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Original article

Aberrations in sperm DNA methylation patterns are associated with abnormalities in semen parameters of subfertile males

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ABSTRACT

Infertility affects about 15% of couples worldwide, with approximately 7% of males suffering from infertility problems. This study was designed to assess the relationship between alterations in sperm DNA methylation patterns and semen parameters in subfertile males. Of a total of 108 males, 30 samples were subjected to 450 K BeadChip arrays to evaluate the variation in DNA methylation level between cases and controls. Three CpG sites showed the highest difference in methylation levels (cg09737095, cg14271023, and cg17662493), which are located in the *KCNJ5*, *MLPH*, and *SMC1β* genes, respectively; these were selected for further analysis using deep bisulfite sequencing in 78 independent samples (21 proven fertile "controls", and 57 subfertile "cases"). The results of a validation study showed that variation in methylation levels was found in more than one CpG site: there was a significant decrease in methylation levels at six CpGs (CpG1, CpG3, CpG4, CpG6, CpG7, and CpG8) in the *KCNJ5* gene-related amplicon ($p \leq 0.001$, $p \leq 0.009$, $p \leq 0.007$, $p \leq 0.007$, $p \leq 0.020$, and $p \leq 0.016$, respectively), and at (CpG1, CpG2, and CpG4) in the *MLPH* gene-related amplicon ($p \leq 0.003$, $p \leq 0.005$, and $p \leq 0.0001$, respectively), while there was a significant increase in the methylation level at six out of eight CpGs in the *SMC1β* gene-related amplicon in cases compared to controls. Our results show that three CpGs have a significant difference in sperm DNA methylation levels in subfertile males compared to proven fertile males.

1. Introduction

Infertility is a complex disease related to several factors such as hormonal, biological, genetic, and lifestyle factors [1–3]. Infertility affects about 15% of couples worldwide [4]. Besides, approximately 7% of males suffer from infertility problems [5]. A common reason for infertility in human couples is male subfertility [6], and approximately 15–30% of couples are diagnosed with unexplained infertility after a routine analysis [7]. Several known causes lead to infertility like genetic causes, which constitute nearly 30% of male infertility cases [8], and there are other causes; however, about 50% of cases of male infertility are still diagnosed as unexplained [9]. Interestingly, several recent studies have shown a relationship between epigenetic alterations and male subfertility [10–13]. Therefore, epigenetics appears to be a promising research field for studying and explaining some of the causes of male infertility.

Epigenetics is defined as alterations to DNA that turn genes "on" or "off" without changes to the DNA sequence [14,15]. Epigenetics includes modifications to histone proteins and DNA methylation; here, we focused on DNA methylation and subfertility in males. DNA methylation is a major epigenetic modification involving the addition of a methyl group to the 5 position of cytosine by DNA methyltransferase to form 5-methylcytosine (5-mC) [16,17]; this addition leads to transcriptional silencing of mammalian genes [18]. The CpG dinucleotides can be found in clusters that have been termed CpG islands and characterised by less methylation than non-CpG islands [17]. It is worth mentioning that CpG islands are found in about 60–70% of gene promoters, and these promoters can be divided according to their CpG density [19,20]. The anomalies of sperm DNA methylation patterns are still under experimental investigation. In contrast, several previous studies have reported that these abnormalities can be transmitted to offspring and can influence the offspring's susceptibility to disease [21,22]. Therefore, alterations in sperm DNA methylation patterns of subfertile males may potentially be responsible for the increased risk of imprinting disorders, perinatal mortality, and several other pregnancy-related complications that are seen in assisted reproductive technology (ART) babies [23]. This study is designed to further evaluate the association between anomalies in sperm DNA methylation patterns and semen parameters of subfertile males.

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For this purpose, the present study tested the differences in sperm DNA methylation patterns between proven fertile males and subfertile males, who have been unable to have children after ten years of attempting childbearing. Also, this study will evaluate the relationship between changes in sperm DNA patterns and semen parameters in subfertile males.

2. Material and methods

2.1. Sample collection and semen analysis

Institutional review board approval (No. PHRC/HC/13/14) was obtained from the Ethics Committee of Helsinki before the initiation of this study. Besides, all participants gave written consent before participation in this study. All samples were analysed in the laboratory of the Molecular Biology, Genetics & Epigenetics Department at the University of Saarland, and the samples were analysed according to standard operating procedures. Briefly, one hundred and eight samples with a mean age of 38.5 ± 4.7 were collected (36 samples from proven fertile males as a "control group" and 72 samples from subfertile males, who have been unable to have children after ten years of attempting childbearing as the "case group"). The exclusion criteria for participation in the study were as follows: diabetes mellitus, the consumption of alcohol, the presence of anti-sperm antibodies, varicocele, and Y chromosome microdeletions, smoking, abnormal hormonal parameters, abnormal body mass index, and infertility problems related to the female partner. In contrast, the participants included in this study have no direct contact with environmental pollutants (e.g. carbon monoxide, toxic metals, radioactive pollutants, lead, and other heavy metals); males had the same nationality, ethnicity, and food supplementation. Briefly, semen samples were collected by masturbation after three days of sexual abstinence, allowed to liquefy at 37 °C for 30 min, and sperm count was assessed immediately using a Meckler counting chamber (Sefi-Medica, Haifa, Israel). The sperm parameters were analysed (sperm counts, a percentage of total sperm motile, and percentage of progressive motility) according to World Health Organisation guidelines [24]. Before DNA extraction from sperm, somatic cells were removed from all semen samples through the use of Somatic Cell Lysis Buffer (SCLB) which is widely used for sperm cell purification [25,26]. First, pure populations of spermatozoa were obtained through a 50% gradient (20 min at $300 \times g$), and then pure spermatozoa were incubated with Somatic Cell Lysis Buffer (SCLB) on ice for 30 min, and washed twice with phosphate-buffered saline (10 min at $500 \times g$). The absence of somatic cell contamination was confirmed by microscopic examination.

2.2. Sperm DNA isolation and sodium bisulfite treatment

Sperm DNA was extracted using the Isolate II genomic DNA kit according to the standard protocol provided by the manufacturer (Bioline, UK). The concentration and purity of extracted DNA were determined with the use of a Nanodrop spectrophotometer ND-2000c (Thermo Scientific). Five hundred nanograms of extracted sperm DNA was treated with sodium bisulfite using the Epitect bisulfite conversion kit (Qiagen, Germany) that converts unmethylated cytosines to uracil, while 5-methylcytosine (5MeC) remains unaltered, as previously described [27]. To confirm the effectiveness of the protocol used in removing somatic cells from the semen samples that was entered in this study, the publicly available data (GEO # GSE41169) were used to define sample purity and based on a known significantly differentially methylated region (DMR) between somatic cells (White blood cell) and spermatozoa. We assessed DNA from whole blood, DNA from round cell-contaminated sperm samples, known pure sperm DNA, and compared these with sperm DNA of the study population. The data showed that the samples evaluated in this study were free from round cell contamination.

2.3. Screening study by infinium 450 K BeadChip array

Thirty semen samples were used in the array screening study with a mean age of 39.4 ± 3.5; this included 15 samples from proven fertile males "controls" and 15 from subfertile males who have been unable to have children after ten years of attempting childbearing "cases". After bisulfite treatment, the DNA of these samples was subjected to Infinium 450 K BeadChip arrays (Illumina, San Diego, CA, USA) following the manufacturer's recommendations [28], and the arrays were scanned using the Illumina iScan. β-values were then generated by analysing the intensities for methylation or no methylation at each CpG tiled on the array using the calculation: β-value = methylated/(methylated + unmethylated). β-values ranged from 0 to 1 and indicate the methylation level for each CpG. A value of 1 represents a completely methylated CpG and 0 represents a completely unmethylated CpG. Raw intensity values obtained from Illumina were used to generate β-values, and the bioinformatic processing and evaluation were performed with the RnBeads program package [29]. The methylation level in each CpG was considered as being differentially methylated CpG (DMC) when the absolute difference of the means of the average β-values between two groups was ≥20%. To determine differentially methylated CpGs (DMC) with possible biological and statistical significance, a Benjamini-Hochberg corrected *t*-test FDR (false discovery rate) of 0.05 was applied, and all CpG sites with a coverage (no. of beads) of ≤5 were excluded from the analysis. Findings were considered significant when $p \leq 0.01$. Referring to the technical results of hybridisation, the gene call rate above 98% per sample and a detection value $p < 0.01$ per CpG site were set as the internal quality criteria. All CpGs that covered a common SNP site in the dbSNP137 database were excluded.

2.4. Validation study by bisulfite profiling (Bi-PROF)

In this study, and according to the results of screening study, three CpG sites that have the greatest difference in methylation level between the case and control groups were subjected to further analysis using local deep bisulfite sequencing (**Bi-PROF**) [30], according to the manufacturer's instructions. In the validation study, 78 samples (independent samples) were used and distributed as follows: 57 samples as cases and 21 samples as controls. Briefly, 500 nanograms of sperm DNA from each sample were subjected to bisulfite treatment using the Epitect bisulfite conversion kit (Qiagen, Germany). PCR reactions were performed in a 50 μl total volume reaction using the "MyTaq™ HS Red Mix" with 2 x concentration (Bioline, UK), according to the manufacturer's protocol. For amplification, fusion primers that consisted of a specific 3′-portion (listed in Table 1, together with respective annealing temperatures and a number of CpGs present within the amplicon sequence) and a universal 5′-portion containing the necessary nucleotide sequences for Illumina sequencing were used. Primers were designed using the BiSearch primer design tool (http://bisearch.enzim.hu/?m=search) with the following criteria: max length of PCR 400, primer concentration 0.167 μmol, potassium concentration 50 mmol, magnesium concentration 2.5 mmol, primer length 20 – 30, max Tm difference 2.0. By using BiSearch, placing primers onto common SNPs could be eliminated. Five microlitres of PCR products were loaded on a 2% agarose gel stained with ethidium bromide, including the DNA ladder (Biolabs, NE). PCR products were purified using Agencourt® AMPure XP