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Methylation loss at *H19* imprinted gene correlates with *methylenetetrahydrofolate reductase* gene promoter hypermethylation in semen samples from infertile males

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Abbreviations: MTHFR, methylenetetrahydrofolate reductase; DMR, differentially methylated regions; COBRA, combine bisulfite restriction analysis

Aberrant methylation at the *H19* paternal imprinted gene has been identified in different cohorts of infertile males. The causes of *H19* methylation errors are poorly understood. In this study, we investigated the methylation status of the *H19* gene in semen DNA samples from infertile males affected by *MTHFR* gene promoter hypermethylation. DNA from normal and abnormal semen samples harbouring *MTHFR* gene promoter hypermethylated, hmMTHFR-nor and hmMTHFR-abn, and without *MTHFR* methylation, MTHFR-nor and MTHFR-abn, were investigated for methylation status in the *H19* locus using bisulfite-treated DNA PCR, followed by cloning and sequencing. The prevalence of *H19* hypomethylated clones was 20% in hmMTHFR-nor and 0% in MTHFR-nor semen samples ($P < 0.05$), and 28% in hmMTHFR-abn compared with 16% in MTHFR-abn semen samples ($P > 0.05$). These results underscore the association between *H19* methylation defects and hypermethylation of the *MTHFR* gene promoter in normal semen samples and suggest that aberrant methylation at *H19* may occur in the normal sperm of infertile males affected by *MTHFR* gene dysfunction. These findings provide new insights into the mechanisms causing abnormal methylation in imprinted genes and, in turn, male infertility.

Introduction

Genomic imprinting is a mechanism that regulates gene expression in a parental origin-dependent way. DNA methylation is the chemical process that controls genomic imprinting. It consists in the attachment of methyl groups to cytosine residues at CpG dinucleotides, CpG islands, in specific DNA regions of imprinted genes, known as differentially methylated regions (DMRs), allowing for mono-allelic parent-specific gene expression.[1]

Correctly imprinted maternal and paternal genes are needed to regulate major functions at the fetomaternal interface, such as nutrient transport, trophoblast proliferation, invasion and angiogenesis.[2] In rodents and humans, impaired methylation imprints generate small placentae with abnormalities in proliferation, apoptosis and trophoblast differentiation and miscarrige.[3-8]

In male gametes, DNA methylation at imprinted genes is established prior to entry into meiosis and is maintained throughout development.[9,10] Three genetic loci have been found to have paternal imprinting in sperm cells, *IGF2/H19, RASGRF1,* and *GTL2*.[11] The best-characterized of these genes is *IGF2/H19*, which displays reciprocal paternal *IGF2* and maternal *H19* gene expression.[9] *IGF2/H19* genes share common enhancers, located downstream of *H19*, whose activity is regulated by a DMR upstream of the *H19* gene. In the maternal allele, *H19* is unmethylated, allowing the CTCF insulator protein (CCCTC-binding factor) to bind to the DMR. This process prevents *IGF2* from accessing common enhancers, thus inhibiting *IGF2* and promoting *H19* expression. In the paternal allele, *H19* is methylated and CTCF binding is blocked, thus inactivating *H19* and promoting *IGF2* expression.[12]

Recent studies have indicated that appropriate establishment of *H19* imprinting plays a critical role in maintaining fertility, since *H19* methylation defects have been identified in several groups of males experiencing infertility.[13-16] The causes inducing *H19* imprinting disorders in spermatogenetic cells are currently poorly understood.

In a recent study, we found that *methylenetetrahydrofolate reductase* (*MTHFR*) gene promoter hypermethylation is strongly associated with semen samples from infertile couples affected

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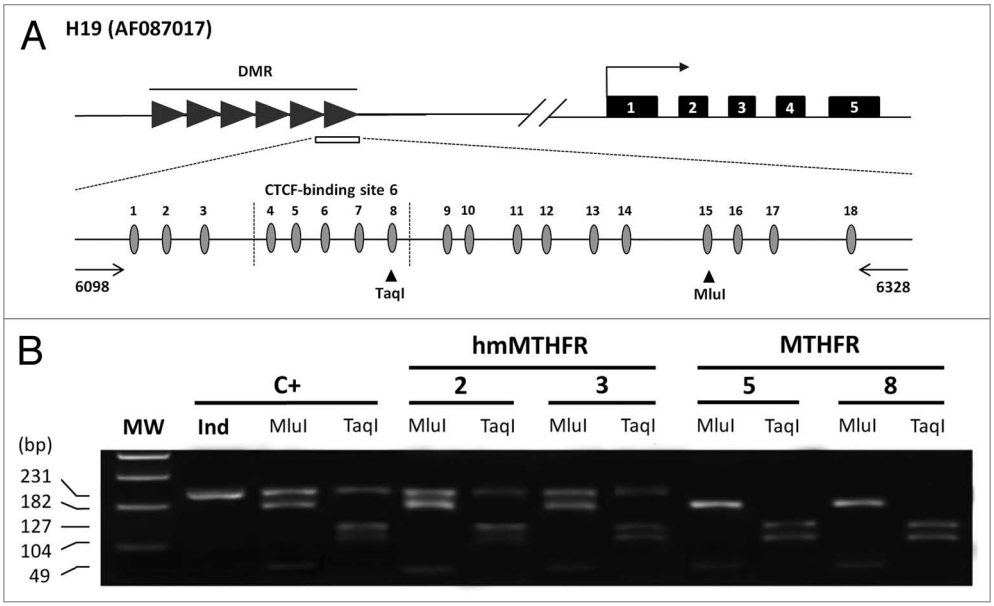

Figure 1. Methylation status of the *H19* imprinted gene in hmMTHFR and MTHFR semen DNA samples from infertile males. (**A**) Genomic structure of *H19* locus and the GenBank accession number. Upper line: filled-in boxes and horizontal arrow indicate gene exons and orientation, respectively; filled-in horizontal arrows represent *H19* DMR region and the white box represents the DMR sequence which was analyzed in this study. Lower line: DMR sequence includes 18 CpG islands (the CTCF-binding site 6 region from 4 to 8 CpG island is also shown). The horizontal arrows represent the primers. Vertical arrowheads indicate the unique bisulfite-PCR restriction enzyme sites, which were analyzed with Mlu I and Taq I. (**B**) Overall methylation status of *H19* detected by COBRA assay in sperm from hmMTHFR and MTHFR semen DNA samples and in the control leukocyte DNA. The same bisulfite-treated DNA amplified by PCR and used for cloning and sequencing, was digested with Mlu I and Taq I restriction enzymes, which were cut only if the restriction site was methylated. Cases 2 and 3 from hmMTHFR semen samples show the unmethylated band with both Mlu I and Taq I enzyme digestion. MW, Molecular weight; C+, human leukocyte DNA.

by the recurrence of spontaneous abortion (RSA) compared with semen samples from controls.[17] Since MTHFR is one of the key regulatory enzymes maintaining the bioavailability of endogenous methyl groups,[18] it was suggested that spermatogonial cells harboring *MTHFR* gene dysfunctions, due to promoter hypermethylation, resulted in a low methyl donor pool giving rise to spermatozoa affected by methylation errors at the paternal imprinted genes which, in turn, accounted for spontaneous abortion.[17]

Taking advantage of our previous results, in this study we have investigated whether methylation defects at the *H19* imprinted gene occurred in DNA semen samples from infertile males affected by *MTHFR* gene promoter hypermethylation.

Results

COBRA analysis of H19 PCR products. In order to confirm that the sequencing results from PCR templates accurately reflected the overall methylation pattern of the amplified *H19* from the sperm cells and leukocytes, a restriction analysis (COBRA) of the PCR products was performed. About half of the methylated and half of the unmethylated templates were obtained after DNA digestion with Taq I and Mlu I restriction enzymes in leukocytes in order to represent paternal and maternal alleles, indicating a lack of bias in the PCR reaction (**Fig. 1**). *H19* PCR products, from DNA sperm belonging to the MTHFR group, yielded digested bands with both Taq I and Mlu I restriction enzymes, suggesting that most of the DNA in each semen sample was methylated at this locus (**Fig. 1**). In the hmMTHFR group, 7/10 (70%) semen DNA samples produced methylated and unmethylated bands: 3/5 (60%) from hmMTHFR-nor (1 with Taq I, 1 with Mlu I, and 1 with both Taq I and Mlu I enzymes) and 4/5 (80%) from hmMTHFR-abn (2 with Taq I, and 2 with both Taq I and Mlu I enzymes) (**Fig. 1**; **Table 1**).

Methylation status of *H19* locus in hmMTHFR and *MTHFR* semen DNA samples. The methylation status of the *H19* locus was investigated by sequencing analysis of the cloned PCR products. We reasoned that spermatozoa carrying *MTHFR* gene dysfunctions contained extensive methylation defects at the *H19* locus, so that only *H19* hypomethylated clones, i.e., clones showing 50% or more than 50% unmethylated CpG islands, were taken into account in this analysis.

A total of 200 clones were investigated for the DNA methylation status of *H19* locus in sperm DNA samples from infertile males: 100 clones from the hmMTHFR group and 100 from the MTHFR group. We examined a total of 18 CpG sites in a 231 bp fragment of the *H19* gene (**Fig. 1**).

In each semen sample, clones with *H19* hypomethylation and complete methylation were analyzed (**Fig. 2**).

During the first step, the overall distribution of the different clones within the hmMTHFR group and the MTHFR group was evaluated. *H19* hypomethylation was detected in 24% of the clones from the hmMTHFR group and in 8% of the clones from the MTHFR group ($P = 0.0038$) (**Fig. 3**). Complete *H19*

Table 1. COBRA analysis of H19 PCR products

Samples	MluI digestion		TaqI digestion		Aberrant methylation of H19
	Complete	Not complete	Complete	Not complete	
MTHFR-group	10/10	-	10/10	-	none
hmMTHFR-group	-	7/10	-	7/10	7/10 (70%)
hmMTHFR-nor		-	1/5	-	
	-	1/5	-	-	3/5 (60%)
	-	1/5	-	1/5	
hmMTHFR-abn	-	2/5	-	2/5	4/5 (80%)
	-	2/5	-	-	

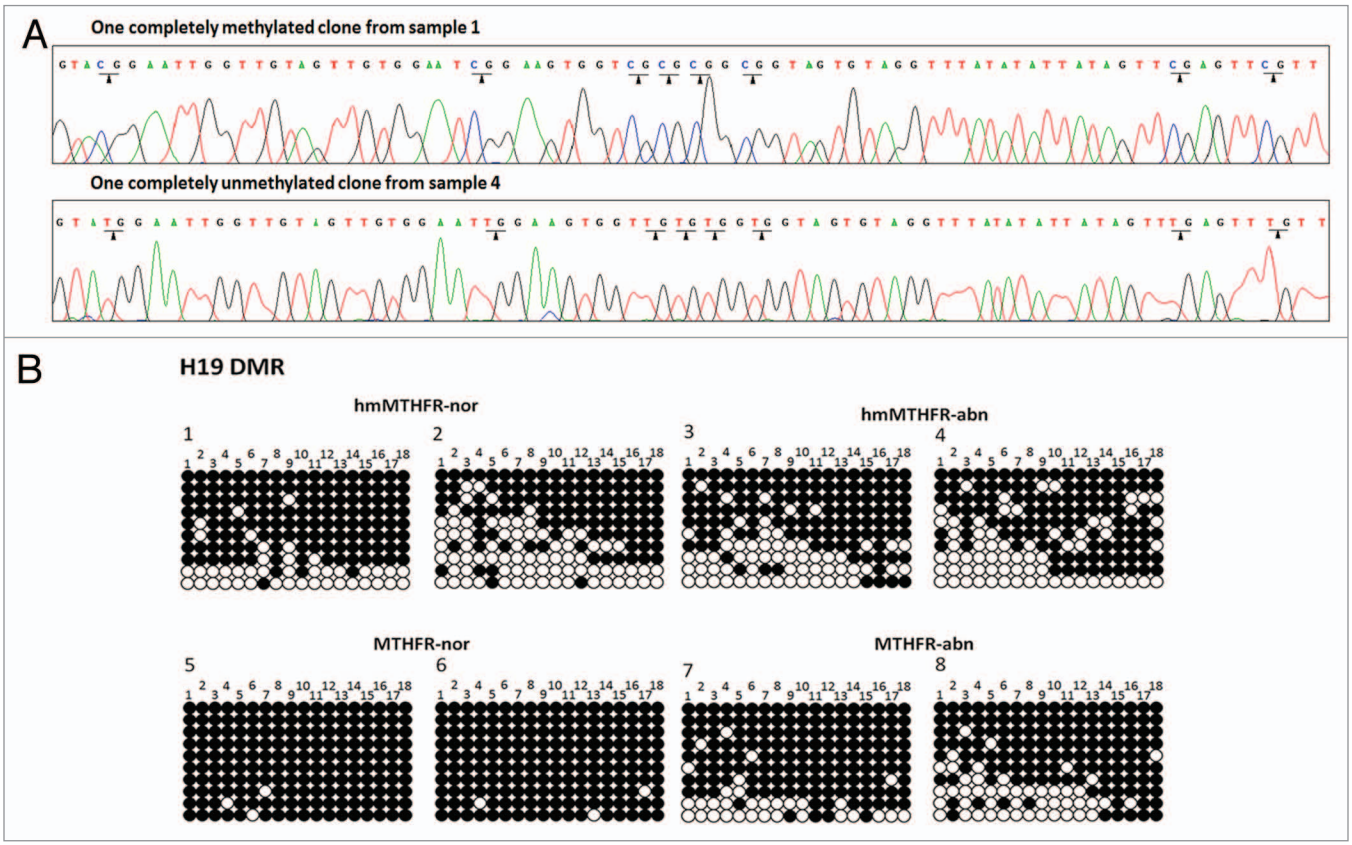

Figure 2. Sequencing analyses of clones from hmMTHFR and MTHFR semen samples. (**A**) DNA sequencing of *H19* in two different clones hmMTHFR-nor (sample 1) and hmMTHFR-abn (sample 4). Arrowheads indicate the methylated CpG islands in sample 1 and unmethylated CpG islands in sample 4. (**B**) Bisulfite-PCR sequencing for *H19* in two representative semen samples endowed with the lowest and the highest prevalence of *H19* hypomethylated clones, respectively, from hmMTHFR-nor and hmMTHFR-abn, as well as MTHFR-nor and hmMTHFR-abn semen samples. Filled-in and clear circles represent methylated and unmethylated CpG islands, respectively. The 18 CpG islands within the *H19* locus are numbered on the upper side of the circles.

methylation was found in 14% of the clones from the hmMTHFR group and in 45% of the clones in the MTHFR group and (P = 0.0001) (**Fig. 3**).

In the second step of our analysis, the methylation status of *H19* gene was investigated in hmMTHFR-nor and hmMTHFR-abn semen samples as well as MTHFR-nor and MTHFR-abn semen samples. The prevalence of *H19* hypomethylated clones was 20% in hmMTHFR-nor compared with 0% in MTHFR-nor semen samples (P = 0.0027) and was 28% in hmMTHFR-abn compared with 16% in MTHFR-abn semen samples (P = 0.2274) (**Fig. 3**). The frequency of completely methylated *H19* clones was 28% in hmMTHFR-nor compared with 72% in MTHFR-nor semen samples (P < 0.0001) and was 10% in hmMTHFR-abn compared with 28% in MTHFR-abn semen samples (P < 0.0001).

Comparative statistical analyses between hmMTHFR-nor and hmMTHFR-abn, as well as MTHFR-nor and MTHFR-abn are reported in **Figure 3**.

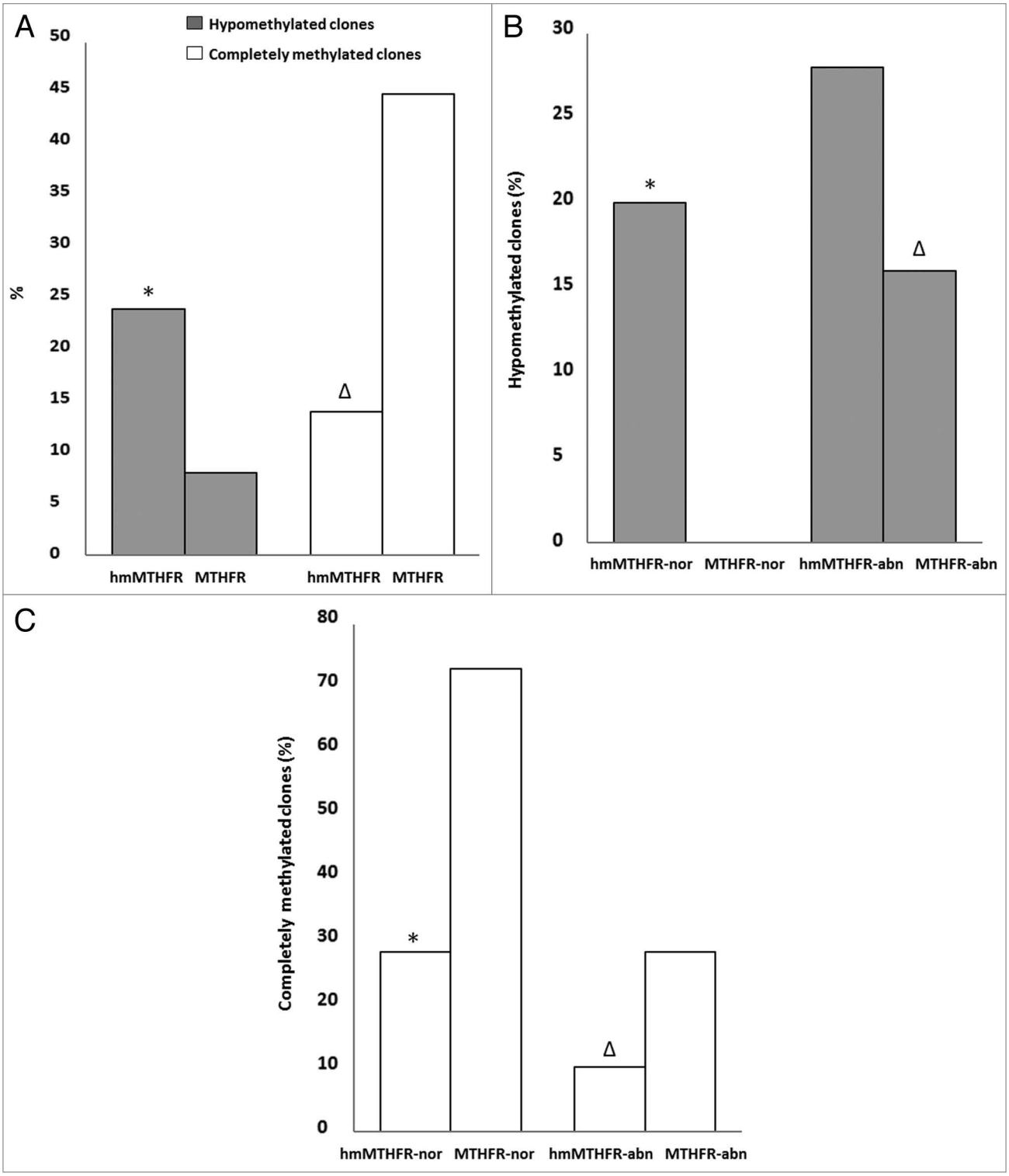

Figure 3. Frequency of *H19* hypomethylated and completely methylated clones. (**A**) Frequency of hypomethylated (gray bars) and completely methylated (white bars) clones of *H19* in hmMTHFR and MTHFR groups. * *H19* frequency significantly different from MTHFR group; $^{\Delta}$*H19* frequency significantly different from MTHFR group. (**B**) Frequency of *H19* hypomethylated clones in hmMTHFR-nor and MTHFR-nor groups and in hmMTHFR-abn and MTHFR-abn groups. **H19* frequency significantly different from MTHFR-nor group; $^{\Delta}$*H19* frequency significantly different from MTHFR-nor group; (**C**) Frequency of *H19* completely methylated clones in hmMTHFR-nor and MTHFR-nor groups and in hmMTHFR-abn and MTHFR-abn groups. **H19* frequency significantly different from MTHFR-nor and hmMTHFR-abn groups; $^{\Delta}$*H19* frequency significantly different from MTHFR-abn group.

Methylation status of CTCF-binding site 6. The CTCF-binding site 6 includes 5 CpG islands, from 4 to 8 CpG, within the *H19* locus. Hypomethylation (i.e., almost 3 unmethylated CpG islands) and complete non-methylation of the CTCF-binding site 6 region of *H19* locus was evaluated in each single clone from the hmMTHFR and MTHFR group. In the hmMTHFR group and MTHFR group, the CTCF-binding site 6 region was hypomethylated in 24% and 5% of the clones, respectively ($P = 0.0002$), whereas the CTCF-binding site 6 was completely unmethylated in 8% of the clones from the hmMTHFR group and in 3% of the clones from the MTHFR group ($P = 0.2134$).

In hmMTHFR-nor and in MTHFR-nor semen samples, the prevalence of clones with hypomethylated CTCF-binding site 6 was 20% and 0% ($P = 0.0027$), respectively, whereas the frequency of completely unmethylated clones was 2% in hmMTHFR-nor and 0% in MTHFR-nor semen samples ($P = 0.3149$). In hmMTHFR-abn and MTHFR-abn semen samples, hypomethylation occurred in 28% and in 6% of the clones, respectively ($P = 0.0078$), whereas completely unmethylated clones were found in 14% of hmMTHFR-abn and in 6% of MTHFR-abn semen samples ($P = 0.3173$) (**Fig. 4**).

Discussion

Methylation loss at the *H19* imprinted gene has been detected in semen samples from infertile males suggesting that *H19* imprinting errors represent a risk factor for male infertility.[13-16,19] The causes inducing aberrant methylation at *H19* are poorly understood.

This study shows for the first time that extensive methylation defects at the *H19* imprinted gene occur in sperm DNA of infertile males harboring hypermethylation of *MTHFR* gene promoter. The methylation status of *H19* locus was investigated in two different groups of sperm DNA samples, one with hypermethylated *MTHFR* gene promoter, the hmMTHFR group, and one without promoter methylation of the *MTHFR* gene, the MTHFR group.

The prevalence of *H19* hypomethylated clones was higher in the hmMTHFR group than in the MTHFR group ($P < 0.05$). On the contrary, the prevalence of completely methylated *H19* clones was higher in the MTHFR group than in the hmMTHFR group ($P < 0.05$). These results indicate that methylation defects at the *H19* locus frequently occur in semen samples harboring hypermethylation at the *MTHFR* gene promoter.

Previous data has indicated that *H19* methylation loss results in abnormal spermatogenesis.[13-16] Complete demethylation or a low level of methylation at the *H19* locus have been detected in semen samples with abnormal semen parameters such as oligozoospermia, astenozoospermia and teratozoospermia, suggesting that aberrant methylation of this gene may represent a risk factor for male infertility.[13-16] In this study, the hmMTHFR and *MTHFR* group included semen samples with normal and abnormal semen parameters, thus allowing the prevalence of *H19* hypomethylated clones to be determined in normal and abnormal sperm. In accordance with previous studies,[13-16] the prevalence of *H19* hypomethylated clones was higher in MTHFR-abn than in MTHFR-nor semen samples ($P < 0.05$). On the contrary, the frequency of *H19* hypomethylated clones was approximately equal in hmMTHFR-abn (28%) and in hmMTHFR-nor (20%) semen samples ($P > 0.05$). To our knowledge, this is the first study showing extensive methylation defects, i.e., hypomethylated clones, at *H19* locus in normal sperm samples from infertile males. While some previous studies have reported methylation errors at the *H19* locus in normal sperm, these errors usually involved only a few CpG sites within the *H19* gene. Furthermore, comparative analyses of the prevalence of *H19* hypomethylated clones between semen samples from the hmMTHFR group and MTHFR group showed that *H19* hypomethylated clones occurred only in hmMTHFR-nor when compared with MTHFR-nor semen samples ($P < 0.05$) and equally in hmMTHFR-abn compared with MTHFR-abn semen samples ($P > 0.05$). These results clearly underscore the association between *H19* methylation defects and the hypermethylation of the *MTHFR gene* promoter in normal semen samples, suggesting that the methylation status of *H19* gene in normal sperm could be affected by *MTHFR gene* dysregulation. Nevertheless, despite statistical analysis between hmMTHFR-abn and MTHFR-abn semen samples being non-significant, probably due to the low sample size used in this study, we cannot exclude that imprinting defects of *H19*, even in abnormal sperm, may be due to *MTHFR gene* dysfunction since prevalence of *H19* hypomethylated clones in hmMTHFR-abn was almost 2-fold greater than in MTHFR-abn semen samples. Finally, the detection of *H19* hypomethylated clones in MTHFR-abn semen samples indicates that other different mechanisms are involved in *H19* methylation defects in abnormal sperm as previously suggested.[20-25]

Infertility is a reproductive health problem that affects approximately 15% of couples. Half of these cases are due to male factors,[26] and about 30% of male infertility cases are idiopathic, since the molecular mechanisms underlying the defects remain unknown.[27] In these patients, sperm with normal semen parameters is frequently observed. Our results indicate that normal sperm harbouring dysfunctions of the *MTHFR gene*, hmMTHFR-nor, is affected by wide-ranging methylation defects at the *H19* locus. Moreover, we verified that most of these methylation defects occurred within the CTCF-binding site 6 region which was hypomethylated or completely unmethylated in 7% and 23% of the clones, respectively. As is already known, CTCF-binding site methylation allows the IGF2 maternal allele to be expressed which is required for normal pre-implantation of the embryo as well as fetal growth and survival.[28-30] Therefore, spermatozoa with abnormal methylation of CTCF binding site 6 have a high risk of causing biallelic inactivation of the IGF2 gene in the human embryo, which could lead to negative effects on embryo development and pregnancy outcome.

Consistently, hypomethylation or complete unmethylation at the CTCF-binding site 6 region has been previously reported in semen samples from normozoospermic males in infertile couples affected by RSA.[31] Interestingly, in our previous study we detected that hypermethylation of the *MTHFR gene* frequently occurred in normal semen samples from RSA males compared with controls. These data strongly support the hypothesis that one possible

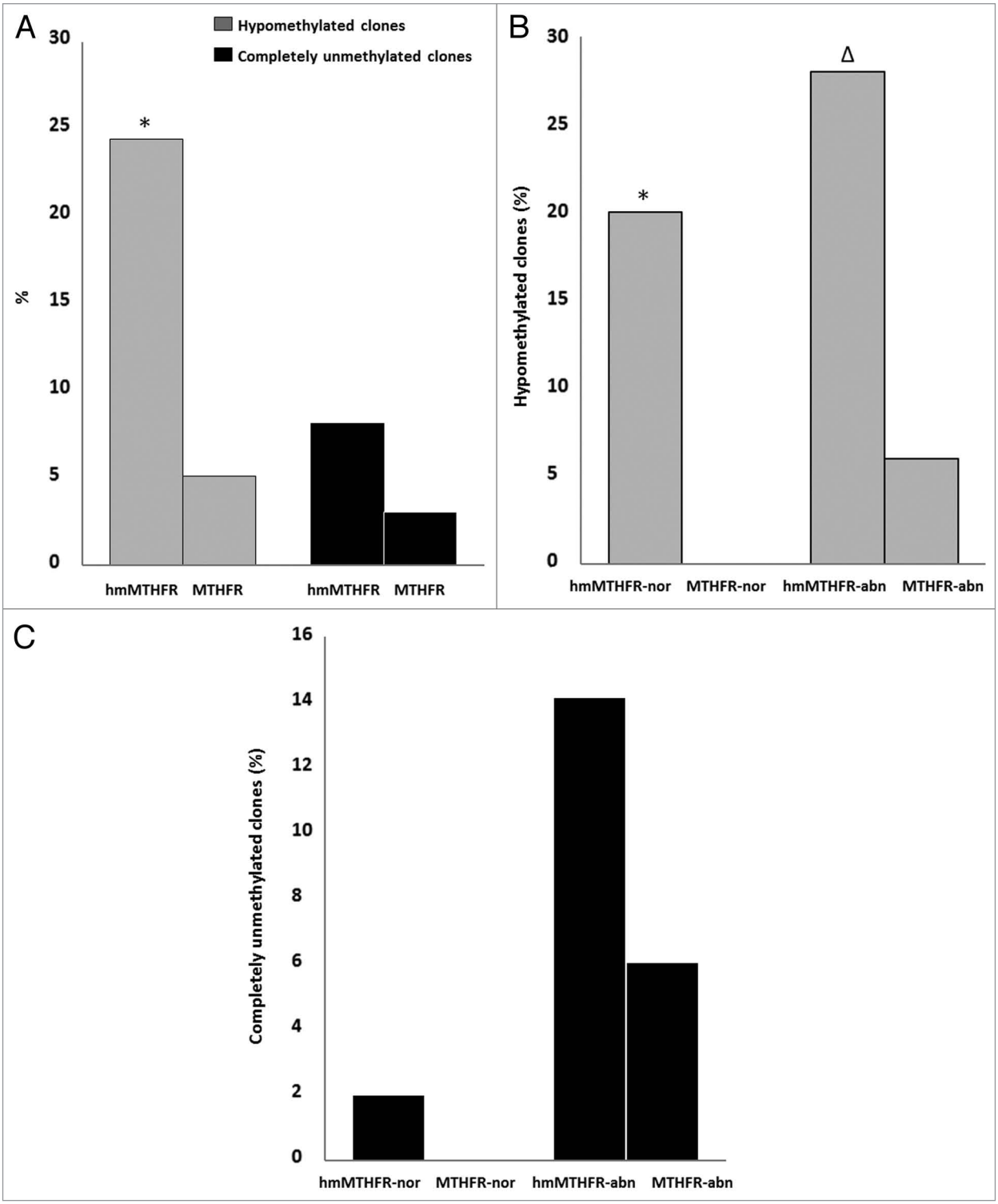

Figure 4. Frequency of clones with hypomethylation and complete unmethylation of CTCF-binding site 6. (**A**) Frequency of clones with hypomethylation (gray bars) and complete unmethylation (black bars) of CTCF binding site 6 in hmMTHFR and MTHFR groups. *The frequency of clones with hypomethylation of CTCF binding site 6 is significantly different from MTHFR group; (**B**) Frequency of clones with hypomethylation of CTCF binding site 6 in hmMTHFR-nor and MTHFR-nor groups and in hmMTHFR-abn and MTHFR-abn groups. * The frequency of clones with hypomethylation of CTCF binding site 6 is significantly different from MTHFR-nor group; $^{\Delta}$The frequency of clones with hypomethylation of CTCF binding site 6 is significantly different from MTHFR-abn group; (**C**) Frequency of clones with complete unmethylation of CTCF binding site 6 in hmMTHFR-nor and MTHFR-nor groups and in hmMTHFR-abn and MTHFR-abn groups.

mechanism involved in some cases of idiopathic male infertility is the lack of *MTHFR gene* activity leading to unmethylation of the CTCF-binding site which in turn affects the fertilizing potential of normal sperm. In addition, low levels of methyl donor pools in spermatogenetic cells may also compromise the methylation status of some other paternal imprinted genes. In this context, we speculate that spermatozoa with imprinting errors could be selectively discarded and may not be able to fertilize oocytes or give rise to embryos that would fail to develop normally, as previously suggested by studies performed in mice using azacytidine to induce hypomethylation in sperm.[32]

In conclusion, this study has shown that extensive methylation defects at the *H19* imprinted gene occur in semen samples from infertile males harboring hypermethylation of the *MTHFR* gene. This association was strongly evident in semen samples with normal semen parameters suggesting that the methylation status of the *H19* gene in normal sperm may be affected by dysfunctions of the *MTHFR* gene. These findings provide new insights into the mechanisms causing abnormal methylation at imprinted genes and, in turn, in some cases of idiopathic male infertility.

Material and Methods

Semen samples. The 20 semen samples used in this study had been collected from males (men aged 36.1 ± 1.2) who were in couples reporting fertility problems. DNA from these 20 semen samples had been previously analyzed for the methylation status of *MTHFR* gene promoter:[17] 10 semen DNA samples had *MTHFR* promoter hypermethylation (i.e., nearly 100% of CpG islands were methylated within the promoter region), hereafter indicated as hmMTHFR group, and 10 semen DNA samples had *MTHFR* promoter region unmethylated hereafter indicated as MTHFR group. In both the hmMTHFR and MTHFR group, 5 semen samples were with normal semen parameters (≥15 × 10^6 sperm/ml; ≥32% sperm rapid progressive motility and ≥4% normal sperm morphology), hmMTHFR-nor and MTHFR-nor, and 5 semen samples were with abnormal semen parameters, hmMTHFR-abn (1 oligoastenozoospermic, 1 teratozoospermic, 2 astenozoospermic and 1 oligozoospermic), and MTHFR-abn (1 oligoteratozoospermic, 1 teratozoospermic, 1 astenozoospermic and 2 oligozoospermic).

Bisulfite treated DNA PCR of H19 locus. The methylation assay was performed at the DMR of the *H19* imprinted gene. 150 ng of sodium bisulfite-treated sperm DNA was amplified at the *H19* locus by hemi-nested Methylation-Specific PCR using the primers F6005, 5'-AGGTGTTTTA GTTTTATGGA TGATGG-3', R6326, 5'-TCCTATAAAT ATCCTATTCC CAAATAACC-3' and F6115, 5'-TGTATAGTAT ATGGGTATTT TTGGAGGTTT-3', which amplify a sequence of 231 bp (GenBank accession number AF087017, position 6098–6328) containing 18 CpG islands (**Fig. 1**). The PCR program was: 10 min of denaturation at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C and a final extension for 5 min at 72 °C.[10,15]

The *H19* amplified sequence contains the CTCF-binding site 6 region, which includes 5 CpG islands, from 4 to 8 CpG island (**Fig. 1**).

Combine Bisulfite Restriction Analysis (COBRA). To confirm that sequencing results did not reflect a cloning bias, bisulfite-treated DNA PCR samples from sperm cells and human leukocytes (positive control of the reaction) were subjected to restriction analysis (COBRA) with the Taq I and Mlu I enzymes.[13,19] These enzymes can only cleave to methylated DNA sequences so that the undigested and digested products indicated unmethylated and methylated templates, respectively. Digestion products were visualized by DNA electrophoresis on a 2.5% agarose gel.

DNA cloning and sequencing. Amplified products were purified with the QIAquick PCR Purification Kit (Qiagen) and then cloned with the TOPO TA cloning kit (Invitrogen), using the Turbo Competent *E. coli* bacteria strain (EuroClone) and the pCR 2.1-TOPO vector (Invitrogen), according to manufacturer's instructions. Selection of bacterial clones containing the fragment of interest was performed using selective LB growth medium with ampicillin (100 μg/ml, Sigma-Aldrich). For each DNA sample, 10 positive clones were selected for sequencing analysis. Single clones were sequenced using automated ABI Prism Genetic (Analyzer Applied Biosystems).

Statistical analysis. Chi-square trend test was performed with Yates' correction to analyze the association between methylation patterns of *MTHFR* and *H19*. *P* values < 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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