0.001),
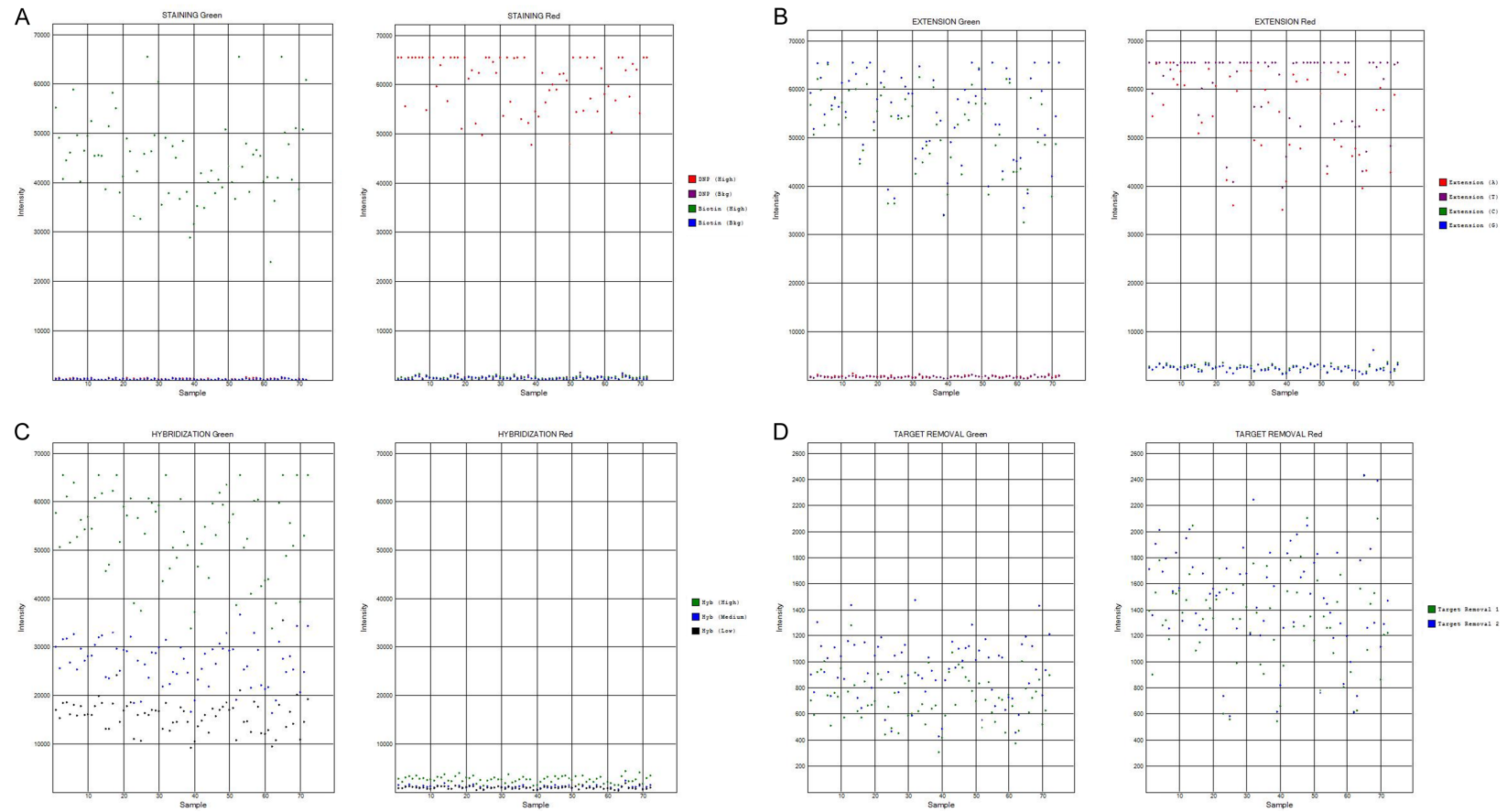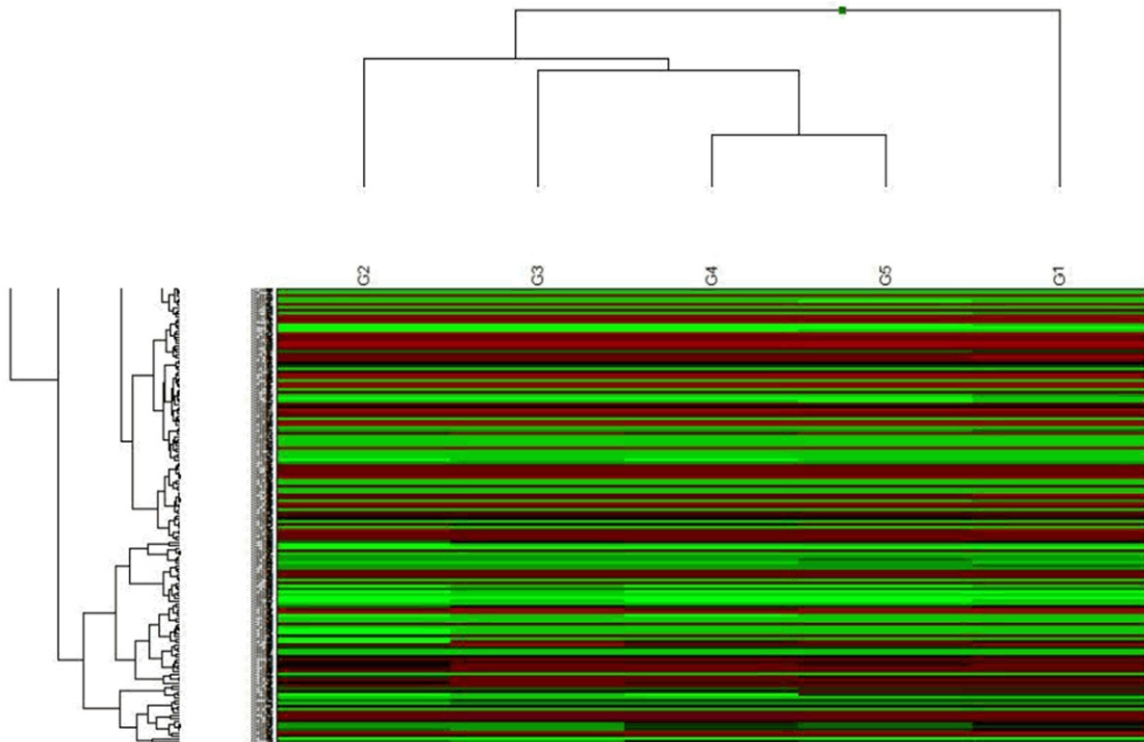
Figure 1. Quality Control of Methylation Chips: A. Staining Control; B. Extension Controls; C. Hybridization Controls; D. Target Removal Controls.
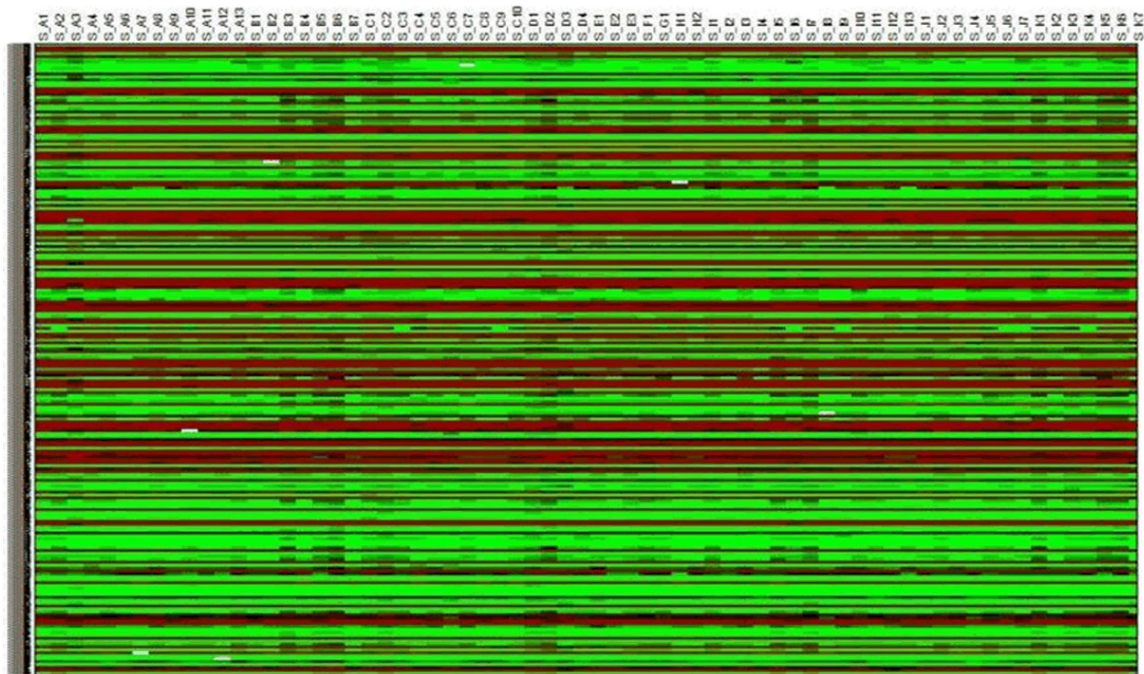
Figure 2. Deviation plot for all CpG sites studied in sperm samples showing the variability of methylation values.
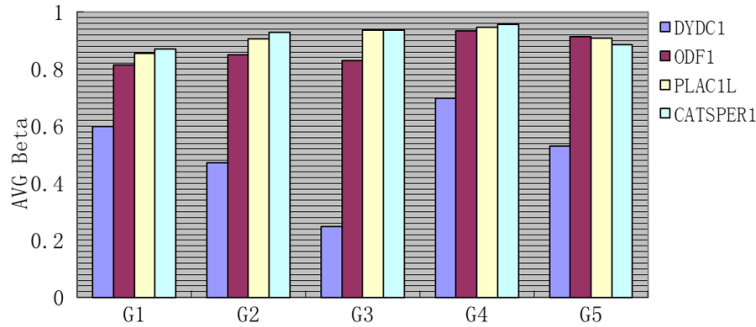

Figure 3. Cluster analysis of differential DNA methylation among five groups: G1 Control (n = 24 volunteers of proven fertility); G2 (n = 12 fertile people with smoking or alcohol use); G3 (n = 15 oligospermia patients); G4 (n = 20 asthenozoospermia patients); G5 (n = 11 oligoasthenozoospermia patients).

including 589 genes with increased methylation and 187 genes with reduced methylation. In comparison with the control group, respectively, a total of 1,427 differential genes were screened out. It was found that the severe oligospermia group included 958 abnormally

Figure 4. Histogram showing the differences in methylation of four genes (restricted expression toward testis) within the five groups: G1 Control (n = 24 volunteers of proven fertility); G2 (n = 12 fertile people with smoking or alcohol use); G3 (n = 15 oligospermia patients); G4 (n = 20 asthenozoospermia patients); G5 (n = 11 oligoasthenozoospermia patients).

mally hypermethylated genes and 607 abnormally hypomethylated genes. The moderate asthenozoospermia group included 1,991 abnormally hypermethylated genes and 648 abnormally hypomethylated genes, while 2,003 abnormally hypermethylated genes and 636 abnormally hypomethylated genes were found in the mild asthenozoospermia group. Cluster analysis of the groups showed that the differences in methylation in the G2 (severe) group were most significant when compared with the G1 (control) group (**Figure 6A**). The average level of methylation in the methylation ratio box plot was also the highest in the G2 (severe) group (**Figure 6B**). Cluster analysis of the candidate genes showed that abnormally methylated genes in men suffering from asthenozoospermia were mainly located on chromosomes 1, 6, 7, and 12 (**Figure 6C**). These genes were mainly linked to ion channels and transport proteins, cytoskeleton, flagellum motility, protein signal transduction, and regulation of transcription (**Table 3**).

Alterations of sperm DNA methylation found in fertile individuals with smoking or alcohol use

Results revealed 189 genes with differential methylation patterns in people that were accustomed to smoking or alcohol use (Diff Score > 30, P < 0.05). Of these, 113 genes were abnormally hypermethylated, while 76 genes were abnormally hypomethylated. Cluster analysis of the differentially methylated genes revealed that these genes were involved in transcriptional regulation, RNA/DNA polymerase activity, associated with molecular chaperones, cytoskeleton, or were apoptosis-related and cyclin-related. Of these, abnormally hypermethylated genes were mainly associated with molecular protein chaperones, including participation in the folding of primary proteins, secondary structure of proteins, and formation of tertiary spatial structures. Abnormally hypomethylated genes were mainly involved in transcriptional regulation, RNA polymerase activity, and synthesis of various RNAs, including messenger RNA, ribosomal RNA, and other small RNAs.

hypermethylated genes and 469 abnormally hypomethylated genes. The moderate oligospermia group included 968 abnormally hypermethylated genes and 459 abnormally hypomethylated genes, while 940 abnormally hypermethylated genes and 487 abnormally hypomethylated genes were observed in the mild oligospermia group. Cluster analysis of the groups showed the most significant differences in methylation in the G3 (moderate) group, compared with the G1 (control) group (**Figure 5A**). The average level of methylation in the methylation ratio box plot was also the highest in the G3 (moderate) group (**Figure 5B**). Cluster analysis of the candidate genes showed that abnormally methylated genes in men suffering from oligospermia were mainly located on chromosomes 1, 2, 6, 7, 10, and 17 (**Figure 5C**). These genes were mostly associated with apoptosis, cell cycle proteins, differentiation, protein signal transduction, and regulation of transcription (**Table 2**).

Comparative methylation analysis between the asthenozoospermia group and fertile control group

Compared with the fertile control group, there were 2,142 genes displaying different methylation levels in the asthenozoospermia group (Diff Score > 50, P < 0.001). A total of 1,612 genes demonstrated increased methylation, while 530 genes showed reduced methylation. Compared with the control group, respectively, a total of 2,639 differential genes were screened out. It was found that the severe asthenozoospermia group included 2,032 abnor-

Table 1. Main signal pathways associated with differentially methylated genes in the infertile group

Term: Pathway	Genes	Count	P Value
GO: 0019882~antigen processing and presentation	RAET1E, HLA-DRB1, TAP2, HLA-DRB5, HLA-C, HLA-DPB1, HLA-DMB, HLA-DOA, HLA-DQA1, LOC285830	10	< 0.0001
hsa04514: Cell adhesion molecules (CAMs)	CADM1, CLDN4, HLA-DRB1, NRXN3, HLA-DRB5, HLA-C, HLA-DPB1, HLA-DMB, HLA-DOA, CDH5, HLA-DQA1	11	0.0003
hsa05320: Autoimmune thyroid disease	HLA-DRB1, HLA-DRB5, HLA-C, HLA-DPB1, HLA-DMB, HLA-DOA, HLA-DQA1	7	0.0005
GO: 0000904~cell morphogenesis involved in differentiation	PRKCA, COL18A1, EFNB3, NRXN3, CTNNA2, SLIT3, SEMA5A, KAL1, TNR, NOTCH4, BAI1, TGFBR3, DST	13	0.0030
GO: 0043067~regulation of programmed cell death	CADM1, GPR109A, TCF7L2, MCF2L, TNFRSF18, DNAJC5, DLG5, ALX4, PCSK6, DNAJA3, NET1, COL18A1, PRKCA, KCNMA1, NGEF, ABR, MCF2, BIRC7, SKP2, SMAD3, ACTN3, VAV2, GAL, GIMAP1, SERPINB9, GSK3B, HIPK2, PLCG2	28	0.0047
GO: 0006936~muscle contraction	KCNMA1, SLC8A1, DYSF, MAP2K3, MYH11, GAA, GAL, CACNA1C, KCNIP2	9	0.0103
GO: 0051056~regulation of small GTPase mediated signal transduction	NGEF, DAB2IP, ABR, MCF2, PSD3, IQGAP2, AGAP1, VAV2, MCF2L, ARHGEF10, ADAP1, NET1	12	0.0107
GO: 0031175~neuron projection development	PRKCA, SEMA5A, EFNB3, NRXN3, MCF2, TNR, KAL1, BAI1, STRN, DST, SLIT3, CTNNA2	12	0.0119
GO: 0035023~regulation of Rho protein signal transduction	NGEF, ABR, MCF2, VAV2, MCF2L, ARHGEF10, NET1	7	0.0129
GO: 0032940~secretion by cell	CPLX2, SCFD2, NRXN3, RPH3AL, SYTL2, PCSK6, GAL, CACNA1C, PCSK5, LLGL2	10	0.0203
GO: 0005976~polysaccharide metabolic process	OVGP1, SPACA3, EGFLAM, XYLT1, GSK3B, GAA, CHI3L2	7	0.0216
GO: 0007338~single fertilization	PLCZ1, OVGP1, SERPINA5, ZAN, ATP8B3	5	0.0295
GO: 0045893~positive regulation of transcription, DNA-dependent	E2F3, ELF1, MAP2K3, GLIS1, RBM4, SMAD3, SIX4, PRDM16, TCF7L2, PRDM7, NCOA2, HIPK2, NOTCH4, YAP1, NR5A2, ALX4	16	0.0474
GO: 0006955~immune response	GALNT2, CPLX2, CADM1, HLA-DRB1, IL1F10, SMAD3, HLA-C, HLA-DMB, HLA-DQA1, RAET1E, DEFA1B, TAP2, LILRA5, PLCG2, HLA-DRB5, TGFBR3, DEFA1, HLA-DPB1, HLA-DOA, DNAJA3, DMBT1, LOC285830	21	0.0405

Figure 5. A. Cluster analysis of differential DNA methylation between varying degrees of oligospermia and the fertile control group; B. Box plot of average methylation percentage in the four groups: G1 Control (n = 24 volunteers of proven fertility), G2 (n = 5, severe oligospermia or azoospermia), G3 (n = 5, moderate oligospermia), G4 (n = 5, mild oligospermia); C. Chromosome distribution of aberrantly methylated genes in the oligospermia group.

imprinted regions, focusing only on a single symptom or disease. In contrast, the current study compared, in a large cohort and at a genome-wide level, DNA methylation patterns of sperm from fertile individuals with the sperm from people suffering from different types of spermatogenic disorders, including oligospermia, asthenozoospermia, or both. Subsequently, this study continued to subdivide and compare each subgroup with the control group, respectively. Differences were found between groups that had never been reported before. This study, also for the first time, evaluated the influence of certain risk factors (smoking or alcohol use) on sperm DNA methylation.

The 450K Infinium Methylation BeadChip (Illumina HD), used in this study, is designed to analyze more than 450,000 methylation sites, covering 96% of the CpG islands, based on methylated high-density chip technology for preliminary screening of aberrantly methylated genes. The chip does not rely on Me-DIP (methylated DNA immunoprecipitation) and covers all NCBI-annotated genes, including 3' and 5' promoter regions. Thus, it avoids defects in areas with high CpG content but an inability to cover the whole CpG islands [35, 36]. After the processed DNA is hybridized to the chip, the methylation status can be obtained by calculating the ratio of the scan fluorescence signal. In the course of this experiment, quality control results showed that no abnormalities in biotin sensitivity monitoring. Positive control signals, such as external standard and internal standard, were normal, while the negative control test was negative (**Figure 1**). This indicates that the data obtained from the chips was reliable, with no impurities or pollution affecting the read out. The detection rate was normal as well.

Many genes which displayed aberrant methylation in infertility patients and fertile individuals

Cluster analysis of the groups showed a difference in methylation between the two groups (**Figure 7A**). The average methylation level of all differentially methylated genes was higher in the smoking or alcohol use group (**Figure 7B**). Abnormally methylated genes were found to be evenly distributed on the chromosome (**Figure 7C**). When the two groups were compared, some genes were found to overlap with most differentially methylated CpGs (**Table 4**).

Discussion

Current research in spermatogenic disorders involves the comparison of sperm and testicular DNA methylation profiles of specific genes or genomic regions from fertile and infertile human males. Several studies [23, 24, 33] have been reported but these analyses were either restricted to a small number of genes or

Table 2. Genes with significant differences in methylation levels between the oligospermia group and control group

Indexes	Genes	Gene description	Delta Beta	Diff Score
High methylation	HLA-DRB5	Major histocompatibility complex, class II, DR beta 5	0.57	374.34
	AKAP13	A kinase (PRKA) anchor protein 13	0.48	374.34
	TMPRSS4	Transmembrane protease, serine 4	0.36	374.34
	NDRG1	N-myc downstream regulated 1	0.35	374.34
	STRN	Striatin, calmodulin binding protein	0.33	374.34
	KCNQ2	Potassium voltage-gated channel, KQT-like subfamily, member 2	0.32	374.34
	DYSF	Dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	0.32	374.34
	ZNF319	Zinc finger protein 319	0.31	374.34
Low methylation	ECE1	Endothelin converting enzyme 1	-0.80	-371.33
	HDAC4	Histone deacetylase 4	-0.68	-371.33
	MYO10	Myosin X	-0.62	-371.33
	CARD11	Caspase recruitment domain family, member 11	-0.62	-371.33
	DYDC1	DPY30 domain containing 1	-0.48	-371.33
	FSCN2	Fascin homolog 2, actin-bundling protein, retinal	-0.46	-371.33
	DMBT1	Deleted in malignant brain tumors 1	-0.45	-275.70
	GPR133	G protein-coupled receptor 133	-0.37	-371.33
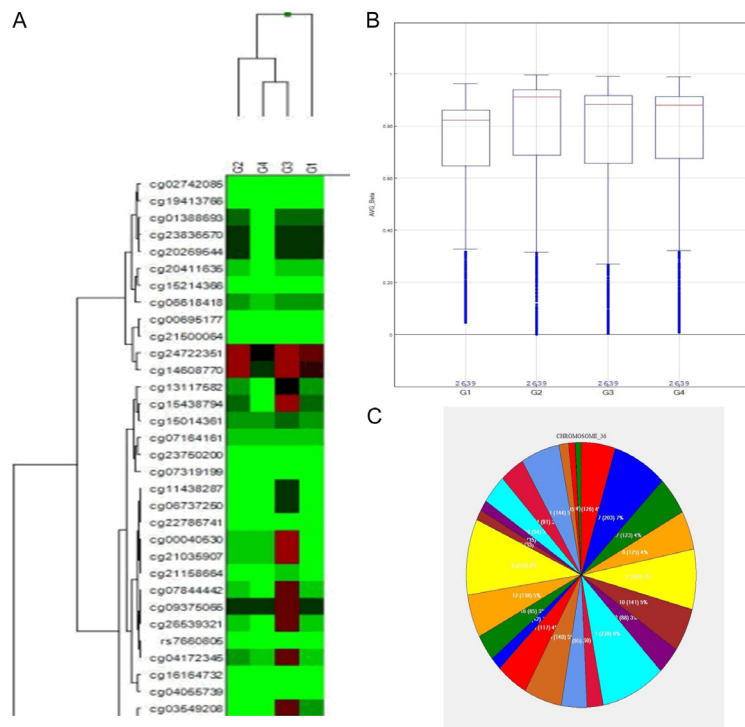
Figure 6. A. Cluster analysis of differential DNA methylation between varying degrees of asthenozoospermia and the fertile control group; B. Box plot of average methylation percentage in the four groups: G1 Control (n = 24 volunteers of proven fertility), G2 (n = 6, severe asthenozoospermia or necrospermia), G3 (n = 7, moderate asthenozoospermia), G4 (n = 7, mild asthenozoospermia); C. Chromosome distribution of aberrantly methylated genes in the asthenozoospermia group.

were screened out in this study. Some were imprinted genes, with significantly elevated me-

thylation in infertile patients, similar to findings of other scholars. The current study found a small number of CpGs showing aberrant DNA methylation associated with 26 imprinted genes in infertile patients. Specifically, present results were compared with data obtained in a previous study [32], where imprinted genes were analyzed in patients with low sperm motility. The current study only found three common CpG sites aberrantly methylated. Reports exist of the imprinted genes *IGF2/H19* [25], *H19,* and *MEST/PEG1* [26] to possess defective methylation and interfere with spermatogenesis. The currents tudy did not find alterations in DNA methylation patterns of these genes previously found to be associated. However, some genes which were rarely studied before were screened out. These genes require further research.

As an example, *PEG3*, which is a maternal imprinting gene, reached 0.21 Delta Beta, 275.70 Diff Score (P < 0.001) in the infertility group, compared

Table 3. Genes with significant differences in methylation levels between the asthenozoospermia group and control group

Indexes	Genes	Gene description	Delta Beta	Diff Score
High methylation	MRPL1	Mitochondrial ribosomal protein L1	0.47	374.34
	MMEL1	Membrane metallo-endopeptidase-like 1	0.44	374.34
	TEKT5	Tektin 5	0.44	374.34
	ATP4B	ATPase, H^+/K^+ exchanging, beta polypeptide	0.43	374.34
	CLDN4	Claudin 4	0.39	374.34
	OPCML	Opioid binding protein/cell adhesion molecule-like	0.37	374.34
	CAPZB	Capping protein (actin filament) muscle Z-line, beta	0.37	374.34
	HLA-DPB1	Major histocompatibility complex, class II, DP beta 1	0.36	374.34
Low methylation	CMPK2	Cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	-0.65	-371.33
	HDAC4	Histone deacetylase 4	-0.515	-371.33
	ADARB2	Adenosine deaminase, RNA-specific, B2	-0.41	-367.92
	TTC9	Tetratricopeptide repeat domain 9	-0.35	-207.04
	SLC1A7	Solute carrier family 1 (glutamate transporter), member 7	-0.35	-212.50
	GRAMD4	GRAM domain containing 4	-0.34	-211.72
	ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif, 2	-0.34	-198.51
	MGAT5	Mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase	-0.32	-371.33
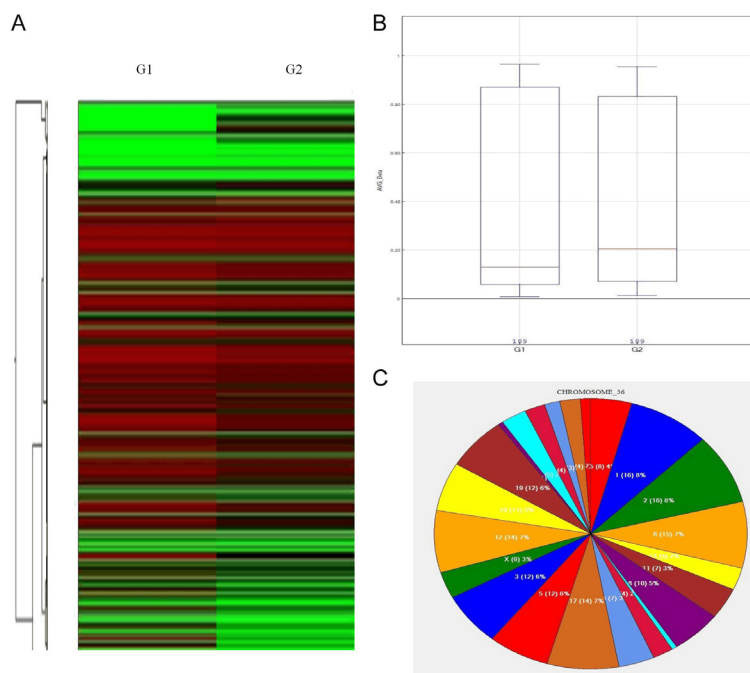
Figure 7. A. Cluster analysis of differential DNA methylation between smoking or alcohol use group and control group; B. Box plot of average methylation percentage in the two groups; C. Chromosome distribution diagram of aberrantly methylated genes in smoking or alcohol use group.

[37]. Ab-normal apoptosis in spermatogenesis might lead to oligospermia [38]. Hammoud SS [39] first reported alterations in sperm DNA methylation patterns at the imprinted loci of *PEG3*. He found that *PEG3* showed significant hypermethylation in oligospermic patients, compared with fertile controls. El Hajj N's study [40] revealed that aberrant methylation of *PEG3* was found in infertile males. However, no significant methylation differences were proven in the results (P > 0.05). Currently, there remains a lack of research on *PEG3* and male infertility. More experiments are needed to verify previous studies.

On the other hand, methylation abnormalities in non-imprinted genes need to be further verified. The current study found a lot of genes with aberrantly methylated CpGs in infertile patients, such as *GSTM1, DNMT3L*, and *CYP1A1*, which have been shown to be potentially associated with oligospermia. *CATSPER1, CRISP2, SEPT4, TCTE3, TEKT4,* and *DNAH1* have been associated with asthenozoo-

with controls. The establishment of sperm DNA methylation marks involved in genomic imprinting is critical during spermatogenesis. Thus, *PEG3* may become a key factor in understanding male infertility. *PEG3* may also play a role in cell proliferation and p53-mediated apoptosis

Table 4. Genes with significant differences in methylation levels between the smoking or alcohol use group and control group

Indexes	Genes	Gene description	Delta Beta	Diff Score
High methylation	ODF1	Outer dense fiber of sperm tails 1	0.21	43.25
	DCUN1D2	DCN1, defective in cullin neddylation 1, domain containing 2 (*S. cerevisiae*)	0.14	31.01
	CASP3	Caspase 3, apoptosis-related cysteine peptidase	0.14	36.52
	PLAC1L	Placenta-specific 1-like	0.12	33.12
	POF1B	Premature ovarian failure, 1B	0.11	30.10
	DUSP12	Dual specificity phosphatase 12	0.09	31.46
	CT45A6	Cancer/testis antigen family 45, member A6	0.08	38.08
	ZNF836	Zinc finger protein 836	0.07	33.69
Low methylation	F2RL3	Coagulation factor II (thrombin) receptor-like 3	-0.12	-40.12
	IRF7	Interferon regulatory factor 7	-0.06	-32.40
	GPC1	Glypican 1	-0.05	-33.01
	SCARF1	Scavenger receptor class F, member 1	-0.05	-31.09
	FGF17	Fibroblast growth factor 17	-0.04	-33.13
	SULT6B1	Sulfotransferase family, cytosolic, 6B, member 1	-0.03	-33.09
	SLC6A12	Solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12	-0.03	-35.70
	GLI2	GLI family zinc finger 2	-0.03	-34.69

spermia [41]. Barau J [42] showed that *DNMT3C* was the enzyme responsible for methylation of the promoters of evolutionarily young retrotransposons in the male germ line and that this specialized activity was required for mouse fertility. Inoue K [43] also suggested that hypomethylation caused by the *DNMT3L* mutation results in increased retrotransposon expression in meiotic spermatocytes. This was proven through comprehensive studies of DNA methylation and polyA (+) RNAs (transcriptome) in developing male germ cells from *Dnmt3l* knockout mice. Research showed that expression of *TEKT4* was significantly decreased in the ejaculated sperm of idiopathic asthenozoospermia patients. This might be one of the causes of idiopathic asthenozoospermia, though further examination is necessary to elucidate the molecular mechanisms relating to the origin of these alterations [44]. These studies are consistent with the results of the current study, which verified the reliability and significance of chip screening technology, to some extent. Of course, there are also some genetic studies with results inconsistent with present experiments. Zhou JH [45] carried out a study focusing on *CRISP2* reduction and its roles in asthenozoospermia, finding no methylation in the *CRISP2* promoter, suggesting *CRISP2* expression may be regulated in the sperm at the post-transcriptional level. However, methylation in the *CRISP2* was observed in present experiments, which reached 104.24 Diff Score (P < 0.001) in the infertile group. Thus, additional

studies are needed to replicate findings presented here and to evaluate the generalizability of present findings in a larger cohort of fertile and infertile population.

In addition, the current study discovered some meaningful new genes in the aberrantly methylated gene group. Given that testis is critical in spermatogenesis, this study compared these dmCpGs with those genes that had restricted expression toward testis or were predominantly expressed in the testis. Nine genes were found, of which four genes had restricted expression toward the testis. They showed differential methylation patterns in patients suffering from oligospermia, asthenozoospermia, and oligoasthenozoospermia. *PLAC1L, DYDC1, CATSPER1,* and *ODF1* had restricted expression toward the testis, while *MMEL1, SDK1, MCF2, CRISP2,* and *GPC1* were predominantly expressed in the testis. Some genes have been reported, while others have not yet been studied. *CATSPER1, ODF1,* and *CRISP2* have been considered to be associated with male infertility [46-48]. *PLAC1L,* also named as *OOSP2* (oocyte secreted protein 2), whose function is still unknown, was only reported in one study [49]. It may be a novel gene for male infertility research. *DYDC1,* which encodes a member of a family of proteins that contains a *DPY30* domain, is involved in acrosome formation during spermatid development. Li S [50] first verified that *DYDC1* plays a crucial role during acrosome biogenesis using a novel approach that involved germ cell trans-

plantation and RNA interference. He found that knockdown of endogenous *Dydc1* interfered with the formation of acrosomes, promoting spermatid differentiation during mouse spermiogenesis. There have been a few such studies about *DYDC1* and spermiogenesis since. *MMEL1* is a member of the neutral endopeptidase (*NEP*) or membrane metallo-endopeptidase (*MME*) family. This protein family plays important roles in pain perception, arterial pressure regulation, phosphate metabolism, and homeostasis. Studies have reported that it may be associated with certain diseases, such as rheumatoid arthritis [51, 52], primary biliary cholangitis [53], and celiac disease [54]. However, its association with male infertility has not been commonly reported. Since *MMEL1* is mainly expressed in the testis, it may be involved in sperm function, possibly by modulating the processes of fertilization. Pinto [55] first reported that *MMEL1* was present in human spermatozoa and might participate in the regulation of sperm motility by regulating tachykinin activity. The mechanisms remain unclear. Other genes, like *SDK1, are* involved in prostate cancer [56], glomerulosclerosis [57], and hypertension [58]. *MCF2* is related to borderline personality disorder [59]. *GPC1* has been associated with biliary atresia [60]. However, each of these lack studies concerning their effects on male infertility. Present findings provide an exciting and potentially clinically useful metric for assessment of male infertility.

Analyzing the outcomes of subgroups in oligospermia and asthenozoospermia, the current study found a high degree of methylation in the sperm genome of the infertile group, compared with the normal group, related to semen parameters. In the oligospermia subgroup, this study found the most significant methylation differences in the moderate oligospermia group, which had the highest average level of methylation in the methylation ratio box plot. Similar observations were made in the asthenozoospermia subgroup where the severe group had the most significant methylation differences, as well as the highest level of methylation. There was a negative correlation between semen sperm motility and methylation levels between groups. Prompt sperm genome methylation levels may be a predictor of semen parameters. In a recent study, Montjean [61]

found a significant positive association between sperm global DNA methylation levels and conventional sperm parameters (sperm concentration and motility). They also identified significant inverse relationships between sperm global DNA methylation, and both, DNA fragmentation index (DFI) and sperm denaturation index (SDI). Abnormal methylation levels of the gene promoter region, which affect the quality of semen, may be one of the underlying mechanisms giving rise to male infertility.

Since certain risk factors like smoking and/or alcohol use [2] may cause male infertility, the current study compared methylation patterns in people accustomed to smoking or alcohol use. There were 189 genes which showed differential methylation patterns in these subjects (Diff Score > 30, P < 0.05). These genes were associated with transcriptional regulation, RNA/DNA polymerase activity, molecular chaperones, cytoskeleton, apoptosis-related, and cyclin-related. The most meaningful gene *ODF1*, which has restricted expression in the testis, has been found to be related to asthenozoospermia [47]. Unfortunately, though, most of the genes got a low Delta Data, indicating that there was not much difference between the experimental and control groups. This may be due to the subjects chosen in the fertile group. Since smoking or alcohol use was seen to have caused abnormal methylation in fertile people, more investigations are necessary to prove the association between smoking or alcohol use and male infertility, especially asthenozoospermia.

Cluster analysis of the candidate genes showed that differentially methylated genes were distributed on each chromosome, mainly located on chromosome 1, 6, and 7. These genes mainly participated in cell adhesion, cell morphogenesis, differentiation, apoptosis, muscle contraction, protein signal transduction, regulation of transcription, fertilization, immune response, and so forth (**Table 1**). Abnormally methylated genes in oligospermic men were mainly associated with apoptosis and cell cycle proteins, while abnormally methylated genes in asthenozoospermic men were mainly involved in ion channels and transport protein, cytoskeleton, and flagellum motility. Thus, methylation of these genes might affect one or more of the above functions giving rise to male infertility.

Genes studied in this article are associated with numerous signaling pathways. One such pathway is involved in antigen processing and presentation during immune response, involving genes including *HLA-DRB1, HLA-DRB5, HLA-C, HLA-DPB1, HLA-DMB, HLA-DOA, HLA-DQA1, TGFBR3, DEFA1, DNAJA3,* and *DMBT1*. The HLA (human leukocyte antigen) system is the most complex polymorphic system known in the human body [62]. It is conservatively estimated that there are at least 1,300 different haplotypes in HLA, corresponding to about 17×10^7 genotypes. This means that two people cannot have the same HLA status, except for identical twins. Thus, HLA can be regarded as an individual's "identity card". It is also considered to be a key molecule in allogeneic transplantation [62]. The current study found abnormally methylated genes involved in this signaling pathway, providing a new idea for examination of the causes of male infertility, especially unexplained infertility. Whether aberrant methylation of *HLA* genes can affect embryo implantation, as an allogeneic transplantation process or involve some other mechanisms, leading to some kinds of male infertility, is yet unknown. This might also provide a clue to the mechanisms underlying male infertility.

Spermatogenesis is a continuous process of cell division and differentiation. Its goal is to maintain the stable transmission of paternal genetic information. This study systematically analyzed DNA methylation profiles of different types of spermatogenic disorders at a genome-wide resolution. Furthermore, this study identified several genes which display abnormally methylated CpGs in infertile and fertile individuals. These important functional genes, which are related to spermatogenesis, suggest that DNA methylation is involved in the control of the functional capacity of germ cells. Further studies are necessary to elucidate the molecular mechanisms related to the origin of these alterations, determining their significance and functional consequences in male infertility.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xiang-Cai Wei, Jinan University, Guangzhou 510632, Guangdong, China. Tel: 020-39151663; Fax: 020-86505085; E-mail: dxcwei@126.com

References

[1] Lindsay TJ and Vitrikas KR. Evaluation and treatment of infertility. Am Fam Physician 2015; 91: 308-314.

[2] Povey AC, Clyma JA, McNamee R, Moore HD, Baillie H, Pacey AA and Cherry NM. Modifiable and non-modifiable risk factors for poor semen quality: a case-referent study. Hum Reprod 2012; 27: 2799-2806.

[3] Esteves SC. Clinical relevance of routine semen analysis and controversies surrounding the 2010 World Health Organization criteria for semen examination. Int Braz J Urol 2014; 40: 443-453.

[4] Gunes S, Arslan MA, Hekim GNT and Asci R. The role of epigenetics in idiopathic male infertility. J Assist Reprod Genet 2016; 33: 553-569.

[5] Jenkins TG, Aston KI, James ER and Carrell DT. Sperm epigenetics in the study of male fertility, offspring health, and potential clinical applications. Syst Biol Reprod Med 2017; 63: 69-76.

[6] Fernandez AF, Assenov Y, Martin-Subero JI, Balint B, Siebert R, Taniguchi H, Yamamoto H, Hidalgo M, Tan AC, Galm O, Ferrer I, Sanchez-Cespedes M, Villanueva A, Carmona J, Sanchez-Mut JV, Berdasco M, Moreno V, Capella G, Monk D, Ballestar E, Ropero S, Martinez R, Sanchez-Carbayo M, Prosper F, Agirre X, Fraga MF, Grana O, Perez-Jurado L, Mora J, Puig S, Prat J, Badimon L, Puca AA, Meltzer SJ, Lengauer T, Bridgewater J, Bock C and Esteller M. A DNA methylation fingerprint of 1628 human samples. Genome Res 2012; 22: 407-419.

[7] Tanaka S, Nakanishi MO and Shiota K. DNA methylation and its role in the trophoblast cell lineage. Int J Dev Biol 2014; 58: 231-238.

[8] O'Sullivan E and Goggins M. DNA methylation analysis in human cancer. Methods Mol Biol 2013; 980: 131-156.

[9] Jung M and Pfeifer GP. Aging and DNA methylation. BMC Biol 2015; 13: 7.

[10] Xu C and Song N. Epigenetic regulation in spermatogenesis. Zhonghua Nan Ke Xue 2014; 20: 387-391.

[11] Yao C, Liu Y, Sun M, Niu M, Yuan Q, Hai Y, Guo Y, Chen Z, Hou J and He Z. MicroRNAs and DNA methylation as epigenetic regulators of mitosis, meiosis and spermiogenesis. Reproduction 2015; 150: R25-34.

[12] Barlow DP and Bartolomei MS. Genomic imprinting in mammals. Cold Spring Harb Perspect Biol 2014; 6.

[13] Cotton AM, Price EM, Jones MJ, Balaton BP, Kobor MS and Brown CJ. Landscape of DNA methylation on the X chromosome reflects CpG density, functional chromatin state and X-chromosome inactivation. Hum Mol Genet 2015; 24: 1528-1539.

[14] Dinh TT, Gao L, Liu X, Li D, Li S, Zhao Y, O'Leary M, Le B, Schmitz RJ, Manavella PA, Weigel D, Pontes O, Ecker JR and Chen X. DNA topoisomerase 1α promotes transcriptional silencing of transposable elements through DNA methylation and histone lysine 9 dimethylation in arabidopsis. PLoS Genet 2014; 10: e1004446.

[15] Jimenez-Useche I, Nurse NP, Tian Y, Kansara BS, Shim D and Yuan C. DNA methylation effects on tetra-nucleosome compaction and aggregation. Biophys J 2014; 107: 1629-1636.

[16] Choy JS, Wei S, Lee JY, Tan S, Chu S and Lee TH. DNA methylation increases nucleosome compaction and rigidity. J Am Chem Soc 2010; 132: 1782-1783.

[17] Oakes CC, La Salle S, Smiraglia DJ, Robaire B and Trasler JM. Developmental acquisition of genome-wide DNA methylation occurs prior to meiosis in male germ cells. Dev Biol 2007; 307: 368-379.

[18] Romero Y, Meikar O, Papaioannou MD, Conne B, Grey C, Weier M, Pralong F, De Massy B, Kaessmann H, Vassalli JD, Kotaja N and Nef S. Dicer1 depletion in male germ cells leads to infertility due to cumulative meiotic and spermiogenic defects. PLoS One 2011; 6: e25241.

[19] Dada R, Kumar M, Jesudasan R, Fernandez JL, Gosalvez J and Agarwal A. Epigenetics and its role in male infertility. J Assist Reprod Genet 2012; 29: 213-223.

[20] Jenkins TG and Carrell DT. The sperm epigenome and potential implications for the developing embryo. Reproduction 2012; 143: 727-734.

[21] Rivera RM and Ross JW. Epigenetics in fertilization and preimplantation embryo development. Prog Biophys Mol Biol 2013; 113: 423-432.

[22] Aston KI, Uren PJ, Jenkins TG, Horsager A, Cairns BR, Smith AD and Carrell DT. Aberrant sperm DNA methylation predicts male fertility status and embryo quality. Fertil Steril 2015; 104: 1388-1397, e1381-1385.

[23] Kitamura A, Miyauchi N, Hamada H, Hiura H, Chiba H, Okae H, Sato A, John RM and Arima T. Epigenetic alterations in sperm associated with male infertility. Congenit Anom (Kyoto) 2015; 55: 133-144.

[24] Urdinguio RG, Bayon GF, Dmitrijeva M, Torano EG, Bravo C, Fraga MF, Bassas L, Larriba S and Fernandez AF. Aberrant DNA methylation patterns of spermatozoa in men with unexplained infertility. Hum Reprod 2015; 30: 1014-1028.

[25] Marques CJ, Francisco T, Sousa S, Carvalho F, Barros A and Sousa M. Methylation defects of imprinted genes in human testicular spermatozoa. Fertil Steril 2010; 94: 585-594.

[26] Poplinski A, Tuttelmann F, Kanber D, Horsthemke B and Gromoll J. Idiopathic male infertility is strongly associated with aberrant methylation of MEST and IGF2/H19 ICR1. Int J Androl 2010; 33: 642-649.

[27] Rahiminia T, Yazd EF, Fesahat F, Moein MR, Mirjalili AM and Talebi AR. Sperm chromatin and DNA integrity, methyltransferase mRNA levels, and global DNA methylation in oligoasthenoteratozoospermia. Clin Exp Reprod Med 2018; 45: 17-24.

[28] Karaca MZ, Konac E, Yurteri B, Bozdag G, Sogutdelen E and Bilen CY. Association between methylenetetrahydrofolate reductase (MTHFR) gene promoter hypermethylation and the risk of idiopathic male infertility. Andrologia 2017; 49.

[29] Nanassy L and Carrell DT. Abnormal methylation of the promoter of CREM is broadly associated with male factor infertility and poor sperm quality but is improved in sperm selected by density gradient centrifugation. Fertil Steril 2011; 95: 2310-2314.

[30] Sarova N, Ahlawat S, Grewal A, Sharma R and Arora R. Differential promoter methylation of DAZL gene in bulls with varying seminal parameters. Reprod Domest Anim 2018; 53: 914-920.

[31] Jenkins TG, Aston KI, Trost C, Farley J, Hotaling JM and Carrell DT. Intra-sample heterogeneity of sperm DNA methylation. Mol Hum Reprod 2015; 21: 313-319.

[32] Pacheco SE, Houseman EA, Christensen BC, Marsit CJ, Kelsey KT, Sigman M and Boekelheide K. Integrative DNA methylation and gene expression analyses identify DNA packaging and epigenetic regulatory genes associated with low motility sperm. PLoS One 2011; 6: e20280.

[33] Heyn H, Ferreira HJ, Bassas L, Bonache S, Sayols S, Sandoval J, Esteller M and Larriba S. Epigenetic disruption of the PIWI pathway in hu-

man spermatogenic disorders. PLoS One 2012; 7: e47892.

[34] Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, Haugen TB, Kruger T, Wang C, Mbizvo MT and Vogelsong KM. World Health Organization reference values for human semen characteristics. Hum Reprod Update 2010; 16: 231-245.

[35] Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, Delano D, Zhang L, Schroth GP, Gunderson KL, Fan JB and Shen R. High density DNA methylation array with single CpG site resolution. Genomics 2011; 98: 288-295.

[36] Sandoval J, Heyn H, Moran S, Serra-Musach J, Pujana MA, Bibikova M and Esteller M. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics 2011; 6: 692-702.

[37] Hong LZ, Zhao XY and Zhang HL. p53-mediated neuronal cell death in ischemic brain injury. Neurosci Bull 2010; 26: 232-240.

[38] Dong WW, Huang HL, Yang W, Liu J, Yu Y, Zhou SL, Wang W, Lv XC, Li ZY, Zhang MY, Zheng ZH and Yan W. Testis-specific Fank1 gene in knockdown mice produces oligospermia via apoptosis. Asian J Androl 2014; 16: 124-130.

[39] Hammoud SS, Purwar J, Pflueger C, Cairns BR and Carrell DT. Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. Fertil Steril 2010; 94: 1728-1733.

[40] El Hajj N, Zechner U, Schneider E, Tresch A, Gromoll J, Hahn T, Schorsch M and Haaf T. Methylation status of imprinted genes and repetitive elements in sperm DNA from infertile males. Sex Dev 2011; 5: 60-69.

[41] Gao K, Wang ZQ, Liu XC, Dong ZL and Shan WS. [Genetic genes associated with oligospermia, asthenospermia and teratospermia: advances in studies]. Zhonghua Nan Ke Xue 2017; 23: 367-371.

[42] Barau J, Teissandier A, Zamudio N, Roy S, Nalesso V, Herault Y, Guillou F and Bourc'his D. The DNA methyltransferase DNMT3C protects male germ cells from transposon activity. Science 2016; 354: 909-912.

[43] Inoue K, Ichiyanagi K, Fukuda K, Glinka M and Sasaki H. Switching of dominant retrotransposon silencing strategies from posttranscriptional to transcriptional mechanisms during male germ-cell development in mice. PLoS Genet 2017; 13: e1006926.

[44] Wu WB, Li YS, Ji XF, Wang QX, Gao XM, Yang XF, Pan ZH and Feng XX. [Expression of TEKT4 protein decreases in the ejaculated spermatozoa of idiopathic asthenozoospermic men]. Zhonghua Nan Ke Xue 2012; 18: 514-517.

[45] Zhou JH, Zhou QZ, Lyu XM, Zhu T, Chen ZJ, Chen MK, Xia H, Wang CY, Qi T, Li X and Liu CD.

The expression of cysteine-rich secretory protein 2 (CRISP2) and its specific regulator miR-27b in the spermatozoa of patients with asthenozoospermia. Biol Reprod 2015; 92: 28.

[46] Tamburrino L, Marchiani S, Vicini E, Muciaccia B, Cambi M, Pellegrini S, Forti G, Muratori M and Baldi E. Quantification of CatSper1 expression in human spermatozoa and relation to functional parameters. Hum Reprod 2015; 30: 1532-1544.

[47] Hetherington L, Schneider EK, DeKretser D, Muller CH, Hondermarck H, Velkov T and Baker MA. Deficiency in outer dense fiber 1 is a marker and potential driver of idiopathic male infertility. Mol Cell Proteomics 2016; 15: 3685-3693.

[48] McCarthy NS, Melton PE, Cadby G, Yazar S, Franchina M, Moses EK, Mackey DA and Hewitt AW. Meta-analysis of human methylation data for evidence of sex-specific autosomal patterns. BMC Genomics 2014; 15: 981.

[49] Li D, Xu Z, Liu J, Pu X, Luo Y and Zheng X. [Restriction landmark genomic scanning for screening aberrant CpG methylations in prostate cancer]. Nan Fang Yi Ke Da Xue Xue Bao 2016; 36: 103-108.

[50] Li S, Qiao Y, Di Q, Le X, Zhang L, Zhang X, Zhang C, Cheng J, Zong S, Koide SS, Miao S and Wang L. Interaction of SH3P13 and DYDC1 protein: a germ cell component that regulates acrosome biogenesis during spermiogenesis. Eur J Cell Biol 2009; 88: 509-520.

[51] Danoy P, Wei M, Johanna H, Jiang L, He D, Sun L, Zeng X, Visscher PM, Brown MA and Xu H. Association of variants in MMEL1 and CTLA4 with rheumatoid arthritis in the Han Chinese population. Ann Rheum Dis 2011; 70: 1793-1797.

[52] Huang H, Huang SC, Hua DJ, Sun QQ, Cen H and Xin XF. Interaction analysis between BLK rs13277113 polymorphism and BANK1 rs-3733197 polymorphism, MMEL1/TNFRSF14 rs3890745 polymorphism in determining susceptibility to rheumatoid arthritis. Autoimmunity 2017; 50: 403-408.

[53] Kus A, Arlukowicz-Grabowska M, Szymanski K, Wunsch E, Milkiewicz M, Ploski R, Shums Z, Norman GL, Milkiewicz P, Bednarczuk T and Krawczyk M. Genetic risk factors for autoimmune thyroid disease might affect the susceptibility to and modulate the progression of primary biliary cholangitis. J Gastrointestin Liver Dis 2017; 26: 245-252.

[54] Saadah OI, Shaik NA, Banaganapalli B, Salama MA, Al-Harthi SE, Wang J, Shawoosh HA, Alghamdi SA, Bin-Taleb YY, Alhussaini BH, Elango R and Al-Aama JY. Replication of GWAS coding SNPs implicates MMEL1 as a potential sus-

ceptibility locus among Saudi Arabian celiac disease patients. Dis Markers 2015; 2015: 351673.

[55] Pinto FM, Ravina CG, Subiran N, Cejudo-Roman A, Fernandez-Sanchez M, Irazusta J, Garrido N and Candenas L. Autocrine regulation of human sperm motility by tachykinins. Reprod Biol Endocrinol 2010; 8: 104.

[56] Zhang Y, Mao XY, Liu X, Song RR, Berney D, Lu YJ and Ren G. High frequency of the SDK1: AMACR fusion transcript in Chinese prostate cancer. Int J Clin Exp Med 2015; 8: 15127-15136.

[57] Kaufman L, Potla U, Coleman S, Dikiy S, Hata Y, Kurihara H, He JC, D'Agati VD and Klotman PE. Up-regulation of the homophilic adhesion molecule sidekick-1 in podocytes contributes to glomerulosclerosis. J Biol Chem 2010; 285: 25677-25685.

[58] Oguri M, Kato K, Yokoi K, Yoshida T, Watanabe S, Metoki N, Yoshida H, Satoh K, Aoyagi Y, Nozawa Y and Yamada Y. Assessment of a polymorphism of SDK1 with hypertension in Japanese individuals. Am J Hypertens 2010; 23: 70-77.

[59] Knoblich N, Gundel F, Bruckmann C, Becker-Sadzio J, Frischholz C and Nieratschker V. DNA methylation of APBA3 and MCF2 in borderline personality disorder: potential biomarkers for response to psychotherapy. Eur Neuropsychopharmacol 2018; 28: 252-263.

[60] Ke J, Zeng S, Mao J, Wang J, Lou J, Li J, Chen X, Liu C, Huang LM, Wang B and Liu L. Common genetic variants of GPC1 gene reduce risk of biliary atresia in a Chinese population. J Pediatr Surg 2016; 51: 1661-1664.

[61] Montjean D, Zini A, Ravel C, Belloc S, Dalleac A, Copin H, Boyer P, McElreavey K and Benkhalifa M. Sperm global DNA methylation level: association with semen parameters and genome integrity. Andrology 2015; 3: 235-240.

[62] Geneugelijk K, Thus KA and Spierings E. Predicting alloreactivity in transplantation. J Immunol Res 2014; 2014: 159479.