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GENETICS

Lack of association of *MTHFR* rs1801133 polymorphism and *CTCFL* mutations with sperm methylation errors in infertile patients

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Abstract
Purpose To find out whether the *MTHFR* rs1801133 polymorphism is a risk factor for male infertility in the Spanish population. To determine if a pattern of sperm DNA hypomethylation at the paternally imprinted loci *H19*-ICR and/or IG-DMR is related to the *MTHFR* rs1801133 polymorphism and/or *CTCFL* mutations.
Methods One hundred and seven samples from individuals who sought consultation for fertility problems and twenty-five semen samples from sperm donors were analyzed. The *MTHFR* rs1801133 SNP was analyzed in all samples by the PCR-RFLP method. We compared the distribution of the genotypes between control and infertile populations and among the groups of patients with altered seminal parameters. In those patients with the most severe hypomethylation pattern (n=12) we also analyzed the *CTCFL* protein-coding exons by sequencing.
Results There were no significant differences in the distribution of the genotypes among the control and infertile populations. Moreover, none of the genotypes were associated, neither to the characteristics of the seminogram, nor to the presence of sperm DNA hypomethylation. We did not identify frameshift, nonsense or missense mutations of the *CTCFL* gene.
Conclusions The *MTHFR* rs1801133 polymorphism is not associated with male infertility in the Spanish population. Neither the *MTHFR* polymorphism, nor *CTCFL* mutations explain a pattern of sperm hypomethylation at paternally imprinting loci.

Keywords *MTHFR* polymorphism · CTCFL · DNA methylation · Male infertility · Imprinting errors

Capsule The *MTHFR* rs1801133 polymorphism is not associated with male infertility in the Spanish population. Neither the *MTHFR* polymorphism, nor *CTCFL* mutations explain a pattern of sperm hypomethylation at paternally imprinting loci.

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Introduction

In previous years, several reports have described an association between male infertility and methylation abnormalities at imprinted loci in spermatozoa [4, 8, 12, 14, 15, 17–20, 23]. Imprinting is a specific epigenetic mark consisting of parental-specific DNA methylation at CpG dinucleotides and histone post-translational modifications. This mechanism controls the expression of a gene on the basis of its parental origin resulting in monoallelic expression. In order to achieve a specific gender epigenetic mark, imprinting is erased in primordial germ cells and established de novo during gametogenesis in a sex-specific manner. There are several histone post-translational modifications leading to a

specific expression profile. One of these is methylation at lysines or arginines catalyzed by histone methyltransferases (HMT). On the other hand, DNA methyltransferases (DNMT) are the enzymes that add the methyl groups to CpG dinucleotides. DNMT3A and DNMT3B are those implicated in de novo DNA methylation, and DNMT3L is a co-factor specifically expressed in the germline [2].

In the testis, the specific factor CCCTC-binding factor-like protein (CTCFL/BORIS) cooperates with Protein Arginine Methyltransferase 7 (PRMT7) in establishing symmetrical dimethyl modification of arginine residues in histones H2A and H4. This action happens close to the *H19*-Imprinting Control Region (*H19*-ICR) leading to the DNA methylation of this ICR [13]. Thus, CTCFL-deficient activity could cause imprinting errors at *H19/IGF2* as well as in other paternally methylated ICR, such as the one that controls *MEG3/DLK1*.

Methyl groups needed by methyltransferases are provided by S-adenosyl-L-methionine (SAM) through the folate pathway. The availability of 5,10-methylenetetrahydrofolate (5,10-methylene-THF) influences the pool of methyl groups and it is derived from folic acid. The 5,10-methylenetetrahydrofolate reductase (MTHFR) is a key regulatory enzyme that plays an important role in the metabolic cycle of methionine, since it catalyzes the reduction of 5,10-methylene-THF to 5-methylenetetrahydrofolate (5-methyl-THF). The 5-methylene-THF is the methyl donor for homocysteine (Hcy) methylation to methionine by the methionine synthase. Methionine is the precursor of SAM, which is the direct methyl donor for the methylation of histones and DNA by HMT and DNMT. The cycle is completed by the conversion of the product resulting from the methyl donation of SAM, the S-adenosylhomocysteine (SAH) to Hcy (Fig. 1).

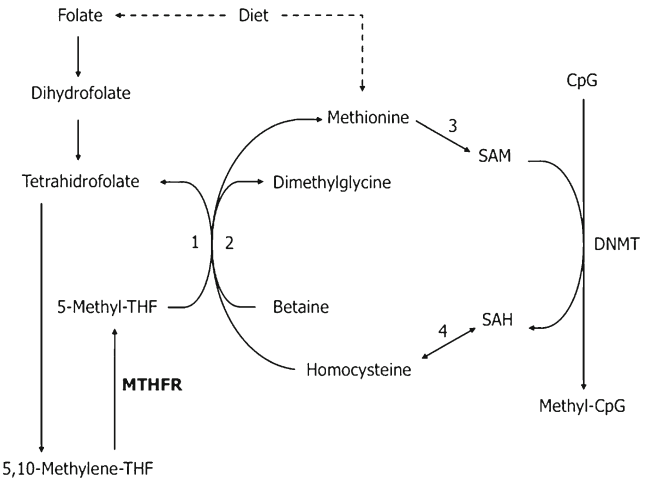

Fig. 1 Folate and methionine pathway. *DNMT* DNA methyltransferase; *MTHFR* 5,10-methylenetetrahydrofolate reductase; *1* Methionine-synthase (vitamin B12 and zinc dependent); *2* Betaine-homocysteine-methyltransferase (zinc dependent); *3* Methionine-adenosyltransferase; *4* S-adenosylhomocysteine hydrolase. Adapted from Ebisch et al. [7]

In humans, only two studies have tried to relate mutations in genes involved in DNA methylation to the presence of imprinting errors in spermatozoa. Kobayashi et al. [14] reported *DNMT3A* (GeneID: 1788) and *DNMT3L* (GeneID: 29947) variations in some infertile patients with sperm DNA methylation anomalies at specific imprinted loci. In relation to the *CTCFL* gene (GeneID: 140690), Poplinski et al. [23], they did not identify mutations in somatic DNA from infertile patients that showed sperm DNA hypomethylation at *IGF2*/*H19*-ICR or severe *MEST* hypermethylation.

On the other hand, the common single nucleotide polymorphism (SNP) rs1801133 of the *MTHFR* gene (GeneID: 4524), which corresponds to a C > T transition causing an alanine-to-valine substitution at position 222 (p.A222V), compromises the enzyme activity [9], and it has been described as associated with male infertility [1, 3, 10, 16, 21, 22, 25, 26]. Nevertheless, to our best knowledge, sperm DNA methylation analysis of infertile men together with rs1801133 genotyping has not yet been investigated.

In this report, we have explored the possibility that DNA hypomethylation at the paternally imprinted ICR's *H19*-ICR and IG-DMR is the consequence of deficient MTHFR activity because of the presence of the rs1801133 polymorphism or due to mutations in the *CTCFL* gene. We have also compared the frequency of *MTHFR* rs1801133 genotypes between the control and the infertile populations. Finally, we have analyzed if there is any relationship between the distribution of the genotypes and specific alterations of the seminal parameters.

Material and methods

Sperm samples

Twenty-five semen samples from sperm donors were selected as a control population. All controls met the following characteristics: 1) Normal karyotype, 2) Proven fertility, 3) More than 90×10^6 of total motile progressive sperm in the raw ejaculates, 4) More than 14 % of normal forms (strict criteria), and 5) More than 10×10^6 of progressive forms per ml after a post-thawing survival test. The average age of the control group was 26±6.15 years of age (range: 19–45).

One hundred and seven samples from individuals who sought consultation for fertility problems were analysed (Supplemental Table 1): 15 normozoospermic (N), 1 oligozoospermic (O), 8 asthenozoospermic (A), 30 teratozoospermic (T), 1 oligoastenozoospermic (OA), 5 oligoteratozoospermic (OT), 31 asthenoteratozoospermic (AT) and 16 oligoasthenoteratozoospermic (OAT). The average age of the infertile individuals was 36±5.50 years of age (range: 26–53). Seminal classification was carried out following the criteria of the World Health Organisation [29].

Table 1 Number and percentage of infertile patients with hypomethylation or normal methylation at *H19*-ICR and IG-DMR [5]

Region	Hypomethylation	Normal methylation
H19-ICR	12/107 (11.21 %)	95/107 (88.78 %)
IG-DMR	40/107 (37.38 %)	67/107 (62.61 %)

Donors and patients gave their informed consent to participate in the study, which was approved by our Institutional Ethics Committee.

The spermatozoa were separated from the rest of the cells of the ejaculate by the direct swim-up technique [27]. The extraction of genomic DNA from the spermatozoa was performed with the commercial extraction kit PUREGEN (Gentra Systems; Minneapolis, MN, USA).

In a previous report by our group, the sperm DNA samples from the infertility patients (n=107) were classified according to the presence of normal methylation or hypomethylation at the paternally imprinted ICR *H19*-ICR and IG-DMR [5]. Among the group of 12 cases with hypomethylation at *H19*-ICR and the group of 40 cases with hypomethylation at IG-DMR (Table 1), we have selected those samples with the most severe pattern of hypomethylation (Tables 2 and 3).

MTHFR rs1801133 analysis

The *MTHFR* rs1801133 SNP was analyzed in all samples (25 from sperm donors and 107 from infertile patients) by the PCR-RFLP method using primers and conditions described by Frosst et al. [9]. Briefly, the C > T transition creates a HinfI recognition sequence. The digested PCR product yields two fragments of 175 and 23 bp for the T allele whereas the wild C allele remains undigested and yields a single 198-bp amplicon.

To evaluate whether *MTHFR* rs1801133 SNP genotypes CC, CT and/or TT showed different frequencies between the control (n=25) and the infertile (n=107) populations, a contingency analysis was performed. This analysis was carried out considering whether the control and infertile individuals presented the genotype: CC, non-CC; CT, non-CT and TT, non-TT.

A second contingency analysis was performed to determine whether some rs1801133 SNP genotypes are preferably associated with normozoospermia, oligozoospermia, asthenozoospermia or teratozoospermia in the infertile population (n=107). For this purpose, the analysis was done considering whether the infertile individuals presented the variables of: normozoospermia, non-normozoospermia; oligozoospermia, non-oligozoospermia; asthenozoospermia, non-asthenozoospermia; teratozoospermia, non-teratozoospermia. The finality of this separation allowed us to determine if a specific component of the spermiogram was preferentially linked to any genotype.

We also checked in the infertile population (n=107) a possible relationship between the presence of hypomethylation at imprinted loci and rs1801133 SNP

Table 3 Percentage of methylation of each analyzed CpG at the IG-DMR locus in the eight infertile patients showing the most severe pattern of hypomethylation

Case	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5
6	91	93	***57***	***79***	***63***
18	94	93	***56***	*77*	***60***
37	85	**82**	**48**	**62**	**55**
39	93	90	***58***	***78***	***64***
59	94	93	***57***	***80***	***63***
79	90	93	***56***	*77*	***61***
104	92	93	***56***	***79***	***64***
Range of reference	97–83	100–83	69–55	89–76	72–59

Hypomethylated CpGs are indicated in bold. CpGs with methylation values close to the lowest threshold of the normal referred range are indicated in bold/italics

Table 2 Percentage of methylation of each analyzed CpG at the *H19*-ICR locus in the six infertile patients showing the most severe pattern of hypomethylation

Case	CpG 1	CpG 2	CpG 3	CpG 4	CpG 6	CpG 7	CpG 8	CpG 9	CpG 10	CpG 11	CpG 12	CpG 13	CpG 14
37	**84**	**84**	**78**	67	**77**	85	**82**	**83**	**74**	**82**	79	**89**	**80**
50	**83**	**84**	**74**	71	**73**	**81**	**80**	**83**	**74**	**76**	75	**85**	**83**
80	**86**	92	85	**56**	**82**	88	**79**	**81**	83	84	**73**	**84**	88
91	**83**	88	**81**	70	**74**	90	87	**84**	**74**	**81**	**73**	**88**	**80**
95	**9**	**8**	**11**	**10**	**10**	**8**	**7**	**8**	**7**	**11**	**10**	**10**	**12**
102	**70**	**73**	**68**	64	**66**	**74**	**71**	**75**	**67**	**74**	**70**	**78**	**76**
Reference range	86–96	87–97	84–93	61–88	82–88	83–100	85–97	85–97	74–92	83–92	73–95	89–99	84–96

Hypomethylated CpGs are indicated in *bold*. All patients showed hypomethylation affecting more than 50 % of the CpGs

genotypes. Again, a contingency table was performed contrasting the presence of a normal or hypomethylation pattern at *H19*-ICR and IG-DMR with every single genotype.

Data of the three contingency tables were analyzed using a Chi-squared test. The level of statistical significance was established as 0.05. The statistical analysis was performed with the specialized support of Servei d'Estadística Aplicada (Applied Statistics Service) of the Universitat Autònoma de Barcelona.

CTCFL sequence analysis

According to GenBank accession number DQ778111, the protein-coding exons of the *CTCFL* gene (exons 3 to 13) were analyzed in sperm DNA samples. This analysis was done exclusively in 12 infertile individuals selected for showing the most severe pattern of hypomethylation in the paternal germline/primary differentially methylated regions; 50 % of the analyzed CpGs hypomethylated or with

Table 4 Primers and amplification conditions used for *CTCFL* sequence analysis. Exon numbering according to the GenBank accession number DQ778111

EXON	Primer	Sequence (5′–3′)	Amplicon length	Annealing
E3	CTCFL E3F	ggccagaccttgtttcaactcc	717 bp	64 °C
	CTCFL E3R	gcaactgcaaaatacctttgtcc		
	nCTCFL E3F[a]	ctcataccaccccttctcc	676 bp	58 °C
	nCTCFL E3R[a]	ttgcataaaagccgacttga		
E4	CTCFL E4F[a]	gcttcatccaggcaaaagtc	355 bp	64 °C
	CTCFL E4R[a]	accatcacctgcccataaag		
	nCTCFL E4F	caggcaaaagtctaattatac	338 bp	58 °C
	nCTCFL E4R	tgcccataaagaaaaacagca		
E5	CTCFL E5F[a]	ttgaaaggacgatgtgctga	396 bp	58 °C
	CTCFL E5R[a]	tggaaattcaagaaaaagaagaca		
	nCTCFL E5F	gatttactttcaagtattta	318 bp	58 °C
	nCTCFL E5R	gaaacaggtggattcacatt		
E6	CTCFL E6F[a]	gaattccttggaatgtttctgg	303 bp	58 °C
	CTCFL E6R[a]	aaaaagcctgtttgtaacagattc		
	nCTCFL E6F	ggttaagtcttgttttgaaa	266 bp	58 °C
	nCTCFL E6R	cagattctactgttagttac		
E7	CTCFL E7F[a]	aatttaaatctcttttacaatgctga	252 bp	58 °C
	CTCFL E7R[a]	aacactgtaggtatcaggccttc		
	nCTCFL E7F	ttaatggaagactctgccat	217 bp	58 °C
	nCTCFL E7R	ggtatcaggccttcagcacc		
E8	CTCFL E8F[a]	ggtgagaaggggttgataa	332 bp	58 °C
	CTCFL E8R[a]	ataggaccacgctccaaaga		
	nCTCFL E8F	aacactttcagcacaggtg	277 bp	58 °C
	nCTCFL E8R	ctccaaagagccagcaaatg		
E9	CTCFL E9F[a]	gaaaccccggtttagaggag	300 bp	58 °C
	CTCFL E9R[a]	cagccctccattcttccata		
	nCTCFL E9F	ggagaggggaagcaggctaa	267 bp	58 °C
	nCTCFL E9R	atacaccactgcctctccaa		
E10	CTCFL E10F	gccttgttcagaatgtgttt	463 bp	58 °C
	CTCFL E10R	aggcatgacagatgctcctgaag		
	nCTCFL E10F[a]	ttggggagggaaataaaagg	323 bp	58 °C
	nCTCFL E10R[a]	gatctttccatgggggattt		
E11	CTCFL E11F[a]	attgccctcgaaagaactca	397 bp	64 °C
	CTCFL E11R[a]	caaatagggctctggacac		
	nCTCFL E11F	tttctttccatgcgtggggtcc	356 bp	58 °C
	nCTCFL E11R	ctggacacatccctggaca		
E12	CTCFL E12F[a]	tgcacagtttataaattccaattcc	492 bp	58 °C
	CTCFL E12R[a]	acctgcaatgtttctttgaaat		
	nCTCFL E12F	ttagctttctgataaatttgct	439 bp	58 °C
	nCTCFL E12R	ctacaaacaggagaacaattcta		
E13	CTCFL E13F	gagttctggagatcagtcatgg	351 bp	58 °C
	CTCFL E13R[b]	GGTCGTTCAGAGGAGTGTGG[b]		
	nCTCFL E13F	tatgttgtgttagtcctttct	235 bp	58 °C
	nCTCFL E13R[b]	TGTGGCGCCTCCCCCTCTCT[b]		

[a]Primers described by Poplinski et al. [23]

[b]3′UTR primers

n nested primers

methylation values close to the lowest threshold of the normal referred range [5], (*H19*-ICR n=6, IG-DMR n=7).

Due to limitations in sperm DNA availability, the analysis was performed by nested PCR using 15 pg of the sperm isolated DNA in a total volume of 25 μl for the first round, and 4 ul of the first purified PCR reaction was used to perform the second round also in 25 μl. The intronic primers and conditions are described in Table 4. First and second round PCR purification was done using ExoSAP-IT™ (Affymetrix Inc/USB products, High Wycombe, United Kingdom) and the final products were sequenced with the forward and reverse primer using the BigDye™ Terminator Sequencing Kit v3.1 (Applied Biosystems, Foster City, USA) on an ABIPRISM 3100.

Results

MTHFR rs1801133 analysis

In the control group (n=25), 8 individuals (32 %) were homozygous CC, 15 (60 %) were heterozygous CT and 2 (8 %) were homozygous TT. In the infertile population (n=107), 47 subjects (44 %) were homozygous CC, 43 (40 %) were heterozygous CT and 17 (15 %) were homozygous TT. The contingency analysis results showed no significant differences (p>0.05) in the distribution of the three genotypes among the control and the infertile populations (Table 5).

Likewise, none of the three rs1801133 genotypes were preferentially associated (p>0.05), neither to the characteristics of the seminogram (Table 6) nor to the presence of an abnormal methylation pattern in paternally methylated *H19*-ICR or IG-DMR (Table 7).

CTCFL sequence analysis

We did not identify nonsense or missense mutations in any case. In addition to several referred single nucleotide polymorphisms, two novel synonymous sequence variations not listed in the dbSNP database were identified. One was present in homozygosis in one patient (Case 18) with IG-DMR slight hypomethylation (c.288G > A;pLeu69Leu), the other one was present in heterozygosis in a patient (Case 102) with *H19*-ICR hypomethylation (c.1308G > A;pGln409Gln).

Table 5 *MTHFR* rs1801133 results in control and infertile populations

	CC	CT	TT	Total
Control	8	15	2	25
Infertile	47	43	17	107
Total	55	58	19	132

Table 6 *MTHFR* rs1801133 results in the infertile population classified according to the presence or absence of normozoospermia, asthenozoospermia, oligozoospermia and teratozoospermia

	CC	CT	TT	Total
Normozoospermia	5	7	3	15
Non-Normozoospermia	42	36	14	92
Asthenozoospermia	21	26	9	56
Non-Asthenozoospermia	26	17	8	51
Oligozoospermia	12	9	2	23
Non-Oligozoospermia	35	34	15	84
Teratozoospermia	40	29	13	82
Non-Teratozoospermia	7	14	4	25

Discussion

The results of *MTHFR* rs1801133 SNP genotyping showed no significant association of the polymorphism neither to the condition of infertility nor to the presence of a hypomethylation pattern at paternally imprinted loci. These results point out that this polymorphism is not a risk factor for male infertility in the Spanish population analyzed. Moreover, the presence of the T allele is not the cause of the lower levels of sperm DNA methylation at the loci that showed hypomethylation. Importantly, even the distribution of the genotypes among the 12 patients with a more marked hypomethylation pattern fits to the values observed in the control population (8 homozygous CC and 4 heterozygous CT).

The association between male infertility and the T allele has mainly been described in Asian population studies [1, 16, 22, 26]. This association has also been described in a population from Brazil [10] and Iran [25]. In European studies, the male infertility-*MTHFR* SNP association is less conclusive. Bezold et al. [3] clearly found this association comparing a large series of infertile and control patients. Paracchini et al. [21] reported a prospective study on men seeking care at the infertility clinic in Milano (Italy), concluding that those subjects carrying the TT genotype were at increased risk of being infertile after the 1-year follow-up. Nevertheless, the rest of the reports from European groups [7, 24, 28] and one from an Indian group [6] did not find any association between male infertility and the *MTHFR* SNP.

Table 7 *MTHFR* rs1801133 results in the infertile population according to the methylation pattern at *H19*-ICR and IG-DMR

		CC	CT	TT	Total
H19-ICR	Normal Methylation	41	38	16	95
	Hypomethylation	6	5	1	12
IG-DMR	Normal Methylation	34	23	10	67
	Hypomethylation	13	20	7	40

Two recent meta-analysis support the results obtained in our infertile population [11, 30]. First, because both studies conclude that the T allele is a risk factor for infertility particularly related to azoospermia; none of infertile patients analyzed in our series were azoospermic. Second, the article from Wu et al. [30] supports that the rs1801133 polymorphism is capable of causing male infertility susceptibility only in Asians, but not in Caucasians infertile patients.

What is still a matter of debate is the origin of this difference according to the geographical localization of the populations. Results suggest that the presence of the T allele affects the reproductive phenotype according to the nutritional characteristics of the population. Thus, in populations without nutritional deficiencies in vegetables, green leafy vegetables, grains and nuts, which are rich in folate, the polymorphism does not represent a risk factor for DNA methylation nor for fertility. However, the effects of the SNP would be detectable in populations where folate nutritional contributions are deficient. Nevertheless, it has to be considered that the presence of other *MTHFR* SNPs together with rs1801133, as well as in other enzymes of the folate pathway, could increase the deleterious effect on infertility and methylation by methyltransferases [7, 21]. That is, the relation between genotype and diet, and the interactions among polymorphisms, has been shown to affect the impact on fertility.

Concerning the *CTCFL* gene sequence analysis, we were unable to relate the pattern of hypomethylation with the presence of *CTCFL* mutations. The imprinting mark is a very complex mechanism that regulates gene expression through the participation of a huge number of molecules in a highly coordinated way. Some of the most important genes involved in this mechanism are the following: *DNMT1, DNMT3A, DNMT3B, DNMT3L, HAT, HDAC, HMT, HDM, HP1, CTCFL, Zfp57, CTCF,* and *PRMT7*.

Like us, other authors have tried to identify the origin of sperm DNA imprinting errors through the analysis of single genes using PCR-based techniques. Overall, the results have not been encouraging enough; Poplinski et al. [23] did not identify any mutation in *CTCFL* while Kobayashi et al. [14] reported the *DNMT3A* and *DNMT3L* variations. However, the imprinting abnormalities were not present in all of the regions analyzed and were related to hypo-, hypermethylation errors and also found in cases with normal sperm methylation patterns. Moreover, the most stand out sequence variations in *DNMT3L* present in homozygosis in patients with hypomethylation at paternally imprinted loci (*H19*-ICR and/or IG-DMR) correspond to a referred SNP (rs75396112) and to a synonymous sequence variation (c.788C > T; pIle98Ile; GenBank accession number AF194032). Accordingly, we do believe that the complexity of the process requires the analysis of the Exome through the application of next-generation sequencing techniques. Unfortunately, we were unable to perform this kind of large-scale analysis in our population due to limitations in the DNA availability.

In conclusion, the *MTHFR* rs1801133 polymorphism is not a risk factor for male infertility in the Spanish population analyzed. Concerning the analysis of the *MTHFR* rs1801133 polymorphism and *CTCFL* mutations as the cause of sperm DNA hypomethylation, the lack of association in patients with the most severe pattern of hypomethylation suggest the implication of other factors.

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