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human reproduction

ORIGINAL ARTICLE *Infertility*

Genome-wide differential methylation analyses identifies methylation signatures of male infertility

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STUDY QUESTION: Do methylation changes in sperm DNA correlate with infertility?

STUDY ANSWER: Loss of spermatogenesis and fertility was correlated with 1680 differentially-methylated CpGs (DMCs) across 1052 genes.

WHAT IS KNOWN ALREADY: Methylation changes in a number of genes have been correlated with reduced sperm count and motility.

STUDY DESIGN, SIZE, DURATION: This case-control study used spermatozoal DNA from 38 oligo-/oligoastheno-zoospermic infertile patients and 26 normozoospermic fertile men.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: Genome-wide methylation analysis was undertaken using 450 K BeadChip on spermatozoal DNA from six infertile and six fertile men to identify DMCs. This was followed by deep sequencing of spermatozoal DNA from 32 infertile patients and 20 fertile controls.

MAIN RESULTS AND THE ROLE OF CHANCE: A total of 1680 DMCs were identified, out of which 1436 were hypermethylated and 244 were hypomethylated. Classification of DMCs according to the genes identified *BCAN, CTNNA3, DLGAP2, GATA3, MAGI2* and *TP73* among imprinted genes, *SPATA5, SPATA7, SPATA16* and *SPATA22* among spermatogenesis-associated genes, *KDM4C* and *JMJD1C, EZH2* and *HDAC4* among genes which regulate methylation and gene expression, *HLA-C, HLA-DRB6* and *HLA-DQA1* among complementation and immune response genes, and *CRISPLD1, LPHN3* and *CPEB2* among other genes. Genes showing significant differential methylation in deep sequencing, i.e. *HOXB1, GATA3, EBF3, BCAN* and *TCERG1L*, are strong candidates for further investigations. The role of chance was ruled out by deep sequencing of select genes.

LARGE-SCALE DATA: N/A.

LIMITATIONS, REASON FOR CAUTION: Genome-wide analyses are fairly accurate, but may not be exactly validated in replication studies across all DMCs. We used the '*t*' test in the genome-wide methylation analysis, whereas other tests could provide a more robust and powerful analysis.

WIDER IMPLICATIONS OF THE FINDINGS: DMCs can serve as markers for inclusion in infertility screening panels, particularly those in the genes showing differential methylation consistent with previous studies. The genes validated by deep sequencing are strong candidates for investigations of their roles in spermatogenesis.

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Key words: male infertility / oligozoospermia / spermatogenesis / DNA methylation / sperm DNA methylation

Introduction

Infertility affects ~10%–15% of reproductively active couples worldwide (Jarow *et al.*, 2002). In about half of them, male factor infertility is the cause. Genetic factors remain the most complex and least understood contributors to variations in fertility (Ferlin *et al.*, 2006; Zorrilla and Yatsenko, 2013). Epigenetics, which constitutes another layer of gene regulation, strongly affects gene expression and function. DNA methylation is one of the best-studied epigenetic modifications that affect gene expression (Lande-Diner *et al.*, 2007) and chromatin packaging (Hammoud *et al.*, 2011) during spermatogenesis. In germ cells, DNA methylation is critically involved in various processes, including paternal genomic imprinting (Houshdaran *et al.*, 2007), silencing of transposable elements (Hollister and Gaut, 2009), silencing and DNA compaction, and numerous aspects of mitosis, meiosis and spermiogenesis (Gaysinskaya *et al.* 2018). The mammalian germ-line undergoes broad epigenetic reprogramming during germ cell maturation and gametogenesis. Widespread erasure of DNA methylation occurs in male primordial germ cells (Seisenberger *et al.*, 2012) and *de novo* DNA methylation occurs during germ cell maturation and spermatogenesis before meiosis (Oakes *et al.*, 2007a). Consequently, the pattern of sperm DNA methylation is unique in comparison with any somatic cell (Reik *et al.*, 2001; Oakes *et al.*, 2007b). In mature sperm, hypomethylated promoters are found in developmental genes and genes encoding signaling factors (Hammoud *et al.*, 2009). As a result, the specific sperm DNA methylation in mammals is suggested to be essential for spermatogenesis, fertilization and early embryonic development (Li *et al.*, 1992; Eckhardt *et al.*, 2006).

Earlier studies on candidate genes have shown a strong association between abnormal semen parameters and aberrant DNA methylation in imprinted, testes-specific and other genes (Houshdaran *et al.*, 2007; Kobayashi *et al.*, 2007; Boissonnas *et al.*, 2010; Hammoud *et al.*, 2010; Marques *et al.*, 2010; Poplinski *et al.*, 2010; Pacheco *et al.*, 2011; Rajender *et al.*, 2011; Sato *et al.*, 2011; Rotondo *et al.*, 2012; Tian *et al.*, 2014). These reports were followed by genome-wide studies utilizing 27 K array, which suggested that methylation could differentiate between high and low sperm count groups (Aston *et al.*, 2012; Schutte *et al.*, 2013; Montjean *et al.*, 2015). Subsequent studies have employed 450 K BeadChip methylation to compare the methylation profile between normozoospermic fertile and normozoospermic infertile patients, finding that in addition to select targets, methylation at several repetitive sequences is lower in sperm than in somatic cells (Urdinguio *et al.*, 2015). Two other similar studies based on 450 K arrays found that differential methylation correlates with sperm motility (Pacheco *et al.*, 2011; Du *et al.*, 2016). Similarly, in a mixed population of infertility patients attending an IVF clinic, Camprubi et al. (2016) found that methylation analysis could differentiate between fertile and infertile individuals. A recent 450 K-based study identified *KCNJ5*, *MLPH* and *SMC1B* as differentially-methylated genes between subfertile and fertile males (Laqqan and Hammadeh, 2017). Most of the above studies provided sufficient strength to the hypothesis that the sperm methylome is associated with sperm count and infertility. Inspite of these reports, the methylation differences between infertile and fertile men remain poorly characterized, particularly due to the lack of replication and a thorough deep analysis around the regions identified by BeadChip analyses.

In the present study, we undertook genome-wide DNA methylation analysis on sperm DNA from infertile individuals and compared them with fertile controls, then performed a validation of the differences in a larger sample size by employing massive parallel sequencing. We found that infertile men differed from fertile controls in 1680 CpG sites, with 1436 CpG sites showing hypermethylation and 244 CpG sites showing hypomethylation in the infertile group. Deep sequencing identified significant differential methylation in a number of genes known to participate in spermatogenesis.

Materials and Methods

Ethical approval

The Institutional Review Board and Ethics Committee of the Central Drug Research Institute (CDRI) approved this study. All subjects were informed about the purpose of sample collection and informed written consents were obtained. Study subjects were recruited at the Department of Endocrinology, Central Drug Research Institute (CDRI), Lucknow and the Institute of Medical Sciences, Banaras Hindu University (BHU), Varanasi.

Semen analysis and sample preparation

For genome-wide DNA methylation, we used samples from normozoospermic fertile controls ($N = 6$) and oligozoospermic infertile patients ($N = 6$). For validation by deep sequencing, 52 semen samples were collected and classified into two groups: normozoospermic fertile controls ($N = 20$) and oligo-/oligoastheno-zoospermic infertile patients ($N = 32$) (Supplementary Tables SI and SII). Inclusion criteria for patients consisted of infertility persisting for longer than an year and the absence of other diseases known to affect fertility, such as mumps, mycoplasma infection, varicosity, prostatitis, epididymitis and orchitis. Individuals with abnormal karyotypes and those with Y-chromosome microdeletions were excluded. All of the participants showed endocrine profiles (serum FSH, inhibin B and testosterone) and testicular volumes within normal range, ruling out congenital hypogonadotropic hypogonadism.

Semen samples were collected after 3–5 days of abstinence and allowed to liquefy at 37°C. Semen parameters were analyzed according to the WHO 2010 recommendations (Cooper *et al.*, 2010). Treatment with somatic cell lysis buffer (SCLB: 0.1% SDS, 0.5% Triton X-100) was used to remove leukocytes (Goodrich *et al.*, 2007), followed by confirmation using light microscopic analysis. In case of controls, swim up, immediately upon liquefaction, was performed to prepare high-quality sperm (Jayaraman *et al.*, 2012), as low quality sperm have been shown to be more inconsistent with respect to methylation (Jenkins *et al.*, 2015).

DNA isolation and quality analysis

High-quality DNA was prepared using MasterPure™ kit (Illumina, San Diego, CA, USA). Samples having minimal shearing were taken for further study and low quality samples were prepared on a second occasion. Isolated DNA samples were quantified by spectrophotometry and fluorometry and 1 μg of DNA was used for bisulphite conversion using EZ DNA methylation-Gold™ Kit (Zymo Research).

Genome-wide (450 K) DNA methylation analysis

Genome-wide methylation analysis was carried out using the Illumina Infinium Human Methylation 450 K BeadChip (Illumina). Genomic DNA (500 ng) from each sample was bisulfite converted using the EZ DNA methylation kit (Zymo Research, California, USA) and 4 μl of it was used for Chip hybridization. Technical control samples of different methylation percentages (0%, 50%, 100%) were spiked-in and repeated across plates to ensure high-quality data.

Gene ontology and pathway enrichment

For gene ontology, the enrichment analysis was performed using the Panther (Panther GO slim) software. Gene names were uploaded and analysis was performed against a human reference genome. Pathway analysis was performed using the KEGG tool and statistical analysis was performed against the same using a Bonferroni multiple test adjustment threshold of $P < 0.05$.

Deep sequencing of select genes

A total of nine genes were selected on the basis of (i) their roles in development, immune response and reproduction from the GO and KEGG analyses (*HOXB1, GPR123, CRISPLD1* and *KDM4C*), (ii) having more than three differentially-methylated CpG (DMC) spots (*HLA-C, HLA-DRB6, HLA-DQA1, TCERG1L and EBF3*) and (iii) being known to participate in spermatogenesis and male fertility (*TP73, GATA3, CPEB2, LPHN2, SMYD3* and *BCAN*). In total, a set of 15 genes was finalized for validation through deep sequencing, which included sequencing of the CpGs adjacent to the target DMCs. PCR conditions for each primer set are detailed in Supplementary Table SIII.

For deep sequencing, we undertook targeted bisulfite amplicon sequencing using MiSeq (Illumina, San Diego, CA, USA) on an independent set of semen samples from oligozoospermic/oligoastheno-zoospermic infertile patients ($n = 32$) and fertile control ($n = 20$) individuals.

The meth primer tool was used to design primer pairs specific for bisulfite converted DNA (Li and Dahiya, 2002), producing PCR amplicons of 300–320 base pairs. PCR amplification was carried out using platinum Taq DNA polymerase (Thermo scientific) and 1 μl of converted DNA template per 25 μl reaction. PCR conditions for each primer set are detailed in Supplementary Table SIII. Amplicons were purified using QIA quick PCR purification kit (Qiagen) as per the manufacturer's suggested protocol. Purified reaction products were run on a 2% agarose gel for confirmation, followed by quantification using the Qubit dsDNA high sensitivity fluorometric assay (Invitrogen). A 200-ng equimolar mix of the 13 amplicons was used as input for library preparation using the TruSeq DNA PCR-free library preparation kit. TruSeq DNA HT adapters (Illumina) were used for indexing. Library quantification was carried out using Kapa library quantification kit (Kapa Biosystems). Equimolar library pools were mixed and diluted to 8 pM for denaturation. PhiX control v3 (Illumina) was spiked in at a 5.0% final concentration, and subsequent cluster generation/sequencing was performed on the MiSeq using MiSeq Reagent Nano Kit (Illumina). A total of 500 cycles of 2 × 250 paired-end sequencing generated over 1820,000 reads.

For the genes where deep sequencing did not produce good or full coverage of the amplicons (TP73 and SMYD3), Sanger sequencing was employed to analyze the methylation level. For this, PCR products were purified and subjected to Sanger sequencing using standard protocols.

Methylation data analysis

For 450 K data analysis, mean Δbeta was calculated for a given CpG site by comparing methylation level in infertile with fertile individuals. Mean Δbeta is referred to as Δbeta across the manuscript. A positive Δbeta indicates relative hypermethylation and negative Δbeta indicates hypomethylation in the cases (Supplementary Table SIV). The *T*-test was used for testing statistical significance ($P < 0.05$). Corrections for multiple testing were made using a Bonferroni's adjustment for all CpG sites passing quality filtering (Dunn, 1961).

In case of deep sequencing, de-multiplexed raw reads were quality assessed using the FastQC tool. Good quality reads were sorted by keeping the Phred score threshold of 20 and adapter trimming was performed with Illumina universal adapter sequence 'GAGATCGGAAGAGC' using trim galore application. QC reports were generated using FastQC for trimming. Good quality processed reads were then aligned using Bismark tool using human genome version hg38 reference sequence. Bismark genome preparation was used for *in silico* DNA bisulphite conversion. Mapping was done using '-non_directional', '-sam-no-hd' and '-sam' commands to create.sam files for further use in downstream processes. The sam file thus created were used for secondary data processing in an R based tool. This alignment was executed in Ubuntu version 16.04 operating system on an one-core/128 Gb RAM work-station.

The methylation percentage at a locus was determined using R based tool called Methylkit by counting the number of 'C' nucleotides aligning to cytosine at a particular location. Bismark generated. sam files were used for obtaining percentage methylation scores in the CpG. This process was run by using 'processBismarkAln' command in the console of R studio. This command generates a tab separated text per Bismark.sam file containing percentages of C and T along with the information of chromosome number and position. In case of Sanger sequencing, the methylation level was estimated by the ratio of two alternate nucleotides at the target loci.

mRNA analysis of differentially-methylated genes

We selected nine genes (*GPR123, HLA-C, EBF3, KDM4C, LPHN3, TCERG1L, BCAN*, *HOXB1* and *TP73*) for estimation of transcript levels in sperm. RNA was isolated from sperm of oligozoospermic infertile and fertile control individuals using Illumina MasterPure complete DNA/RNA purification kit. Equal amount of RNA was converted into cDNA, which was used for estimation of transcript levels using a LightCycler 480 instrument (Roche Life Sciences).

Results

Global differential DNA methylation

Global hypermethylation and hypomethylation were significantly different between cases and controls (Fig. 1A–C). A total of 1680 DMCs were observed, of which 1436 were hypermethylated and 244 were hypomethylated (Fig. 1D). Of these, 1662 DMCs were annotated with a unique CpG identifier number, 12 were single polymorphic sites with rs id and six were denoted only by chromosome number (Supplementary Table SIV). Region-wise distribution of the DMCs

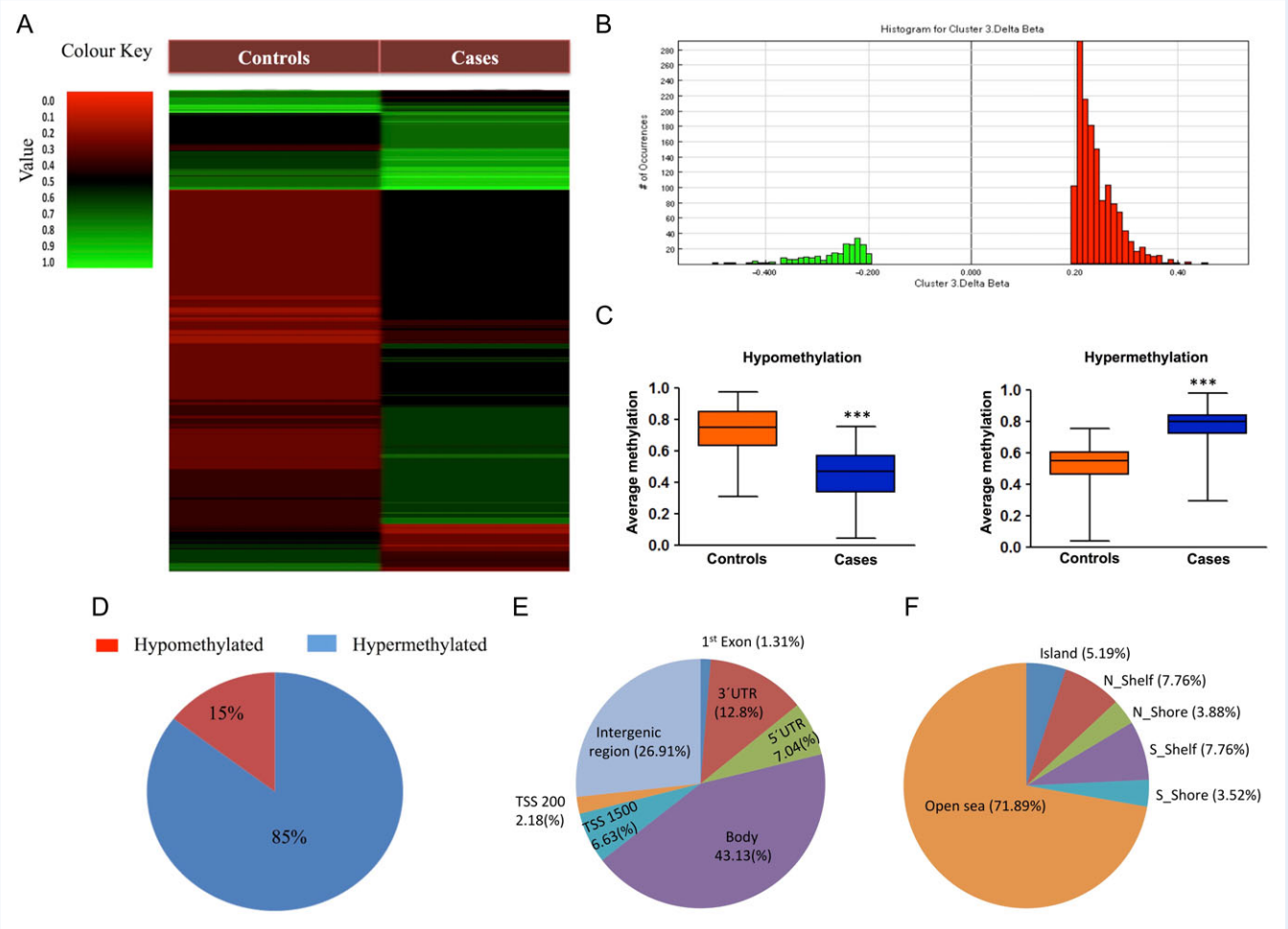

Figure 1 (**A**) Heat map of genome-wide DNA methylation. (**B**) Histogram showing the number of differentially-methylated CpGs (DMCs) across various β-values. (**C**) Box plot comparing mean β-values for hypermethylated and hypomethylated DMCs between cases and controls. (**D**) Percentage of DMCs showing hypermethylation and hypomethylation. (**E**) Distribution of DMCs on the basis of organization of gene transcript structure. (**F**) Distribution of DMCs on the basis of CpG island and neighborhood regions.

showed that methylation differences were more frequent in the gene body (43.13%) and intergenic regions (26.91%) and less frequent around transcriptional start sites (TSS), e.g. TSS1500 (6.63%) and TSS200 (2.18%), untranslated regions, e.g. 5′UTR (7.04%) and 3′UTR (12.8%), and in the first exon (1.31%) (Fig. 1E). Most of the DMCs belonged to open sea regions (71.89%) with a very little frequency in the CpG island (5.19%), N-shelf (7.76%), N-shore (3.88%), S-shelf (7.76%) and S-shore (3.52%) regions (Fig. 1F)

Out of 1662 annotated DMCs, 1230 spots were referred to genes, and other spots were either in the intergenic regions or placed distantly enough with respect to any gene. These 1230 DMCs represented 1052 genes, with four genes (*CACNA2P1*, *HOXB1*, *MED12L* and *RICTOR*) having four, three genes (*HLA-DQA1*, *LMF1*, *RPH3AL*) having five, two genes (*HLA-C*, *TCERG1L*) having six and one gene (*EBF3*) having seven DMCs (Supplementary Table SIV). A number of the genes possessing DMCs have been shown to play important roles in spermatogenesis and fertility (Table I). We have also undertaken an analysis on the regulatory regions to which the DMCs affiliate and found that seven hypermethylated DMCs belonged to the *GTDC1*, *ABCA7*, *FHIT*, *TMEM87A*, *COL11A1*, *DDX43* and *ZNF492* genes and four hypomethylated DMCs belonged to the *ATP1A4*, *HDAC4*, *TEKT5* and *TSNAX-DISC1* genes. An additional analysis on DNase hypersensitive sites and enhancers identified 50 and 532 CpGs, respectively (Supplementary Table SIV). Out of 50 DNase hypersensitivity sites, 20 were hypomethylated and 30 were hypermethylated, and out of 532 enhancer sites, 67 were hypomethylated and 465 were hypermethylated.

Gene ontology and KEGG analysis of genes

We took the above 1052 genes for ontology and KEGG pathway analysis to classify them based on their functions and biological pathways. A total of 971 gene IDs were mapped and used for gene ontology (GO). GO showed 702 hits in molecular function, 589 hits in cellular component and 1391 hits in biological processes (Supplementary Fig. 1SA–C). We took reproduction as our target biological process for gene selection and delve down to the lowest possible level to spermatogenesis (Supplementary Fig. S1C).

Table I DMC harboring genes involved in spermatogenesis or fertility or those with predominant testicular expression.

Gene	Protein	Genomic localization	Methylation change	Function summary	β-value
CPEB2	Cytoplasmic polyadenylation element binding protein	3′UTR	Hypermethylation	Required for spermatogenesis	0.2067
HOXB1	Homeobox B1	Promoter	Hypermethylation	Highest expression in testis	0.2164
RECQL4	RecQ Like Helicase 4	Body	Hypomethylation	Abundantly expressed during spermatogenesis	−0.2698
SOD3	Superoxide Dismutase 3	Promoter	Hypomethylation	Function in fertilization	−0.2698
VRK1	Vaccine Related Kinase 1	Body	Hypermethylation	Spermatogonial proliferation and differentiation	0.2589
SPATA16	Spermatogenesis Associated 16	Promoter	Hypermethylation	Specifically expressed in testis and involved in acrosome formation and sperm–egg fusion.	0.2443
SPATA22	Spermatogenesis Associated 22	Body	Hypermethylation	Predominantly expressed in germ cells and essential for early meiotic prophase	0.2443
EZH2	Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit	Body	Hypermethylation	Involved in spermatogonial differentiation and apoptosis	0.2106
TET2	Tet Methylcytosine Dioxygenase 2	Promoter	Hypermethylation	Required for spermatogenesis	0.2480
NPHP1	Nephrocystin 1	Body	Hypermethylation	Required for sperm morphogenesis	0.2265
JMJD1C	Jumonji Domain Containing 1C	Body	Hypermethylation	Involved in spermatogenesis and fertility	0.2180
PRDM1	PR/SET Domain 1	Body	Hypermethylation	Required for spermatogenesis	0.2321
AKAP9	A-Kinase Anchoring Protein 9	Body	Hypermethylation	Required for spermatogenesis and Sertoli cell maturation	0.2008
AKT3	AKT Serine/Threonine Kinase 3	Body	Hypermethylation	Dominantly expressed in testis and brain	0.2008
DDX4	DEAD-Box Helicase 4	3′UTR	Hypermethylation	Required for germ cells development and spermatogenesis	0.2077
TBL1XR1	Transducin Beta Like 1 X-Linked Receptor 1	Body	Hypermethylation	Involved in chromatin remodeling and sperm differentiation	0.2479
SPATA5	Spermatogenesis Associated 5	Body	Hypermethylation	Required for mitochondrial function and integrity during spermatogenesis	0.2444
SPATA7	Spermatogenesis Associated 7	Body	Hypermethylation	Specifically expressed in testis and retina	0.2444
HLA-DQA1	Major Histocompatibility Complex, Class II, DQ Alpha 1	Body	Hypermethylation	Involved in spermatozoa and oocyte interactions	0.2156
HLA-DRB6	Major Histocompatibility Complex, Class II, DR Beta 6 (Pseudogene)	Body	Hypermethylation	Involved in spermatozoa and oocyte interactions	0.2160

KEGG pathway analysis suggested their participation in various pathways such as phagosome (15 genes, $P = 2.87 \times 10^{-06}$), axon guidance (13 genes, $P = 9.62 \times 10^{-06}$), cell adhesion molecule (12 genes, $P = 6.29 \times 10^{-05}$), MAP kinase signaling pathway (17 genes, $P = 2 \times 10^{-04}$), viral myocardidities (10 genes, $P = 4.59 \times 10^{-06}$), type 1 diabetes mellitus (8 genes, $P = 5.49 \times 10^{-06}$), alograft rejection (7 genes, $P = 1.90 \times 10^{-05}$), pathways in cancer (21 genes, $P = 2.74 \times 10^{-05}$) and graft versus host disease (7 genes, $P = 3.84 \times 10^{-05}$) (Supplementary Fig. S1D). Pathway analysis for genes specific to reproduction is presented in Supplementary Fig. S1E.

Deep sequencing of selected genes in infertile and fertile samples

To validate genome-wide differential methylation data, a targeted bisulphite deep sequencing of 15 genes was undertaken on sperm DNA from 32 oligo-/oligoastheno-zoospermic infertile and 20 normozoospermic fertile controls. Along with target DMCs identified by 450 K analysis, deep sequencing analyzed additional proximal CpGs, which helped us in assessing region-wise methylation pattern. Deep sequencing of the amplicons yielded on an average 60,000 pairs of

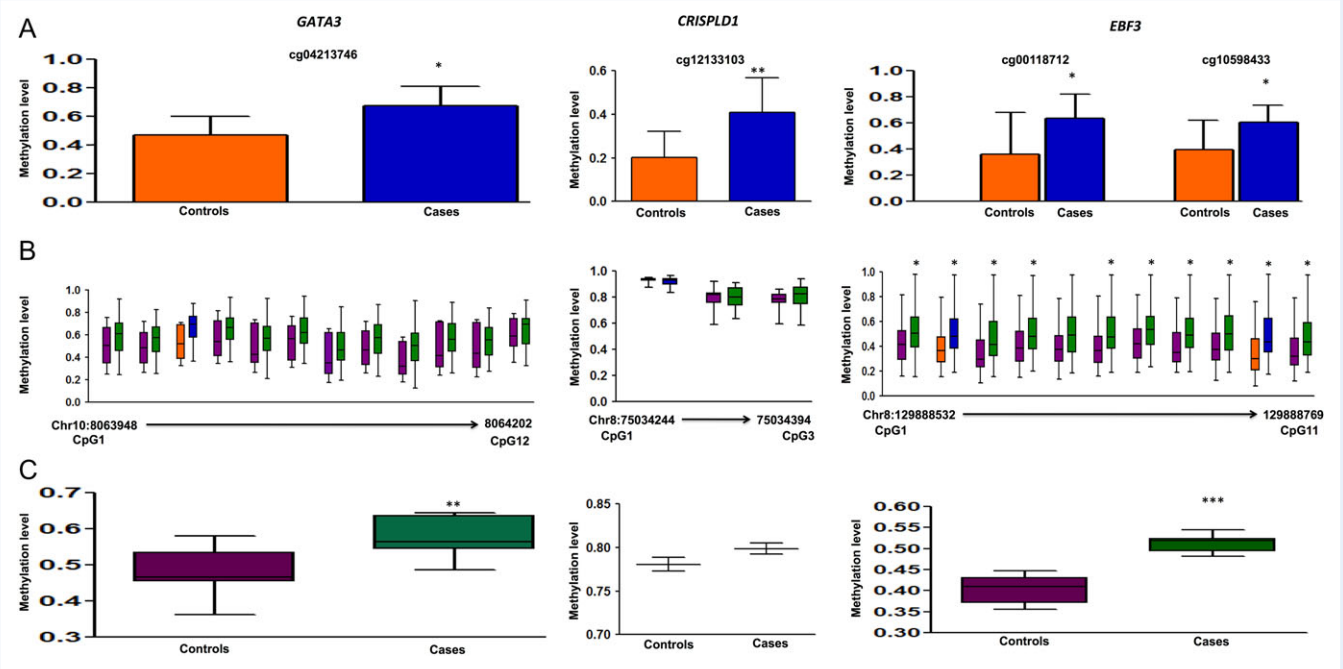

Figure 2 Methylation data for *GATA3, CRISPLD1* and *EBF3* for 450 K DMC (**A**), deep sequencing of neighboring CpGs in the target region (**B**), average methylation in the target region compared between cases and controls (**C**). 450 K DMC in deep sequencing (B) has been denoted using the same colors as in graphical representation of the DMC in panel A. In panel B, chromosome and nucleotide positions of the CpGs analyzed have been shown by numbers on the *X*-axis of the box plot.

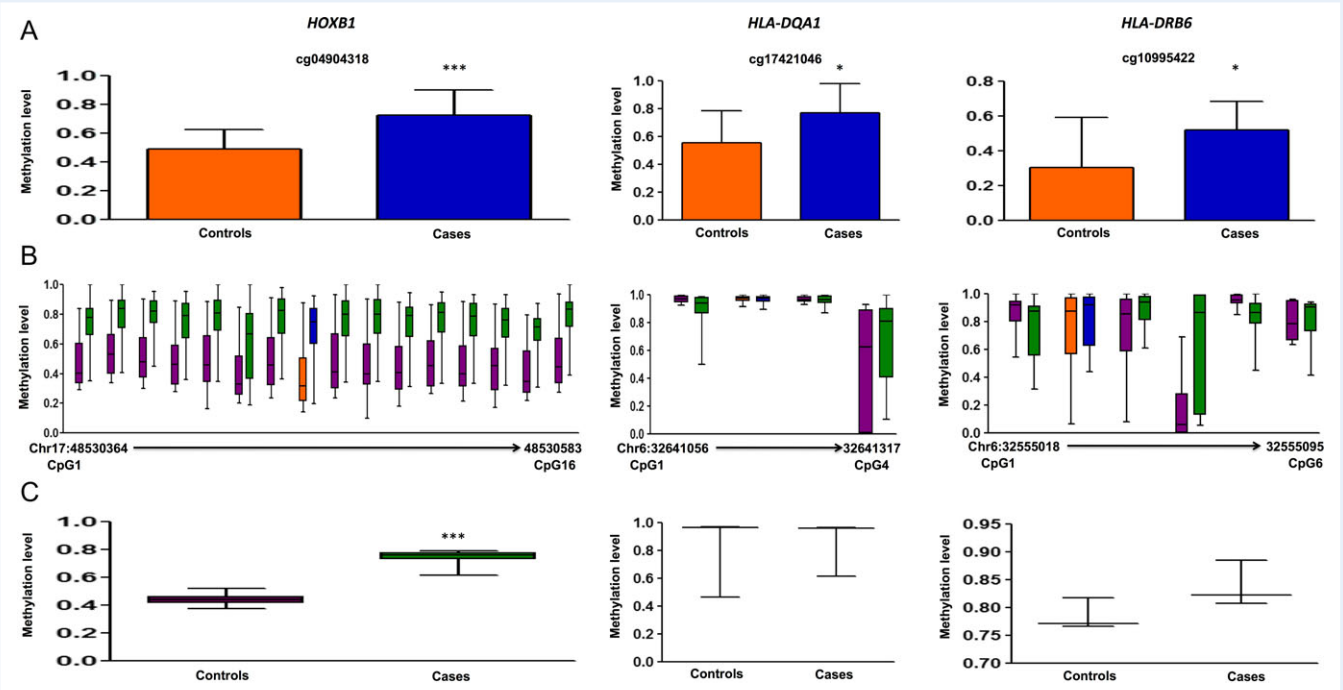

Figure 3 Methylation data for *HOXB1, HLA-DQA1* and *HLA-DRB6* for 450 K DMC (**A**), deep sequencing of neighboring CpGs in the target region (**B**), average methylation in the target region compared between cases and controls (**C**). 450 K DMC in deep sequencing (B) has been denoted using the same colors as in graphical representation of the DMC in panel A. In panel B, chromosome and nucleotide positions of the CpGs analyzed have been shown by numbers on the *X*-axis of the box plot.

reads with mapping efficiency close to an average of 60%. The qualities of reads were exceptionally good with almost 98% of reads with a Q score above 30 (Supplementary Fig. S2). The quality improved slightly after adapter trimming as adapter contamination in the reads was negligible.

In deep/Sanger sequencing, six genes (*HOXB1*, *CRISPLD1*, *HLA-DQA1*, *HLA-DRB6*, *GATA3* and *EBF3*) showed hypermethylation in the cases (Figs 2 and 3), seven genes (*BCAN*, *CPEB2*, *LPHN3*, *SMYD3*, *KDM4C*, *HLA-C* and *TCERG1L*) showed hypomethylation in the cases (Figs 4 and 5) and two genes (*TP73*, *GPR123*) showed no difference in methylation between cases and controls (Figs 5 and 6). With regard to the above, the hypermethylation in *HOXB1*, *GATA3* and *EBF3* and hypomethylation in the *BCAN* and *TCERG1L* genes were statistically significant.

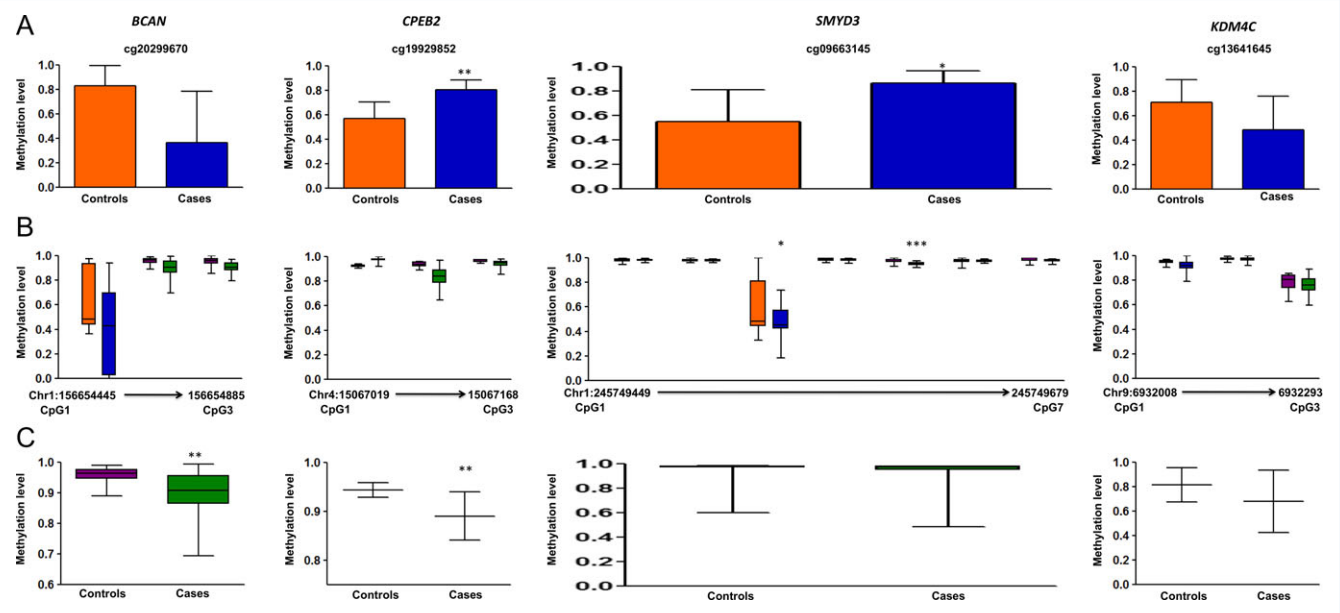

Figure 4 Methylation data for *BCAN, CPEB2, SMYD3* and *KDM4C* for 450 K DMC (**A**), deep sequencing of neighboring CpGs in the target region (**B**), *SMYD3* data was generated using Sanger sequencing. Average methylation in the target region compared between cases and controls (**C**). 450 K DMC in deep sequencing (B) has been denoted using the same colors as in graphical representation of the DMC in panel A. In panel B, chromosome and nucleotide positions of the CpGs analyzed have been shown by numbers on the *X*-axis of the box plot.

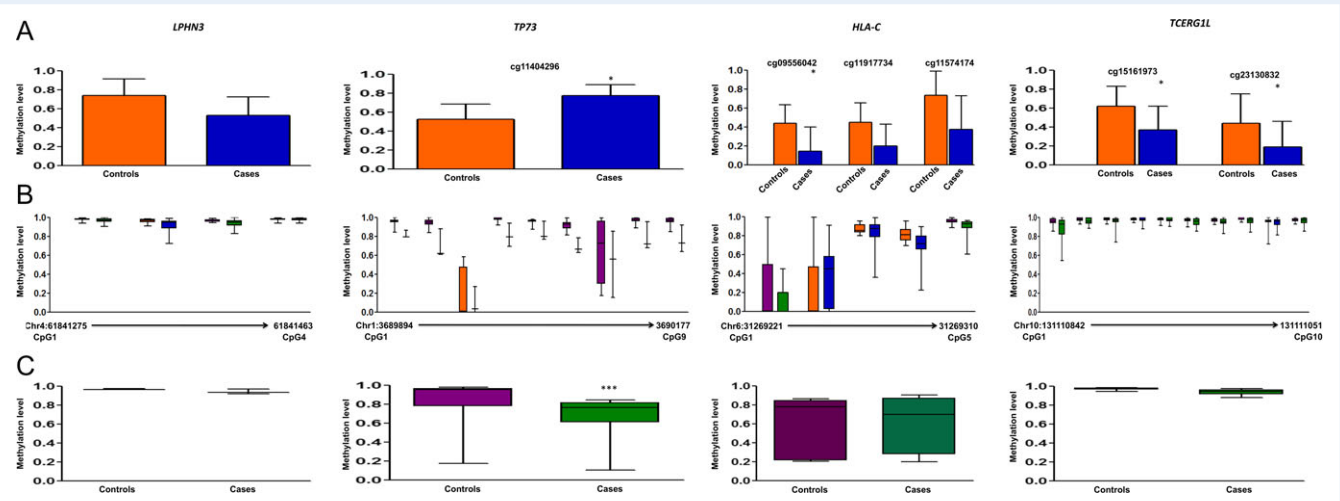

Figure 5 Methylation data for *LPHN3, TP73, HLA-C* and *TCERG1L* for 450 K DMC (**A**), deep sequencing of neighboring CpGs in the target region (**B**), *TP73* data was generated using Sanger sequencing. Average methylation in the target region compared between cases and controls (**C**). 450 K DMC in deep sequencing (B) has been denoted using the same colors as in graphical representation of the DMC in panel A. In panel B, chromosome and nucleotide positions of the CpGs analyzed have been shown by numbers on the *X*-axis of the box plot.

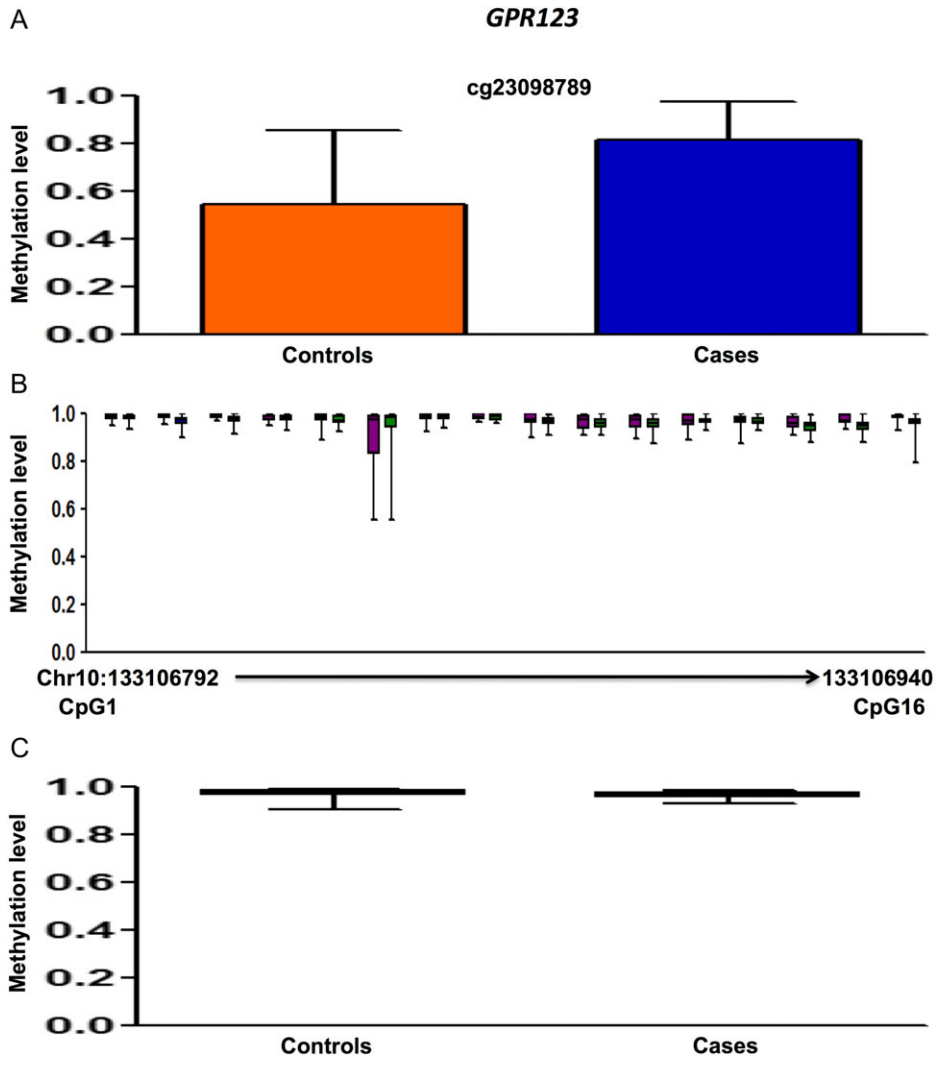

Figure 6 Methylation data for *GPR123* for 450 K DMC (**A**), deep sequencing of neighboring CpGs in the target region (**B**), average methylation in the target region compared between cases and controls (**C**). 450 K DMC in deep sequencing (B) has been denoted using the same colors as in graphical representation of the DMC in panel A. In panel B, chromosome and nucleotide positions of the CpGs analyzed have been shown by numbers on the *X*-axis of the box plot.

Transcript levels in sperm

We compared the expression data with methylation output from the deep sequencing experiment. Out of nine genes tested for expression analysis, six genes *(GPR123, HLA-C, KDM4C, LPHN3, TCERG1L, BCAN)* showed expression concordant with methylation data; however, the expression of three genes *(HOXB1, TP73 and EBF3)* was not concordant with their methylation level (Fig. 7).

Discussion

We found that out of 1680 DMCs, 1436 were hypermethylated and 244 were hypomethylated in the patients group. However, one limitation of our study could be the use of the '*t*' test in the genome-wide methylation analysis, whereas other tests could provide a more robust and powerful analysis.

A majority of the DMCs were in the non-island regions, which is consistent with previous reports stating that the key mark of methylation in germ cells are non-CpG island sequences (Hajkova *et al.*, 2002). Out of the differentially-methylated DNase hypersensitive sites, a majority showed hypermethylation in the patients, which is consistent with overall hypermethylation in this group. Similarly, Camprubi *et al.* (2016) found 696 DMCs, a majority of which were hypermethylated (74%). Another similar study found hypermethylation in sperm with poor morphology in comparison to sperm with good morphology (Cassuto *et al.*, 2016). Differential methylation in *ANK2, PRDM1* (Camprubi *et al.*, 2016), *TP73, GATA3* and *VAX2* (Pacheco *et al.*, 2011), and *MLPH*, *SMC1B* and *KCNJ5* (Laqqan and Hammadeh, 2017) is consistent with previous studies, which makes them strong candidates for inclusion in infertility screening panels. Out of the above, *PRDM1* (PR domain containing 1, with ZNF domain), a repressor of beta-interferon gene expression, plays a role in the primordial germ

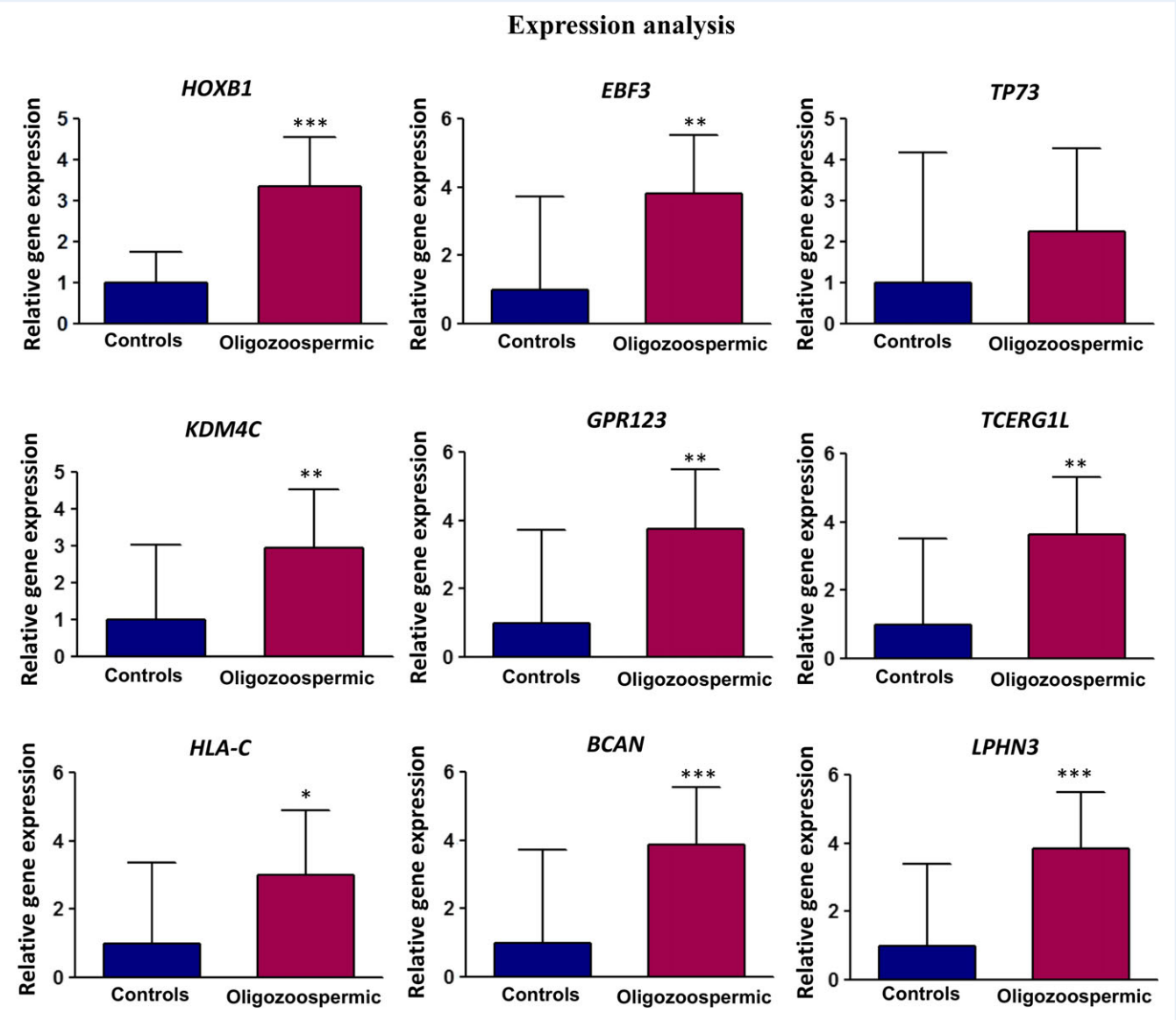

Figure 7 Expression analysis of selected genes. mRNA from sperm was converted into cDNA and subjected to analysis of transcript level by real time PCR.

cell induction (Ohinata *et al.*, 2005) and development (Fang *et al.*, 2018). Similarly, *MLPH* is expressed in testis and plays a role in acrosome biogenesis and the development of spermatid tail (Fukuda and Kuroda, 2002). The importance of *ANK2*, *VAX2*, *SMC1B* and *KCNJ5* genes in spermatogenesis remains unknown.

Among imprinted genes, we found DMCs in *ASB4, BCAN, CTNNA3, DLGAP2, GATA3, MAGI2* and *TP73* genes (Supplementary Table SIV). The ankyrin repeat and SOCS box-containing 4 (*ASB4*) gene is widely expressed in murine testes from the fourth week after birth extending into adulthood with specifically high expression in the pachytene spermatocytes, suggesting its pivotal role in testes development and spermatogenesis (Kim *et al.*, 2008). Interestingly, under acute heat stress conditions, the *CTNNA3* gene is down-regulated in chicken testis (Wang *et al.*, 2015), suggesting its role in the regulation of spermatogenesis. *MAGI2* plays an important role in sperm cell maturation by engaging in the regulation of tight junctions (Ihara *et al.*, 2012). *DLGAP2* is paternally expressed only in testis (Ranta *et al.*, 2000), but requires further investigation for its role in spermatogenesis.

We found significant hypermethylation in spermatogenesis-associated gene family members, *SPATA5, SPATA7, SPATA16* and *SPATA22*, which play various functions in spermatogenesis or fertility (Dam *et al.*, 2007; Dorosh *et al.*, 2013; Tanaka *et al.*, 2015). We also observed differential methylation in testis-specific genes, *TSPY* and *TTTY4B*, which are important for spermatogenesis (Krausz *et al.*, 2010; Lu *et al.*, 2014). *SMYD3* transcript shows 2.3-fold higher expression in adult human testes as compared to fetal testes (Zhou *et al.*, 2005) and mutations in this gene have been reported in patients with azoospermia (Venkatesh *et al.*, 2014). Other genes harboring DMCs included ten eleven translocase (*TET2*), histone demethylases (*KDM4C* and *JPJD1C*), histone deacetlyases (*HDAC4*), histone methyltranferases

(*EZH2*), which play important roles in gene regulation. At least EZH2 has been shown to play a role in the regulation of spermatogonial differentiation (Jin *et al.*, 2017).

Complementation genes constitute another interesting category in which we observed significant differential methylation. We observed that *HLA-C* gene was hypomethylated in infertile subjects while *HLA-DRB6* and *HLA-DQA1* were hypermethylated. Interestingly, van der Ven *et al.* (2000) demonstrated that HLA classes II MHC are strongly associated with gamete quality and embryonic development. *HLA-DQA1* and *HLA-DRB1* are associated with severe andrological problems in infertile men when compared with normozoospermic men. Mori *et al.* (1990) elucidated that HLA antigens are expressed in spermatozoa and are beneficial for spermatozoa and oocyte interactions in the mouse. Interestingly, ~50 differentially-methylated genes belonged to the immune response system, which opens up new avenues for investigating their functional roles in male infertility. It is interesting to note that epigenetic alterations in autoimmune processes affect fertility (Schutte *et al.*, 2013).

Among other differentially-methylatyed genes, *CRISPLD1* may have a role in cellular adhesion, which is essential for fertilization (Gibbs *et al.*, 2008). In addition, *CRISPLD1* was identified as a seminal plasma biomarker, upregulation of which affects sperm motility, acrosome reaction and capacitation (Antoniassi *et al.*, 2016). *LPHN3*, which encodes a member of the G-protein coupled receptors (GPCR), showed differential expression in asthenozoospermia, normozoospermic infertile patients and controls in our previous study (Bansal *et al.*, 2015). *CPEB2*, which encodes the cytoplasmic polyadenylation element binding protein, is expressed post-meiotically in mouse spermatogenesis and may have a role in translational regulation of stored mRNAs in the transcriptionally inactive haploid spermatids (Kurihara *et al.*, 2003). KEGG pathway analysis showed that candidate genes participate in key cellular processes associated with spermatogenesis, such as MAPK, which participates in signal transduction cascades and cell adhesion molecules, which participate in the blood testis barrier and the Sertoli cell dependent maturation of germ cells.

Deep sequencing revealed significant hypermethylation in *HOXB1, GATA3* and *EBF3* genes and hypomethylation in *BCAN* and *TCERG1L* genes, making them excellent candidates for further investigation. Interestingly, *GATA3* in the Sertoli cells has been shown to coordinate with androgen receptor to regulate gene expression (Bhardwaj *et al.*, 2008). Further research is required to understand the importance of *BCAN* and *EBF3* in spermatogenesis. Another research candidate is *TP73* since knockout mice for *TP73* are vulnerable to increased DNA damage and cell death in spermatogonia, disorganized apical ectoplasmic specialization, malformed spermatids and marked hyperspermia, suggesting its importance in spermatogenesis (Inoue *et al.*, 2014). We also found that the association between sperm transcript levels and DNA methylation may not be straightforward as sperm are transcriptionally inactive.

In conclusion, the present study highlights the association between methylation changes and loss of fertility. Mapping of the DMCs with respective genes showed *HOXB1, EBF3, ANK2, PRDM1, TP73, GATA3, VAX2, MLPH, SMC1B, KCNJ5, ASB4, BCAN, CTNNA3, DLGAP2, MAGI2, SPATA5, SPATA7, SPATA16, SPATA22, TET2, KDM4C, TCERG1L, JMJD1C, HDAC4, SMYD3, HLA-C, HLA-DRB6, HLA-DQA1, CRISPLD1, LPHN3* and *CPEB2* genes to be the top candidates showing alterations. The genes consistent with previous methylation studies include *ANK2, PRDM1, TP73, GATA3, VAX2, MLPH, SMC1B* and *KCNJ5*, which makes them excellent candidates for inclusion in infertility screening panels. Besides the above, this study has identified other differentially-methylated genes, *DLGAP2, HLA-C, HLA-DRB6, HLA-DQA1, CPEB2* and *EBF3*, which need further investigation for their roles in spermatogenesis. The genes showing hypermethylated (*HOXB1, GATA3* and *EBF3)* and hypomethylated CpGs (*BCAN* and *TCERG1L)* in deep sequencing are strong candidates for investigation of their roles in spermatogenesis. In addition to identifying new genes that may play important roles in spermatogenesis, this study has identified methylation markers that can become a part of methylation-based infertility screening panels.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Conflict of interest

All authors have declared no conflict of interest.

Authors' contributions

S.R., G.G., S.S., K.M.S. and K.S. conceived the study design. K.M.S., S. S., V.S., R.P. and S.R. conducted experiments. K.M.S., V.S., K.S., N.K. A. and S.T. contributed samples. R.P., G.G., K.S. and S.R. supervised the study. S.S. and K.M.S. analyzed the data. All authors have read and approved the manuscript.

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