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ORIGINAL ARTICLE

Aberrant methylation of the TDMR of the *GTF2A1L* promoter does not affect fertilisation rates *via* TESE in patients with hypospermatogenesis

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Increasing evidence shows a relationship between epigenetic regulation and male infertility. The *GTF2A1L* gene promoter contains the DNA methylation site of a tissue-specific differentially methylated region (TDMR). Eighty-six patients with non-obstructive azoospermia were assessed for the DNA methylation state of CpG islands in the *GTF2A1L* promoter using testicular genomic DNA. Based on histological criteria, 26 of the 86 patients had normal spermatogenesis (controls), 17 had hypospermatogenesis and 26 had a Sertoli cell-only phenotype or tubular sclerosis. *GTF2A1L* TDMR methylation was significantly lower in testes DNA from control samples than from hypospermatogenic samples ($P=0.029$). Patients with hypospermatogenesis were divided into two subgroups: high DNA methylation (HM, $n=5$) and low DNA methylation (LM, $n=12$). The *GTF2A1L* TDMR methylation rate differed significantly between the HM and LM groups ($P=0.0019$), and *GTF2A1L* expression was significantly higher among the LM than in the HM patients ($P=0.023$). High TDMR methylation was correlated with low GTF2A1L gene expression levels. Both groups demonstrated relatively good outcomes with respect to sperm retrieval, fertilisation, pregnancy and childbirth rates. We observed that aberrant *GTF2A1L* gene expression was not correlated with fertilisation rates. The testicular sperm extraction (TESE) technique may be used to overcome male infertility due to aberrant TDMR methylation.
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INTRODUCTION

Male factors of total infertility may account for up to 40%–50% of infertile couples,[1] and many cases of male infertility are unreported. Testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) are commonly used techniques to overcome male infertility, and sperm retrieval is successful in 30%–70% of patients with non-obstructive azoospermia.[2] In hypoplasia, 97.7% of patients had late spermatids or sperm recovered using the assisted reproductive technique (ART).[3] In healthy males, infertility may result from a number of unknown factors including genetic or epigenetic disorders.

Epigenetic modifications, in particular, result in stable or semi-stable changes in gene expression without affecting the DNA nucleotide sequence.[4,5] DNA methylation plays a role in gene regulation during development and has also been involved in genomic imprinting and X-inactivation.

CpG dinucleotides, also known as CpG islands, are located in the promoter regions of many genes and have variable DNA methylation states. CpG islands are generally not methylated during normal cell development, with the exception of imprinted genes. These islands are preferentially located at the start of genes and on the differentially methylated regions of imprinted genes.[6]

However, tissue-specific differentially methylated regions (TDMRs) are in a somewhat different category. These regions can be identified by DNA methylation array methods and are demethylated in some tissues but fully methylated in others.[7,8] Therefore, the DNA methylation of TDMRs in gene loci causes transcriptional repression,[9–11] is interspersed throughout the entire genome and ensures that the DNA methylation status corresponds to specific tissue types.

Methylation analysis performed on testicular DNA has revealed numerous differentially methylated loci in the male germline.[12,13] Therefore, some genes with demethylated TDMRs are expressed in a tissue-specific manner.[14,15] Numerous CpG islands that contain TDMRs are potential methylation sites in normal cells and tissues. The expression of genes associated with identified TDMRs has been shown to correlate with methylation status; thus, aberrant methylation of TDMRs may adversely affect clinical manifestations.

GTF2A1L (NM_006872) encodes the Homo Sapiens general transcription factor IIA, 1-like factor formerly known as *ALF*. The synthesis of eukaryotic mRNA requires the assembly and stability of RNA polymerase II and various general transcription factors. The *GTF2A1L* gene encodes a germ cell-specific counterpart of the large (alpha/beta) subunit of the general transcription factor TFIIA, which is able to stabilize the binding of TATA-binding protein to DNA and may be uniquely important to testis biology.[16–18] The *GTF2A1L* gene is selectively transcribed in reproductive tissues and is coexpressed in late pachytene spermatocytes and in haploid round spermatids with the

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TATA-binding protein-related factor 2, and these proteins form stable complexes in testis extracts.[19]

Aberrant DNA methylation at TDMRs has raised concerns about the status of male fertility. In this study, we have confirmed the TDMR of the *GTF2A1L* promoter by applying matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) to human testicular tissue. We also describe the TDMR methylation patterns of *GTF2A1L* CpG islands, characterize the methylation status of TDMR in some cases of male infertility and discuss the associated ART outcomes.

MATERIALS AND METHODS

Patients and samples

This study was approved by the Ethics Committee of the Kanazawa University Graduate School of Medical Science. All participants granted their informed consent for this study. Patients (n=86) with azoospermia in their ejaculate were recruited between May 2006 and April 2011, and patients with abnormal karyotypes or Y-chromosome microdeletions were excluded from the study, as described previously.[20] Testicular tissue specimens were obtained from patients who underwent either a diagnostic testicular biopsy or a sperm retrieval procedure from testicular tissues to evaluate histology while attempting a testicular biopsy or microdissection TESE. Histological examinations of at least 50 seminiferous tubules were performed as previously described.[21] The most advanced spermatogenetic cell that was identified in each analysis determined the histological classification of the sample. Men with azoospermia who were found to have normal sperm upon histological examination of testis samples were classified as having normal spermatogenesis.

Patient categorisation

Patients were classified into five groups using histological diagnosis as demonstrating normal spermatogenesis (NS, n=26), hypospermatogenesis (HS, n=17), maturation arrest (MA, n=17), Sertoli cell only phenotype (SCO, n=21) and tubular sclerosis (TS, n=5). Sperm were present in the tissues of 26 patients who were thought to have non-obstructive azoospermia due to obstructive azoospermia. We defined these patients as the control group. The mean ages (mean$\pm$s.d.) of NS (control), HS, MA, SCO and TS patients were 36.0$\pm$5.9, 38.2$\pm$3.9, 34.8$\pm$4.1, 35.2$\pm$3.7 and 37.2$\pm$5.0 years, respectively.

Isolation of genomic DNA and total RNA

Human genomic DNA from several normal tissues (kidney, muscle, heart, brain and colon tissue) were kindly provided by Professor Hiroki Nagase, Department of Advanced Medical Science, Division of Cancer Genetics, Nihon University School of Medicine. These samples were obtained by organ donations from autopsy cases at the Pathology Division of the Nihon University School of Medicine in Tokyo, Japan. When the tissues were received through organ donation, the bereaved families or relatives provided their informed, written consent.[22] Premium Total RNA (Clontech, Mountain View, CA, USA) from various normal human tissues was used as a control for the gene expression studies. Total RNA and testicular genomic DNA were isolated from each of the testicular tissue specimens. TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used for RNA and DNA extraction from the same specimens. Total RNA was treated with the TURBO DNA-free kit (Ambion, Austin, TX, USA) to remove any residual genomic DNA. Single-stranded cDNA was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). The additional primer pair sequences used for *GTF2A1L* amplification was 5′-CTGCCTCAACCCGGTGCCTAAAC-3′ and 5′-GCTGAACCACTGAGCACTGACTCCAC-3′ (product size, 798 bp). The primer pair sequences used for *GAPDH* amplification was 5′-GACCACAGTCCATGCCATCA-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ (product size, 453 bp).

Bisulphite treatment and quantitative DNA methylation analysis

Genomic DNA (1 μg) isolated from testicular specimens was treated with sodium bisulphite using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. Quantitative DNA methylation analysis at single CpG dinucleotides was performed using the MassARRAY Compact system (Sequenom, San Diego, CA, USA) to quantify the methylation status of CpG islands in the *GTF2A1L* promoter, as previously described.[13] MALDI-TOF MS was used for the high-throughput quantitative DNA methylation assay, and to analyse the base-specific cleaved amplification products. Each methylation evaluation was conducted using EpiTyper software v1.0 (Sequenom), which was able to generate quantitative results for each cleavage fragment (known as a CpG unit), and includes individual CpG dinucleotides or aggregates of multiple CpG sites.

Putative *GTF2A1L* promoter regions were chosen for this analysis. Arbitrarily chosen genomic regions, approximately 1-kb upstream of the *GTF2A1L* start site, were analysed to determine whether there were promoter-specific changes in DNA methylation. Bisulphite-treated DNA was amplified using polymerase chain reaction (PCR) with a reverse primer (5′-CAGTAATACGACTCACTATAGGGAGAAGGCTTTAAAACAAACCATAACAACACC-3′) tagged with a T7 promoter sequence and a forward primer (5′-AGGAAGAGAGGGATTGAGGAA ATAATTTGTGAA-3′). The amplification products were transcribed *in vitro*, base-specifically cleaved by RNaseA, and subjected to MALDI-TOF MS for the quantitative DNA methylation assay. The graphic data obtained from methylation analysis using MassARRAY were expressed as an epigram. DNA methylation standards (0%, 20%, 40%, 60%, 80% and 100% methylated genomic DNA) and correction algorithms based on the R statistical computing environment were used for data correction and normalisation.[13]

LightCycler amplification

GTF2A1L mRNA was quantified using the LightCycler TaqMan Master (Roche Applied Science, Basel, Switzerland). The Universal probe No. 62 (Roche) with forward primer 5′-TCCTGGTTATCCCATTCATGT-3′ and reverse *GTF2A1L* primer 5′-CTGTCACCATAATTGGTACATTGAC-3′ were used for amplification. As an internal reference standard, *GAPDH* expression was also measured using universal probe No. 60 (Roche) with forward primer 5′-AGCCACATCGCTCAGACA-3′ and reverse primer 5′-TCAGGAAATTTGACTTTCCATTC-3′. Each universal probe was designed according to the Universal Probe Assay Design Centre (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp). All PCR reactions were performed in a total volume of 20 μl comprising 4 μl of 5 × LightCycler TaqMan Master (Roche Diagnostics, Mannheim, Germany), 0.3 μl of 10 μmol l^{-1} TaqMan probe, 1 μl of 10 μmol l^{-1} each primer, 2 μl of sample cDNA and 11.6 μl of DEPC-treated water.

Amplification of *GTF2A1L* and of *GAPDH* was performed in triplicate for each sample. The thermal cycling conditions used were: 10 min at 95 °C, followed by 50 cycles at 95 °C for 10 s and 60 °C for 20 s for both *GTF2A1L* and *GAPDH*. The number of *GTF2A1L* and *GAPDH* transcripts in each sample was calculated with the LightCycler software using the experimentally generated standard curves.

Statistical analysis

The data were analysed using the GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA, USA; http://www.graphpad.com). Non-parametric statistical analyses were used because only a small number of subjects were available, and most variables demonstrated a skewed data distribution. The Mann–Whitney *U* test was used to compare the mean methylation rates for non-parametric data. The Student's *t*-test was used to examine the correlation between *GTF2A1L* expression and hormone levels, and differences were considered to be statistically significant at $P<0.05$.

RESULTS

Confirmation of TDMR in the GTF2A1L of normal tissues

The expression of *GTF2A1L* in several different human tissue types (testis, kidney, muscle, heart, brain and colon) was evaluated using reverse transcription-PCR. The *GTF2A1L* transcript was detected in testis tissue only (**Figure 1**).

The DNA methylation status of the *GTF2A1L* regulatory region in several different human tissue types was investigated using testes genomic DNA. We assayed DNA methylation in testis, kidney, muscle, heart, brain and colon, and focused on the regions upstream of the *GTF2A1L* gene. These amplicons were screened using the MALDI-TOF MS system (MassARRAY) to quantify methylation at CpG islands. Methylation at CpG islands in the *GTF2A1L* promoter was lower in the testis (methylation rate: 21.6%±2.83%) than in the five other tissue types: kidney (85.3%±6.21%), muscle (83.1%±9.31%), heart (84.8%±5.66%), brain (88.9%±5.85%) and colon (84.8%±8.16%). These differences indicate the presence of a TDMR in the 5′-upstream *GTF2A1L* promoter region somewhere between −258 bp and +41 bp of the transcription start site. This region, a 299 bp-amplicon, contains a promoter CpG island with 22 CpG dinucleotides (**Figure 2**). Therefore, the TDMR of *GTF2A1L* was classified as having low methylation at CpG islands in the normal testes.

Quantification of the GTF2A1L methylation state in the testicular genome

We analysed DNA methylation for the *GTF2A1L* gene promoter in genomic DNA samples from testes using MassARRAY. Testes DNA in all samples from the control group ($n=26$) had low methylation at the *GTF2A1L* promoter, and the mean methylation rate was 24.4%±10.6% (95% CI: 20.1%–28.7%). Testis DNA from all samples from the HS group ($n=17$) also demonstrated low DNA methylation, and the mean methylation rate was 35.1%±12.0% (95% CI: 29.0%–41.3%). The mean *GTF2A1L* DNA methylation rates in patients with HS were significantly higher compared with the controls ($P=0.029$). However, the mean methylation rates in patients with MA ($n=17$), SCO ($n=21$) and TS ($n=5$) were 66.5%±0.07% (95% CI: 62.9%–70.1%), 76.5%±0.08% (95% CI: 73.1%–79.9%) and 79.4%±0.07% (95% CI: 70.2%–88.7%), respectively. Testes DNA from all samples from the MA, SCO or TS groups demonstrated high DNA methylation (**Figure 3**). Patients with SCO and TS were extreme cases, because the genomic DNA extracted from those testes biopsies may not present with any germ cells. Although all samples revealed high *GTF2A1L* DNA methylation except NS (control) and some HS samples, differences in methylation levels between control and HS groups were statistically significant in this study.

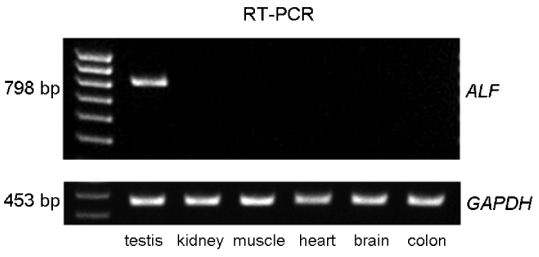

Figure 1 Tissue-specific expression of *GTF2A1L* in normal human tissues. cDNAs were prepared from total RNA isolated from testis, kidney, muscle, heart, brain and colon tissues. *GAPDH* was used as the internal standard for comparison.

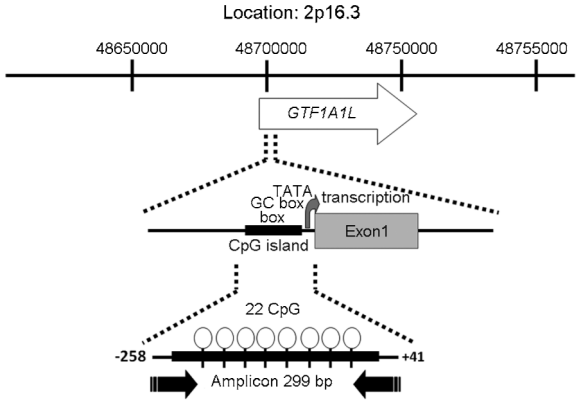

Figure 2 Chromosomal location of *GTF2A1L*. This map is based on the March 2006 human reference sequence (NCBI Build 36.1). The *GTF2A1L* gene is shown as an arrow, with an arrowhead indicating the direction of transcription. CpG islands are shown with the number of CpGs. The amplicon was generated from the −258 bp to +41 bp (299 bp) CpG region near the transcription start site.

Characterisation of patients with hypospermatogenesis

To further characterize the patients with HS, this group was divided into two subgroups according to methylation level: low methylation (LM) and high methylation (HM). The mean CpG methylation rates were 28.6%±6.9% (95% CI: 24.1%–33.0%, $n=12$) in the LM group versus 50.9%±2.3% (95% CI: 48.0%–53.7%, $n=5$) in the HM group, and these means were significantly different ($P=0.0019$, **Figure 4a**). These results indicate that some subjects with HS exhibit relatively high rates of high methylation in the *GTF2A1L* promoter region.

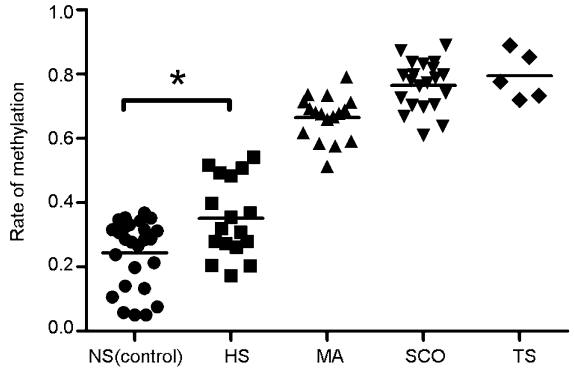

Figure 3 Rate of methylation status in patients characterized histological diagnosis. Normal spermatogenesis controls (NS, closed circles, $n=26$), hypospermatogenesis (HS, closed squares, $n=17$), maturation arrest phenotype (MA, closed triangles, $n=17$), Sertoli cell only phenotype (SCO, inverted closed triangles, $n=21$) and tubular sclerosis (TS, closed diamonds, $n=5$). The asterisk indicates a statistically significant difference between values ($P<0.05$).

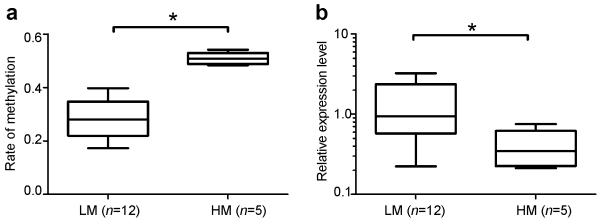

Figure 4 Classification of high- and low-methylation TDMR groups in patients with hypospermatogenesis. (**a**) Box–Whiskers plot (min. to max.) of patients with hypospermatogenesis subdivided according to the *GTF2A1L* methylation rate: low methylation (LM, n=12) and high methylation (HM, n=5). (**b**) The relative expression level of *GTF2A1L* in patients with hypospermatogenesis and low methylation (LM, n=12) or high methylation (HM, n=5). The asterisk indicates a statistically significant difference between values (P<0.05). TDMR, tissue-specific differentially methylated region.

GTF2A1L expression in the HM and LM groups

The *GTF2A1L* mRNA was measured using real-time quantitative reverse transcription-PCR with *GAPDH* mRNA as an internal reference standard. The *GTF2A1L* expression rate in the control group was 0.91±0.42 (95% CI: 0.74–1.08), whereas the rates in the MA, SCO and TS groups were 0.18±0.10 (95% CI: 0.1–0.23), 0.095±0.039 (95% CI: 0.08–0.11) and 0.03±0.02 (95% CI: 0.001–0.060), respectively. Both patients with SCO and TS also exhibited high DNA methylation, and the *GTF2A1L* expression was very low, as expected.

The relative *GTF2A1L* expression level in the HS group is shown in **Figure 4b**. Expression of *GTF2A1L* mRNA was significantly higher in the LM group (median=0.9370, 25% percentile=0.5745, 75% percentile=7.425) than in the HM group (median=0.3460, 25% percentile=0.2251, 75% percentile=0.6211) (P=0.019). These results demonstrated that highly methylated TDMRs correlate with low levels of gene expression. Therefore, the alteration of methylation within the *GTF2A1L* promoter may greatly affect its expression and result in spermatogenesis failure.

Clinical characteristics of patients with LM and HM

The general characteristics and clinical parameters of patients with hypospermatogenesis are summarized in **Tables 1** and **2**. These patients were classified into two groups based on the *GTF2A1L* methylation state as HM (**Table 1**) and LM (**Table 2**); the mean patients' age in each group was 33.8±2.63 years and 37.7±2.1 years, respectively. Levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T) in the LM group were 3.2±0.42 mU ml^{-1}, 2.7±0.24 mU ml^{-1} and 5.7±0.99 ng ml^{-1}, respectively. In contrast, the mean FSH, LH and T levels in the HM group were 5.7±0.77 mU ml^{-1}, 2.7±0.44 mU ml^{-1} and 4.4±0.30 ng ml^{-1}, respectively. No significant difference was found in mean levels of FSH (P=0.08), LH (P=0.96) or T (P=0.12) between the LM and HM groups (**Tables 1** and **2**). Following various ARTs, including TESE–ICSI, both groups had relatively good outcomes with respect to sperm retrieval and fertilisation as well as pregnancy and childbirth rates. We observed that aberrant *GTF2A1L* promoter methylation was not correlated with reproductive outcome following TESE–ICSI procedures. Moreover, the sperm positive control group (n=26) revealed normal endocrinology profile and retrieval, and fertilisation rate are 100%.

DISCUSSION

Numerous CpG islands that contain TDMR are potential methylation sites in normal tissues. In this study, DNA methylation at the *GTF2A1L* CpG island was apparently lower in testes samples with normal spermatogenesis; however, aberrant methylation at the *GTF2A1L* CpG island was associated with hypospermatogenesis in some azoospermic patients. We revealed that the aberrant methylation of TDMRs was associated with human spermatogenesis. However, only five patients exhibited relatively high methylation at the *GTF2A1L* CpG island, but these low numbers are likely not sufficient to generate statistical significance. Other subjects, including patients with MA, SCO and TS, exhibited high *GTF2A1L* promoter methylation, but low *GTF2A1L* promoter methylation is considered to be the normal status for TDMR. Although larger numbers of infertile male subjects may be necessary to detect the influence of aberrant methylation on hypospermatogenesis incidence, the relatively higher *GTF2A1L* gene methylation observed in the five patients may still yield useful data.

Previously, CpG islands were considered to be almost entirely unmethylated except within imprinted regions and on the inactive X chromosome.[23] However, accumulating evidence suggests that methylation of TDMRs is associated with modulated gene expression[24–26] and impaired spermatogenesis.[13,22,27]

Unlike imprinted genes, many TDMRs may play a role in defining cellular identity and tissue-specific regulation of genome function. Moreover, the TDMRs may be broadly distributed in intragenic and intergenic regions that have CpG islands. Approximately 4% of more than 5000 autosomal genes with unmethylated CpG island promoters were methylated in normal peripheral blood.[28] Aberrant TDMR methylation may increase the prevalence of male infertility and might be involved in idiopathic male infertility.

TESE is a powerful technique used to retrieve sperm from patients with azoospermia. ARTs are regularly used to facilitate fertilisation and pregnancy. ART may be associated with epigenetic changes in imprinted genes that can lead to human disease. Various artificial fertilisation procedures may be implicated in the susceptibility to epigenetic defects such as Beckwith–Wiedemann syndrome and Angelman syndrome after ART.[29] However, the absolute incidence of imprinting disorders is very low.[30–32]

Epigenetic regulation of the germline has recently become a clear candidate process that results in male infertility. Tissue-specific CpG island methylation was identified at developmental gene loci, which became demethylated during the differentiation into adult tissues.[33,34] Moreover, high CpG dense regions were mostly unmethylated, and low CpG dense regions were demonstrated as the preferential targets for de novo methylation in a 5-methyl cytosine antibody study.[35]

Table 1 Clinical features of HS patients with high methylation

Age (year)	*FSH (mU ml^{-1})*	*LH (mU ml^{-1})*	*Testosterone (ng ml^{-1})*	*TESE*	*Sperm retrieval*	*ICSI*	*Fertilisation*	*Pregnancy*	*Childbirth*
34	2.25	2.42	4.6	Yes	Yes	Yes	Yes	Yes	Yes
41	3.4	3.29	8.2	Yes	Yes	Yes	Yes	Yes	Yes
42	N/P	N/P	N/P	Yes	Yes	Yes	Yes	Yes	Yes
31	4.23	2.7	3.71	Yes	Yes	Yes	Yes	Yes	Yes
29	2.8	2.2	6.2	Yes	Yes	Yes	Yes	Yes	Yes

Abbreviations: HS, hypospermatogenesis; ICSI, intracytospermic sperm injection; N/P, not performed; TESE, testicular sperm extraction including microdissection.

Table 2 Clinical features of HS patients with low methylation

Age (year)	*FSH (mU ml^{-1})*	*LH (mU ml^{-1})*	*Testosterone (ng ml^{-1})*	*TESE*	*Sperm retrieval*	*ICSI*	*Fertilisation*	*Pregnancy*	*Childbirth*
31	2.8	1	4.1	Yes	Yes	Yes	Yes	No	No
31	3.4	2.2	3.8	Yes	Yes	Yes	Yes	Yes	Yes
32	7.2	5.4	6.9	Yes	Yes	Yes	Yes	Yes	Yes
45	5.4	2.5	4.5	Yes	Yes	Yes	Yes	No	No
35	3	3.1	5.1	Yes	Yes	Yes	Yes	Yes	Yes
38	4.5	1.5	3.7	Yes	Yes	Yes	Yes	No	No
54	5.4	0.8	4.4	Yes	Yes	Yes	Yes	Yes	Yes
36	6.7	4	4.1	Yes	Yes	Yes	Yes	Yes	Yes
58	N/P	N/P	N/P	Yes	Yes	Yes	Yes	Yes	Yes
38	4.93	2.06	4.7	Yes	Yes	Yes	Yes	Yes	Yes
34	11.81	2.42	2.9	Yes	Yes	N/P	N/P	N/P	N/P
41	7.21	4.64	4.2	Yes	Yes	Yes	Yes	Yes	Yes

Abbreviations: FSH, follicle-stimulating hormone; HS, hypospermatogenesis; ICSI, intracytospermic sperm injection; LH, luteinizing hormone; N/P, not performed; TESE, testicular sperm extraction including microdissection.

Additionally, testicular spermatozoa from men with abnormal spermatogenesis often have methylation defects, which further supports an association between ARTs and the occurrence of imprinting errors.[30] Studies of the methylation levels in paternally and maternally imprinted genes and aberrantly methylated imprints revealed a significant association with abnormal semen parameters, but did not appear to influence ART outcomes.[36]

In the present study, sperm retrieval, fertilisation, pregnancy and child birth rates were relatively high. An atypical *GTF2A1L* expression was due to an aberrant methylation of *GTF2A1L* TDMR in testicular DNA; however, these aberrations did not cause serious damage in the testicular spermatozoa recovered *via* TESE procedures. One possible explanation for this observation was that aberrant TDMR methylation was considered to result in weak CpG island promoters, which are the preferential targets for de novo methylation.

The GTF2A1L protein is coexpressed in late pachytene spermatocytes and in haploid round spermatids[19] and may form part of a transcriptional network that is vital for the completion of meiosis and for the preparation of post-meiotic differentiation.[37]

Hypospermatogenesis is characterized histologically by a reduced number of spermatozoa in any seminiferous tubule .[21] Although some post-meiotic failure is believed to result from aberrant methylation in promoters of some testis-specific genes, the genetic regulation of post-meiotic spermiogenesis remains unclear (e.g., germ cell-specific epigenetic processes, including histone to protamine exchange in haploid spermatids or genomic imprinting in the parent). Once germ cells complete meiosis, male infertility caused by aberrant methylation can be overcome using TESE.

In summary, we demonstrated that aberrant TDMR methylation at the *GTF2A1L* promoter may have caused a decrease in *GTF2A1L* expression that was associated with hypospermatogenesis. We suggest that TESE is a valuable procedure for retrieving sperm from patients with hypospermatogenesis. However, this issue must be further examined in a larger patient population to provide adequate data for statistically significant results and to confirm our conclusion.

AUTHOR CONTRIBUTIONS

KS and EK contributed to the study design, performed experiments, analyzed data and wrote the manuscript. MI, MT and YM contributed to the collection of written informed consent from all patients and controls and the purification of the DNAs from all participants. KS and EK analysed the data and assisted with writing the paper. MN designed the study, obtained the funding and revised the paper.

COMPETING FINANCIAL INTERESTS

All authors declare that there are no competing financial interests.

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