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H19 gene methylation status is associated with male infertility

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Abstract. The present study investigated the *H19* gene methylation status in male infertility. Between March 2013 and June 2014, semen samples were collected from 15 normal fertile males and 15 males experiencing infertility, and routine analysis and sperm morphological assessment were performed. The semen samples were subjected to density gradient centrifugation to separate the sperm fraction, and genomic DNA from the sperms was extracted and treated for bisulfite modification. Following *in vitro* amplification by polymerase chain reaction (PCR), the purified PCR products were cloned into pMD®18-T vectors and successful cloning was confirmed by restriction enzyme digestion. Positive clones were sequenced and the DNA methylation status was analyzed. The overall methylation rate in the normal fertile group was 100% (270/270), whereas in the infertile group the methylation rate was lower at 94.1% (525/558), revealing a statistically significant decrease in overall methylation rate in the infertile patients compared with the control group (χ^2=15.12; P<0.001). The average methylation rates of CpG 1, 3 and 6 in the infertile group were statistically different from those in the normal control group (all P<0.05). The abnormal methylation of imprinted gene *H19* is associated with male infertility, suggesting that *H19* may serve as a biomarker for the detection of defects in human spermiogenesis.

Introduction

Infertility is the absence of pregnancy within one year of unprotected normal intercourse, and remains the leading reproductive health problem affecting 15% of couples of reproductive age (1). However, male infertility (MI), defined as the incapability of a male to conceive with a fertile female, accounts for 50% of all infertility cases, and affects 1/20 males worldwide (2,3). Despite the progress in diagnostic methods to identify MI, such as physical examination, reproductive history and semen analysis, the underlying etiology of MI is poorly understood (4,5). In general, MI is associated with non-genetic risk factors such as testicular torsion or trauma, seminal tract infections, hypogonadotropic hypogonadism, cryptorchidism, gonadal dysgenesis, idiopathic oligozoospermia, reproductive channel obstruction, and anti-sperm antibodies (6-8). More recently, genetic risk factors in MI have received significant attention with the identification that single gene mutations and chromosomal aberrations account for 10-15% of MI cases (9-11).

The *H19* gene is one of the first imprinting genes to have been identified and is expressed from the maternal allele in humans and mice, with its expression highly restricted to heart and skeletal muscles in adults (12). The *H19* gene is located on the human 11p15.5 chromosome and on the distal section of mouse chromosome 7, spanning ~2.5 kb and containing 5 exons and 4 intrinsic factors (13,14). The *H19* gene encodes a non-coding RNA lacking an open reading frame and its expression is highly regulated by DNA elements (15,16). *H19* has a role in the regulation of body weight and cell multiplication, and is dysregulated in certain types of cancer (17,18). Recently, it has been shown that the *H19* gene encodes microRNA (miR), namely miR-675 which is important in tumorigenesis (19). *H19* expression was present in numerous types of cancer including hepatocellular carcinoma, choriocarcinoma, breast cancer, bladder cancer, colorectal cancer, testicular cancer, esophageal cancer and ovarian cancer (20). Notably, aberrant methylation in *H19* gene alters the onset of MI and affects individual susceptibility to MI (21,22). The present study hypothesized that aberrant methylation of the *H19* gene is associated with MI.

Materials and methods

Ethical statement. The study was approved by the academic board and the ethics committee of the First Affiliated Hospital of South China University (Hengyang, China). All eligible patients conformed to the study inclusion criteria. Written-informed consent was obtained from each eligible patient, and the residual semen following analysis was used in the present investigation. All experimental procedures were conducted according to the Declaration of Helsinki (23).

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Subjects and grouping. A total of 15 MI patients (age, 35.5±8.5 years) who were admitted to the First Affiliated Hospital of South China University were enrolled in the present study between March 2013 and June 2014 as the experimental group. Semen samples were collected from the patients, with sperm concentration ≤20x10^6/ml, sperm (a+b) concentration ≤50%, and percentage of morphologically normal sperm ≤15%. The inclusion criteria for the present study were as follows: i) Patients who had received a routine seminal fluid analysis ≥2 times at the First Affiliated Hospital of South China University, with consistent results; ii) seminal plasma fructose and neutral α-glucosidase were expressed normally; iii) an anti-sperm antibody test was negative; and iv) white blood cell semen counts wesre <1x10^6/ml. Conversely, patients were excluded if they had a history of hypertension, cardiovascular diseases, metabolic diseases, acquired immune deficiency syndrome, syphilis, hepatitis B and other infectious diseases, and a long-term history of heavy drinking or contact with poison. Furthermore, patients with varicocele, inflammation of the urinary and reproductive system, or genital tract infections, including those caused by *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *Ureaplasma aurealyticum*, were excluded. In addition, patients with infertility caused by diseases such as non-inflammatory sexual dysfunction of accessory sex gland or infection were excluded from the investigation.

A total of 15 fertile males (age, 32.5±6.5 years) with normal semen analyses were enrolled as the control group. The semen samples were determined to be normal in accordance with the standards of the World Health Organization (WHO) (24): Normal seminal liquefaction; no sperm agglutination; volume >2.0 ml; pH>7.2; sperm concentration >20x10^6/ml; total sperm number for one ejaculation ≥40x10^6/ml; sperm progressive motility within 60 min ≥50%, or sperm fast progressive motility≥25%; percentage of morphologically normal sperm ≥15%; survival rate ≥60%; and white blood cell concentration <1x10^6/ml.

Semen samples. The semen samples from MI patients and control subjects were obtained by masturbation following 2-7 days of sexual abstinence, collected in disposable semen cups, and immediately placed at 37˚C. Rapidly following seminal liquefaction, the concentration and motility of the raw semen samples were examined according to the Fourth Edition of the WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction (24). Staining was performed with a modified Papanicolaou staining technique recommended by the WHO (24). Briefly, prepared smears were fixed in 95% (v/v) ethanol for ~15 min, rehydrated in an ethanol gradient and stained with hematoxylin (Beyotime Institute of Biotechnology, Haimen, China) for 4 min. Following washing with pure-water for 30 sec and 4~8 times immersion in acidic ethanol for 1 sec each, the smears were dehydrated using an ethanol gradient and stained with Orange G6 (Beijing Leagene Biotech Co., Ltd., Beijing, China) for 1 min. Subsequently, the semen samples were washed three times for 30 sec each with 95% ethanol, followed by staining with EA-50 Green (Beijing Leagene Biotech Co., Ltd.) for 1 min and washing two times for 30 sec each with 95% ethanol and two times for 15 sec each with 100% (v/v) ethanol. After air drying and mounting with neutral balsam (Sigma-Aldrich, St. Louis, MO, USA), multiple regions were selected on the smears for morphological evaluation under a BH-2 optical microscope (Olympus Corporation, Tokyo, Japan), using the domestic WLJY-9000 WeiLi Color Sperm Analysis System (Beijing Weili New Century Science & Tech. Deve., Co., Ltd., Beijing, China) for assessing sperm quality.

Following the morphological assessment, the remaining sperm were separated using Percoll density gradient centrifugation. Briefly, 2 ml 40% Percoll liquid (Sigma-Aldrich) was added to the bottom of a conical centrifuge tube. A transfer pipette was then plunged into the bottom of the tube and 2 ml 80% Percoll liquid (Sigma-Aldrich) was added below the 40% Percoll liquid to form a two-layer Percoll density gradient. The semen samples were added on top of the 40% Percoll, and centrifugal separation was performed at 400 x g for 20 min at room temperature. Following discarding of the supernatant, 1 ml Earle's balanced salt solution (Sigma-Aldrich) was added to the sediment prior to centrifugation at 1,000 x g for 5 min at room temperature. The resulting supernatant was discarded and 0.1-0.2 ml sperm suspension was maintained for the subsequent procedures.

DNA extraction and polymerase chain reaction (PCR) amplification. Genomic DNA was extracted from the sperm using a TIANamp Blood DNA kit (cat. no. DP304-02; Tiangen Biotech Co., Ltd., Beijing, China) to obtain 30 μl dissolved DNA solution. The purity of the DNA was assessed by the A260/A280 ratio, and the DNA concentration was calculated from the absorption readings obtained from a spectrophotometer (DU 800; Beckman Coulter, Inc., Brea, CA, USA). Bisulfite modification of the DNA was conducted using an EpiTect Bisulfite kit (cat. no. 59104; Qiagen China Co., Ltd., Shanghai, China) in accordance with the manufacturer's protocol, and the DNA samples were then preserved at -20˚C. The *H19*-specific primers used for the reaction were synthesized by 216 bp fragments of imprinted gene *H19* which contained 18 CpG loci (genbank accession no. AFl 25183; nucleotides 7881-809): *H19* forward, 5'-TGGGTATTTTTGGAGGTTTTTTT-3', and reverse, 5'-ATAAATATCCTATTCCCAAATAA-3' (Beijing Liuhe Huada Genetic Science and Technology Co., Ltd., Beijing, China). PCR amplification was performed using bisulfite-modified genomic DNA as template. The total volume of the fundamental reaction system was 25 μl, which consisted of 0.25 μl LA *Taq* polymerase, 2.5 μl 10X LA Taq buffer, 4 μl dNTP (all Takara Biotechnology Co., Ltd., Dalian, China), 3.25 μl deionized water, 5 μl sense primer (2 μM), 5 μl antisense primer (2 μM), and 5 μl DNA template. The PCR was conducted on a Mastercycler Gradient PCR thermal cycler (Eppendorf, Hamburg, Germany), with the following conditions: Denaturation for 15 min at 95˚C, 50 cycles of annealing at 95˚C for 30 sec, 57˚C for 30 sec and 72˚C for 30 sec, and a final extension at 72˚C for 5 min. The amplification products (5 μl) were analyzed by 2% agarose gel electrophoresis.

Cloning and sequencing of PCR products. The PCR products of the *H19* gene in each group were recovered and purified using an EasyPure Quick Gel Extraction kit (cat. no. EG101; Beijing TransGen Biotech Co., Ltd., Beijing, China). All purified PCR products were cloned into pMD18-T vectors

Table I. Comparison of the sperm parameters in the semen of the experimental and control groups.

Sperm parameter	Control group (n=15)	Experimental group (n=15)	P-value
Sperm concentration (x10^6/ml)	113.6±32.1	11.8±7.2	<0.01
Sperm (a+b) (%)	53.7±4.5	18.6±12.1	<0.01
Morphologically normal sperm (%)	19.2±5.3	9.2±2.3	<0.01

Table II. Comparison of the average methylation rate (%) in each *H19* DMR CpG locus between the normal control group and the experimental group.

CpG locus	Experimental group (n=15)	Control group (n=15)	P-value
CpG1	73.33	100.00	**0.03**
CpG2	100.00	100.00	NA
CpG3	73.33	100.00	**0.03**
CpG4	100.00	100.00	NA
CpG5	100.00	100.00	NA
CpG6	66.67	100.00	**0.01**
CpG7	81.82	100.00	0.07
CpG8	100.00	100.00	NA
CpG9	100.00	100.00	NA
CpG10	86.67	100.00	0.14
CpG11	93.94	100.00	0.31
CpG12	80.00	100.00	0.07
CpG13	100.00	100.00	NA
CpG14	93.94	100.00	0.31
CpG15	100.00	100.00	NA
CpG16	100.00	100.00	NA
CpG17	100.00	100.00	NA
CpG18	100.00	100.00	NA

P-values shown in bold represent statistically significant values following the comparison between the normal semen group and the experimental group. NA indicated unavailable comparative results. DMR, differential methylation region; CpG, cytosine and guanine separated by a phosphate; NA, non-available.

(Takara Biotechnology Co., Ltd.) using a TA ligation system (10 μl) containing 0.25 μl pMD18-T vector, 1 μl 10X ligase buffer, 1 μl ligase enzyme (both Takara Biotechnology Co., Ltd.), and 7.75 μl DNA fragments. The plasmid was transformed into *E. coli* DH5α chemically-competent cells (cat. no. CB101-03; Tiangen Biotech Co., Ltd.), which were coated onto a Luria-Bertani agar plate (Sigma-Aldrich) containing 50 μg/ml ampicillin (Promega Corporation, Madison, WI, USA) and cultured overnight at 37˚C until white single colonies were observed. The white single colonies were picked for culturing at 37˚C overnight, after which a small quantity of the plasmids were extracted using the E.Z.N.A.® Plasmid Mini kit I (cat. no. D6943-01, Omega Bio-Tek, Inc., Norcross, GA, USA) and verified by enzyme digestion with *Rsa*I (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). The products of the enzyme digestion were separated by agarose gel electrophoresis and positive clones were identified by staining with ethidium bromide (Omega Bio-Tek, Inc.). The DL 2000 Marker (Takara Biotechnology Co., Ltd.) served as a reference. *H19*-positive bacterial clones were selected from each group and sent to GENEWIZ, Inc. (Beijing, China) for sequencing using a universal primer. The sequence analysis was conducted using a BiQ analyzer 2.0 software for DNA methylation analysis (http://biq-analyzer.bioinf.mpi-sb.mpg.de).

Statistical analysis. Data were presented as means ± standard deviation. The statistical comparison of the overall methylation rate of the *H19* gene between groups was analyzed using a t-test. Statistical analysis was conducted using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant result.

Results

Baseline characteristics. As shown in Table I, sperm concentration in the experimental group was ~10-fold lower compared with that of the control group, a result which was statistically significant (P<0.01). Sperm (a+b) percentage in the experimental group was also significantly lower compared with that in the control group (P<0.05). In addition, statistically significant differences also existed in the percentage of morphologically normal sperm between the control and experimental groups (P<0.05).

PCR amplification of the H19 differential methylation region (DMR). The results of the agarose gel electrophoresis with a DL2000 marker as a reference revealed a single band of 216 bp PCR product, as shown in Fig. 1, representing the amplified DNA fragment of *H19* DMR. DNA amplification products of the *H19* DMR in the normal control and experimental groups are shown in Fig. 1A and B.

Identification of positive H19 DMR clones. The positive clones underwent electrophoresis following enzyme digestion with the DL2000 marker as a reference. As shown in Fig. 2, the results of the PCR indicated the presence of positive clones of *H19* for the normal control and experimental groups, with one band having a fragment size of ~2,692 bp, which is consistent with the molecular size of the pMD18-T vector, and another band having a fragment size of ~216 bp, which is consistent with the fragment size of *H19* DMR. Fig. 2A shows two positive clones corresponding to the normal control group following *H19* DMR PCR product cloning, transfer and enzyme-digestion. Fig. 2B shows one positive clone corresponding to the experimental group.

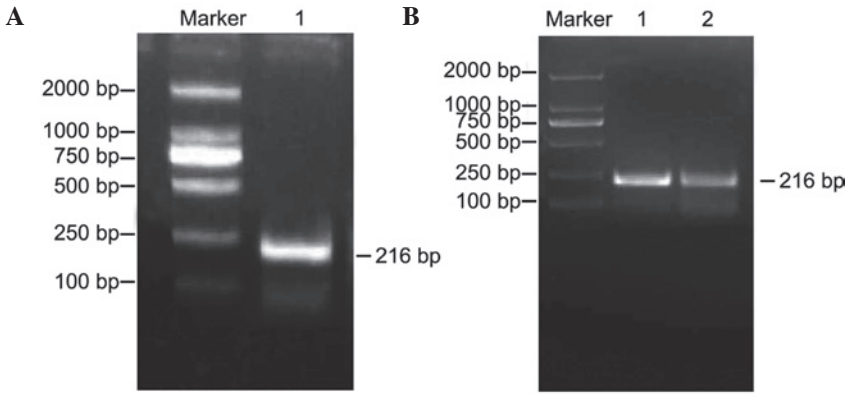

Figure 1. *In vitro* amplification of imprinted gene *H19* by agarose gel electrophoresis. (A) Marker, DNA electrophoresis 100 bp marker (DL 2000); lane 1, DNA amplification fragments of the *H19* DMR in the normal control group. (B) Marker, DNA electrophoresis 100 bp marker (DL 2000); lanes 1 and 2, DNA amplification fragments of the *H19* DMR in the experimental group. DMR, differential methylation region.

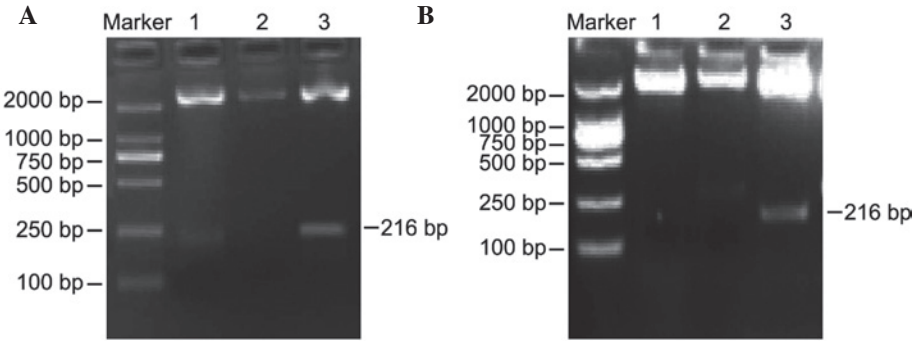

Figure 2. Confirmation of the presence of recombinant plasmid of imprinted gene *H19* by agarose gel electrophoresis. (A) Marker, DNA electrophoresis 100 bp marker (DL 2000); lanes 1 and 3, positive clones in the normal control group following *H19* DMR cloning and enzyme-digestion; lane 2, enzyme digestion results of negative clones. (B) Marker, DNA electrophoresis 100 bp marker (DL 2000); lanes 1 and 2, enzyme digestion results of negative clones; lane 3, positive clones in experimental group following *H19* DMR cloning and enzyme-digestion. DMR, differential methylation region.

Methylation analysis of CpG loci in H19 DMR. The methylation scattergram of each locus in the *H19* DMR was obtained using BIQ analyzer software. Fig. 3A shows 18 methylated CpG loci in 15 clones from 15 fertile males in the normal control group and all sites exhibited 100% methylated status. Similarly, Fig. 3B shows the 18 methylated CpG loci detected in 15 clones from 15 patients of the experimental group. It evident that, among the 18 methylated CpG loci, CpG 1, 3, 6, 7, 10, 11, 12 and 14 revealed individual differences in the methylation pattern, with only 4/31 clones in the experimental group exhibiting 100% methylation at all 18 sites. As seen in Fig. 3, comparison of the overall methylation rate of *H19* DMR between the normal control group and the experimental group was conducted. The methylation rate in the normal control group was 100% (270/270), whereas that in the experimental group was 94.1% (525/558), and the comparative differences were considered statistically significant (P<0.001).

Comparative analysis of the average methylation rate of the H19 DMR CpG locus. Following t-test analysis, the average methylation rate of each *H19* CpG in the experimental group was 66.67-100%, whereas that in the normal control group was constant, namely 100%. In addition, the comparison of the average methylation rate of each CpG locus between the normal control group and the experimental group is shown in Table II. Statistically significant results between the normal control group and the experimental group were observed to be present in CpG1, CpG3 and CpG6 (P=0.032, P=0.032, and P=0.014, respectively).

Discussion

In the present study, the methylation status of the *H19* gene was assessed in both normal males and infertile males, and the results demonstrated that the levels of DNA methylation of the *H19* gene were lower in the infertile males compared with the controls. Therefore, aberrant methylation of the *H19* gene may be correlated with the progression of MI. Genomic imprinting is the allele-specific expression in certain genes that controls gene expression from both paternal and maternal genomes and is critical for normal development due to its significance in placental functions, neurobehavioral processes and embryonic growth (25,26). In addition, DNA methylation is the most important silencing mechanism underlying the regulation of genetic elements, specifically for elements with substantial CpG dinucleotide content (27). Aberrant DNA methylation is usually found in human cancers and may account for chromosomal instability and deregulated gene expression (28). In fertilization, spermatozoa have male DNA methylation patterns that contribute to paternal methylation imprints, and immediately following fertilization the paternal genome is demethylated, including imprinted genes and repeat sequences.

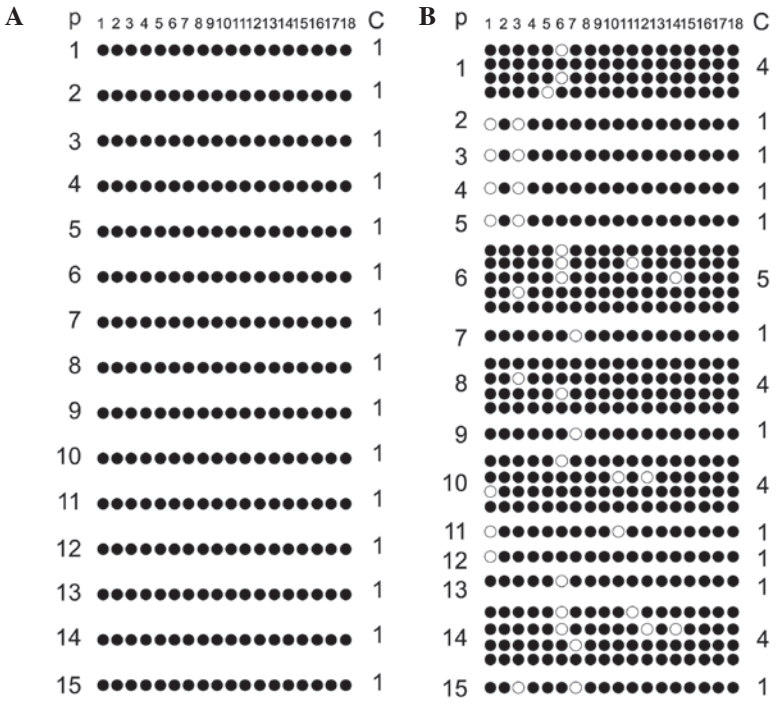

Figure 3. Methylation scattergram of each *H19* DMR CpG locus. Methylation status of the *H19* DMR CpG locus in the (A) fertile and (B) infertile males. Numbers 1-18, CpG locus; P, individual; C, total number of clones in each pattern of methylation; ●, methylated CpG locus; ○, unmethylated CpG; DMR, differential methylation region.

The present study used bisulfate sequencing and PCR to measure the degree of methylation of the *H19* imprinting control region (ICR), which demonstrated that control individuals carried 100% methylation of *H19* ICR, whereas infertile males with asthenospermia and oligozoospermia exhibited *H19* hypomethylation (29). The methylation status of the *H19* ICR may serve as an indicator for aberrant DNA methylation function acting at that locus, and may contribute to altered gene expression, thus contributing to MI. Therefore, the decreased methylation of *H19*, an important imprinted gene in fertile males, indicates that aberrant methylation of the *H19* genes may be correlated with the progression of MI, and may serve as a biomarker for deficiency in human sperm development.

The results of the present study demonstrated that CpG locus 1, CpG locus 3 and CpG locus 6 in the *H19* DMR displayed aberrant methylation with greater frequency, the reason for which remains unknown. DNA methylation in the mammalian genome involves covalent addition of a methyl group at the 5'-end of a cytosine in the context of CpG. Following t-test analysis, the methylation status of a total of 18 CpGs located in the H19 DMR of human sperm in infertile men were compared with the methylation patterns of normal individuals in order to determine the specificity and the extent of the loss of DNA methylation associated with distinct sperm abnormalities. Among the 18 CpGs in infertile men, CpG1, CpG3, CpG6, CpG7, CpG10, CpG11, CpG12 and CpG14 exhibited differences in the unmethylated status compared with the CpGs of healthy men. Analysis of the average methylation rate of *H19* DMR CpG loci and comparison of the degree of unmethylation at each CpG in the clones analyzed indicated that CpG locus 1, CpG locus 3 and CpG locus 6 in the *H19* DMR are associated with aberrant methylation.

There were limitations to the present study. Notably, the study only focused on the analysis of *H19* gene methylation. However, paternal allele expression of IGF is strongly associated with maternal allele expression of *H19*, and the DMR located 2-4-kb upstream of the *H19* gene refers to the ICR of the locus, and its deletion impacts both *H19* and IGF2 expression. In addition, the small sample sizes used in the study may lead to lower statistical significance during the analysis of *H19* gene methylation.

In conclusion, the results of the present study provide evidence that aberrant methylation of the *H19* gene may be correlated with the progression of MI, and CpG locus 1, CpG locus 3 and CpG locus 6 in the *H19* DMR appear associated with aberrant methylation.

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