Methylation defects of imprinted genes in human testicular spermatozoa

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Objective: To study the methylation imprinting marks of two oppositely imprinted genes, *H19* and *MEST/PEG1*, in human testicular spermatozoa from azoospermic patients with different etiologies. Testicular spermatozoa are often used in intracytoplasmic sperm injection for treatment of male factor infertility, but the imprinting status of these cells is currently unknown.
Design: Experimental prospective study.
Setting: University research laboratory and private in vitro fertilization (IVF) clinic.
Patient(s): A total of 24 men, five with anejaculation, five with secondary obstructive azoospermia, five with primary obstructive azoospermia, and nine with secretory azoospermia due to hypospermatogenesis.
Intervention(s): Spermatozoa were isolated by micromanipulation from testicular biopsies.
Main Outcome Measure(s): DNA methylation patterns were analyzed using bisulfite genomic sequencing with cloning analysis.
Result(s): We found *H19* complete methylation was statistically significantly reduced in secretory azoospermic patients with hypospermatogenesis, with one patient presenting complete unmethylation. Hypomethylation also affected the CTCF-binding site 6, involved in regulation of *IGF2* expression. Regarding the *MEST* gene, all patients presented complete unmethylation although this was statistically significantly reduced in the anejaculation group.
Conclusion(s): Testicular spermatozoa from men with abnormal spermatogenesis carry methylation defects in the *H19* imprinted gene which also affect the CTCF-binding site, further supporting an association between the occurrence of imprinting errors and disruptive spermatogenesis. (Fertil Steril® 2010;94:585–94. ©2010 by American Society for Reproductive Medicine.)

Key Words: Azoospermia, DNA methylation, genomic imprinting, *H19*, *MEST*, male factor infertility, testicular spermatozoa

Genomic imprinting leads to monoallelic parental-dependent expression of a subset of genes in mammals. Parental alleles are marked differentially, primarily by DNA methylation of CpG dinucleotides located on the differentially methylated regions of imprinted genes. These regulate the expression of imprinted genes and constitute the imprinting control regions (ICRs) (1). Methylation imprinting marks are erased in primordial germ cells (PGCs) (2) and are later reestablished during germ cell development according to parental sex. In murine spermatogenesis, paternal imprints start to be acquired prenatally and are definitively established before the onset of meiosis (3, 4). It is interesting that paternal imprints seem to be acquired earlier in the paternally inherited allele than in the allele of maternal origin, suggesting that, even when devoid of methylation, parental alleles can retain their identity (3). In humans, establishment of paternal imprints has been shown to be complete by the primary spermatocyte stage (5). On fertilization, a wave of DNA demethylation occurs, active in the paternal pronucleus and passive in the maternal pronucleus, which does not affect the methylation imprinting marks previously set in gametes (6).

There are only three known imprinting control regions that acquire methylation in the paternal germ line: *H19-Igf2*, *Dlk1-Gtl2*, and *Rasgrf1* (7). *H19* is one of the best characterized imprinted genes; it is expressed from the maternal allele, it is imprinted in human (8) and mice (9), and it encodes an untranslated RNA (10) whose function remains unclear. It was previously suggested to have tumor-suppressor activity (11) but also to be an oncofetal RNA (12), and was recently demonstrated to give rise to a microRNA (miR-675) (13). Additionally, *H19* is physically and functionally linked to another imprinted gene, *IGF2* (insulin-like growth factor-2) through the sharing of common enhancers (14, 15). *H19* and *IGF2* are oppositely imprinted because *H19* is expressed

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from the maternal allele and *IGF2* is expressed from the paternal allele. The *H19* DMR (differentially methylated region) is located upstream of the transcription start site and harbors several CTCF (CCCTC-binding factor) binding sites (16, 17); CTCF binds to the maternal unmethylated DMR and prevents *IGF2* from accessing the common enhancers, thus silencing *IGF2* expression. Oppositely, methylation on the paternal DMR prevents the binding of CTCF, and *IGF2* is expressed. After fertilization, the promoter of *H19* becomes methylated and silences the *H19* paternal allele while allowing the continued expression of *IGF2* (18).

In humans, *H19* is located in 11p15.5, a region linked to imprinting associated Beckwith-Wiedemann syndrome. This syndrome is characterized by congenital overgrowth and an increased risk of developing embryonic tumors such as Wilms tumor. In some BWS patients, the genetic cause has been ascribed to the occurrence of *H19* microdeletions and hypermethylation of the maternal alleles, which leads to *IGF2* loss of imprinting and hyperexpression (19). On the other hand, paternal hypomethylation of *H19*, probably leading to *H19* biallelic expression and down-regulation of *IGF2*, has recently been demonstrated to occur in the male germ line, in association with oligozoospermia (20–22). Also, *H19* hypomethylation was recently described as the cause of Silver-Russell syndrome, another congenital disorder characterized by severe intrauterine and postnatal growth retardation (23).

MEST/PEG1 (mesodermal specific transcript/paternally expressed gene 1) is located in human chromosome 7q32, is paternally expressed (maternally methylated), and is imprinted in mice (24) and humans (25). *MEST* deficiency in mice, caused by a target mutation, was shown to cause general growth retardation of embryonic and extraembryonic structures and abnormal maternal response to newborns (26). *MEST* was also found to encode an imprinted antisense RNA (MESTIT1–MEST intronic transcript 1) that is only expressed from the paternal allele which has been suggested to play a role in the regulation of *MEST* expression during development (27). This transcript was found to be predominantly expressed in the testis and in mature motile spermatozoa, indicating a possible role in human sperm physiology and fertilization (28).

The occurrence of imprinting syndromes in children born after assisted reproductive techniques (ART), usually presenting loss of methylation of the maternal alleles, has raised concern about the safety of these techniques regarding the establishment of imprints in the oocyte and maintenance during in vitro embryo culture (7). However, imprinting methylation defects have also been described in the paternal allele, in sperm from patients with oligozoospermia, suggesting an association between imprinting errors and infertility itself (20–22).

Spermatozoa retrieved from testis biopsies are often used in ART, namely in intracytoplasmic sperm injection (ICSI), in cases of severe male factor infertility due to obstructive or secretory azoospermia (29). However, the methylation status of imprinted genes in human testicular spermatozoa of azoospermic patients is currently unknown. Both *H19* and *MEST* were previously shown to be representative imprinted genes for the evaluation of methylation errors present in sperm from oligozoospermic patients (21). Additionally, increased expression of *H19*, *IGF2,* and *MEST* in the embryo and placenta suggests an important function for these genes in preimplantation and postimplantation embryo development (gene expression databases: SymAtlas at http://symatlas.gnf.org and Unigene at http://www.ncbi.nlm.nih.gov).

We describe *H19* and *MEST* methylation patterns in testicular spermatozoa from patients with anejaculation (ANJ), secondary inflammatory obstructive azoospermia (OAZI), obstructive azoospermia due to congenital bilateral absence of vas deferens (CBAVD), and secretory azoospermia due to hypospermatogenesis (HP). We provide further evidence that the occurrence of imprinting errors in the male germ line is associated with impairment of spermatogenesis and discuss the possible implications of these new findings.

MATERIALS AND METHODS

Patient Recruitment and Classification

Under patients' informed consent and according to the guidelines of the medical ethics committee, testicular spermatozoa were retrieved from testicular biopsies of 24 cases, divided into four groups: ANJ (n = 5) mainly due to spinal cord injuries, secondary obstructive azoospermia due to inflammatory epididymal disease (n = 5), primary obstructive azoospermia due to CBAVD (n = 5), and secretory (nonobstructive) azoospermia due to hypospermatogenesis (n = 9). Biopsies from normal fertile males were not included because it was not possible to obtain testicular spermatozoa from these cases. However, the present results were compared with cases with normal spermatogenesis, without (ANJ) or with (OAZI) epididymal obstruction, as well as with data obtained from normoozoospermic ejaculated spermatozoa (22).

All patients had normal karyotypes and absence of Y-chromosome AZF and DAZ microdeletions (30, 31). Ages ranged from 22 to 44 years with the average being of 32.3 (range: 22 to 44 years) in ANJ, 37.2 years (range: 29 to 43 years) in OAZI, 35 years (range: 27 to 44 years) in CBAVD, and 32.8 years (range: 27 to 42 years) in HP.

Isolation of spermatozoa by Micromanipulation from Testicular Biopsies

Testicular biopsy samples were prepared as described previously elsewhere (29). Briefly, each fragment was mechanically tweezed in sperm preparation medium (SPM with HEPES buffer; Medicult, Copenhagen, Denmark), and the resultant fluid was washed with SPM twice (5 minutes, 500 × *g*). The pellet was resuspended in 2 mL of erythrocyte-lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 2 mM EDTA, pH 7.2) (Sigma, Barcelona, Spain) during 5 minutes at 32°C, 5% CO_2 (32). After washing, samples were digested for 1

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