

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

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Title

Aberrant methylation of the tissue-specific differentially methylated region (TDMR) at the *GTF2A1L* promoter does not affect fertilization rates via testicular sperm extraction in patients with hypospermatogenesis

Running title:

Aberrant methylation of TDMR in *GTF2A1L*

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Abstract

There is increasing evidence of a relationship between epigenetic regulation and male infertility. *GTF2A1L* promoters contain the DNA methylation site of a tissue-specific differentially methylated region (TDMR). Eighty-six patients with secretory azoospermia were assessed by the DNA methylation state of CpG islands in the *GTF2A1L* promoter using testicular genome 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 methylation (LM, $n=12$). The *GTF2A1L* TDMR methylation rate differed significantly between HM and LM groups ($P=0.0019$), and *GTF2A1L* expression was significantly higher among LM than HM patients ($P=0.023$). High TDMR methylation was correlated with low levels of gene expression. Both groups had relatively good outcomes with respect to sperm retrieval and fertilization, pregnancy, childbirth rates. We observed that aberrant expression of the *GTF2A1L* gene was not correlated with fertilization rates. The TESE technique may be used to overcome male infertility due to aberrant TDMR methylation.

key words

ALF/ azoospermia/ CpG island/ hypospermatogenesis/ MALDI-TOF MS/ TESE

Introduction

Male factors of total infertility may account for up to 40–50% of infertile couples¹. There are many more cases of male infertility that are unreported. Testicular sperm extraction (TESE) and intracytoplasmic sperm injection (ICSI) are commonly used to overcome male infertility, and sperm retrieval is successful in 30–70% of patients with non-obstructive azoospermia.² In hypoplasia, 97.7% had late spermatids/sperm recovered using assisted reproductive technique (ART).³ In healthy males, infertility may have a number of unknown causes, including genetic or epigenetic disorders.

Epigenetic modifications in particular result in stable or semi-stable changes in gene expression without affecting the nucleotide sequence of DNA.^{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 their DNA methylation state varies. CpG islands are generally not methylated during normal cell development, with the exception of imprinted genes. They are preferentially located at the start of gene transcripts and on the differentially methylated regions (DMRs) of imprinted genes.⁶

However, tissue-specific differentially methylated regions (TDMRs) are a somewhat different category, identified by means of DNA methylation array methods, and are demethylated in some tissues and fully methylated in others.^{7, 8} Therefore, DNA methylation of TDMRs in gene loci causes transcriptional repression.⁹⁻¹¹ TDMRs are interspersed throughout the entire

genome and ensure that 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 containing 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 at TDMRs may adversely affect clinical manifestations.

GTF2A1L (NM_006872) encodes homo sapiens general transcription factor IIA, 1-like, formerly known as *ALF*. Synthesis of eukaryotic mRNA requires the assembly and stability of RNA polymerase II and various general transcription factors. This gene encodes a germ cell-specific counterpart of the large (alpha/beta) subunit of general transcription factor TFIIA that is able to stabilize the binding of TATA-binding protein (TBP) to DNA and may be uniquely important to testis biology.¹⁶⁻¹⁸ The gene is also selectively transcribed in reproductive tissues. *GTF2A1L* is coexpressed in late pachytene spermatocytes and in haploid round spermatids with TBP-related factor 2, and that 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/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to human testicular tissue. We also describe 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 informed consent for this study. Patients (n = 86) with azoospermia in 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 sperm retrieval from testicular tissues to evaluate histology while attempting testicular biopsy or microdissection TESE. Histological examination of at least 50 seminiferous tubules was performed as previously described.²¹ The most advanced spermatogenic 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 categorization

Patients were classified into five groups by histological diagnosis: 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 be secretory azoospermia due to obstructive azoospermia.

We defined these patients as the control group. The mean ages (mean \pm SD) of NS(control), HS, MA, SCO, and TS patients were 36.0 ± 5.9 , 38.2 ± 3.9 , 34.8 ± 4.1 , 35.2 ± 3.7 , and 37.2 ± 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 donation from autopsy cases at the Pathology Division of the Nihon University School of Medicine in Tokyo, Japan. When tissues were received through organ donation, bereaved families or relatives provided 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 the extraction of RNA and DNA 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 a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). An additional primer pair used for *GTF2A1L* expression was 5'- CTGCCTCAACCCGGTGCCTAAAC -3' and 5'- GCTGAACCACTGAGCACTGACTCCAC -3' (product size, 798 bp). The

primer pair used for *GAPDH* expression was 5'- GACCACAGTCCATGCCA TCA-3' and 5'- TCCACCACCCTGTTGCTGTA-3' (product size, 453 bp).

Bisulfite treatment and quantitative DNA methylation analysis

Genomic DNA (1 µg) isolated from testicular specimens was treated with sodium bisulfite 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 described¹³. MALDI-TOF MS was used for the high-throughput quantitative DNA methylation assay. This system analyzed base-specific cleaved amplification products. Each evaluation of methylation was conducted using EpiTyper software v1.0 (Sequenom), which is able to generate quantitative results for each cleavage fragment (known as a CpG unit), and can include individual CpG dinucleotides or aggregates of multiple CpG sites.

Putative 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. Bisulfite-treated DNA was polymerase chain reaction (PCR)-amplified using a reverse primer (5'- CAGTAATACGACTCACTATAGGGAGAAGGCTTT AAAACAAACCATAACAACACC-3') tagged with a T7 promoter sequence. The forward primer used was 5'-AGGAAGAGAGGGATTGAGGAA ATAATTTGTGAA-3'. The amplification products were transcribed *in vitro*,

base-specifically cleaved by RNase A, 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 R statistical computing environment were used for data correction and normalization.¹³

LightCycler amplification

GTF2A1L mRNA was quantified using the LightCycler TaqMan Master (Roche Applied Science, Basel, Switzerland). Universal probe no. 62 (Roche) with forward primer 5'-TCCTGGTTATCCCATTTCATGT-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 Center (<https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp>). All PCR reactions were performed in a total volume of 20 μ L comprising 4 μ L of 5x LightCycler TaqMan Master (Roche Diagnostics, Mannheim, Germany), 0.3 μ L of 10 μ mol/L TaqMan probe, 1 μ L of 10 μ mol/L each primer, 2 μ L of sample cDNA, and 11.6 μ L 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 these standard curves.

Statistical analysis

Data were analysed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Because of the small number of subjects and the skewed distribution of most variable, non-parametric statistics were used. The Mann–Whitney U test was used to compare mean methylation rates for non-parametric data. Student's *t*-test was used to examine the correlation between *GTF2A1L* expression and hormone levels. Differences were considered statistically significant at $P < 0.05$.

Results

Confirmation of TDMR in GTF2A1L in normal tissues

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

DNA methylation status of the *GTF2A1L* regulatory region in several different human tissue types was investigated using testes genomic DNA. We assayed 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: $21.6 \pm 2.83\%$) than in the five other tissue types: kidney ($85.3 \pm 6.21\%$), muscle ($83.1 \pm 9.31\%$), heart ($84.8 \pm 5.66\%$), brain ($88.9 \pm 5.85\%$), and colon ($84.8 \pm 8.16\%$). These differences indicated that there is a TDMR in the 5'-upstream region, somewhere within -253 to 43 bp of the transcription start site; this region, a 301-bp amplicon, contained a promoter CpG island with 22 CpG dinucleotides (Figure 2). Therefore, the TDMR of *GTF2A1L* was classified as low methylation at CpG islands in the normal testes.

Quantification of the GTF2A1L methylation state in the testicular genome

We analyzed methylation at the *GTF2A1L* gene promoter in genomic DNA samples from testes using MassARRAY®. Testes DNA from all samples from the control group (n = 26) was low methylated at the *GTF2A1L* promoter, and the mean methylation rate was $24.4 \pm 10.6\%$ (95% CI 20.1–28.7%). Testis DNA from all samples from the HS group (n = 17) was also low methylated, and the mean methylation rate was $35.1 \pm 12.0\%$ (95% CI 29.0–41.3%). The mean methylation rates in patients with HS were significantly high 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 $66.5 \pm 0.07\%$ (95% CI 62.9–70.1%), $76.5 \pm 0.08\%$ (95% CI 73.1–79.9%), and $79.4 \pm 0.07\%$ (95% CI 70.2–88.7%), respectively. Testes DNA from all samples from the MA, SCO or TS group were high methylated (Figure 3). Patients with SCO and TS were extreme cases, because the genomic DNA extracted from those testes biopsies may not present any germ cell. Although all samples revealed high

methylation except NS (control) and part of HS samples, differences in methylation level between control and HS groups were statistically significant in this study.

Characterization 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 $28.6 \pm 6.9\%$ (95% CI 24.1–33.0%, n = 12) in the LM group versus $50.9 \pm 2.3\%$ (95% CI 48.0–53.7%, n = 5) in the HM group, and these means were significantly different ($P = 0.0019$, Figure 4A). This finding indicated that some subjects with HS exhibited relatively high rates of high methylation in the *GTF2A1L* promoter region.

GTF2A1L expression in the HM and LM groups

GTF2A1L mRNA was measured using real-time quantitative RT-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 rate in the MA, SCO and TS group was 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 methylation, the expression was very low as expected.

On the other hand, the relative *GTF2A1L* expression level in the HS group is depicted 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 correlated with low levels of gene expression. Therefore, the alteration of methylation within the *GTF2A1L* promoter may greatly affect expression and result in failure of spermatogenesis.

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 *GTF2A1L* methylation state: HM (Table 1) and LM (Table 2); the mean level of the patients in each group were 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, 2.7 ± 0.24 mU/mL, and 5.7 ± 0.99 ng/mL, respectively. In contrast, the mean level of FSH, LH, and T in the HM group were 5.7 ± 0.77 mU/mL, 2.7 ± 0.44 mU/mL, and 4.4 ± 0.30 ng/mL, respectively. There was no significant difference 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 fertilization, pregnancy, childbirth rates. We observed that aberrant methylation of the *GTF2A1L* promoter was not correlated with reproductive outcome following TESE-ICSI procedures. Moreover sperm proven control group (n=26) revealed normal endocrinology profile and retrieval and fertilization rate are 100%.

Discussion

Numerous CpG islands containing TDMR are potential methylation sites in normal tissues. In this study, methylation at the *GTF2A1L* CpG island was apparently low 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, there were only five patients exhibiting relatively high methylation at the *GTF2A1L* CpG island. These numbers might be unlikely to provide sufficient statistical power; however, all other subjects, including patients with MA, SCO and TS, exhibited high methylation, and low methylation at the *GTF2A1L* promoter is thought to be the normal status in TDMR. Although larger numbers of male infertility subjects may be necessary to detect the influence of aberrant methylation on hypospermatogenesis incidence, these relatively higher methylation in five patients can yield meaningful aspects.

Previously, CpG islands were thought 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 expression²⁴⁻²⁶ and impairment of spermatogenesis.^{13, 22, 27}

Unlike imprint genes, many TDMRs may play a role in cellular identity and tissue-specific regulation of genome function. Moreover, they 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 be included in idiopathic male infertility.

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

On the other hand, epigenetic regulation of the germline has recently become clearer a candidate process resulted in male infertility. Tissue-specific CpG island methylation was identified at developmental gene loci, which became demethylated during differentiation to adult tissues.^{33, 34} Moreover, high CpG density were mostly unmethylated, and low CpG density were preferential targets for de novo methylation using 5-methyl cytosine antibody study.³⁵

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

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

The *GTF2A1L* protein is co-expressed in late pachytene spermatocytes and in haploid round spermatids¹⁹, which 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 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 a parent). Once germ cells complete meiosis, male infertility caused by aberrant methylation could 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 to retrieve sperm from patients with hypospermatogenesis. However, our conclusion must be explored in a larger patient population to provide adequate statistical power to confirm this conclusion.

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Figure Legends

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). *GTF2A1L* 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 designed from -258 to +41 (299 bp) from the transcription start site.

Figure 3. Rate of methylation status in patients characterized histological diagnosis.

Normal spermatogenesis as control (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). Asterisk indicates statistically significant difference in 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 *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). Asterisk indicates statistically significant difference in values (P<0.05).

RT-PCR

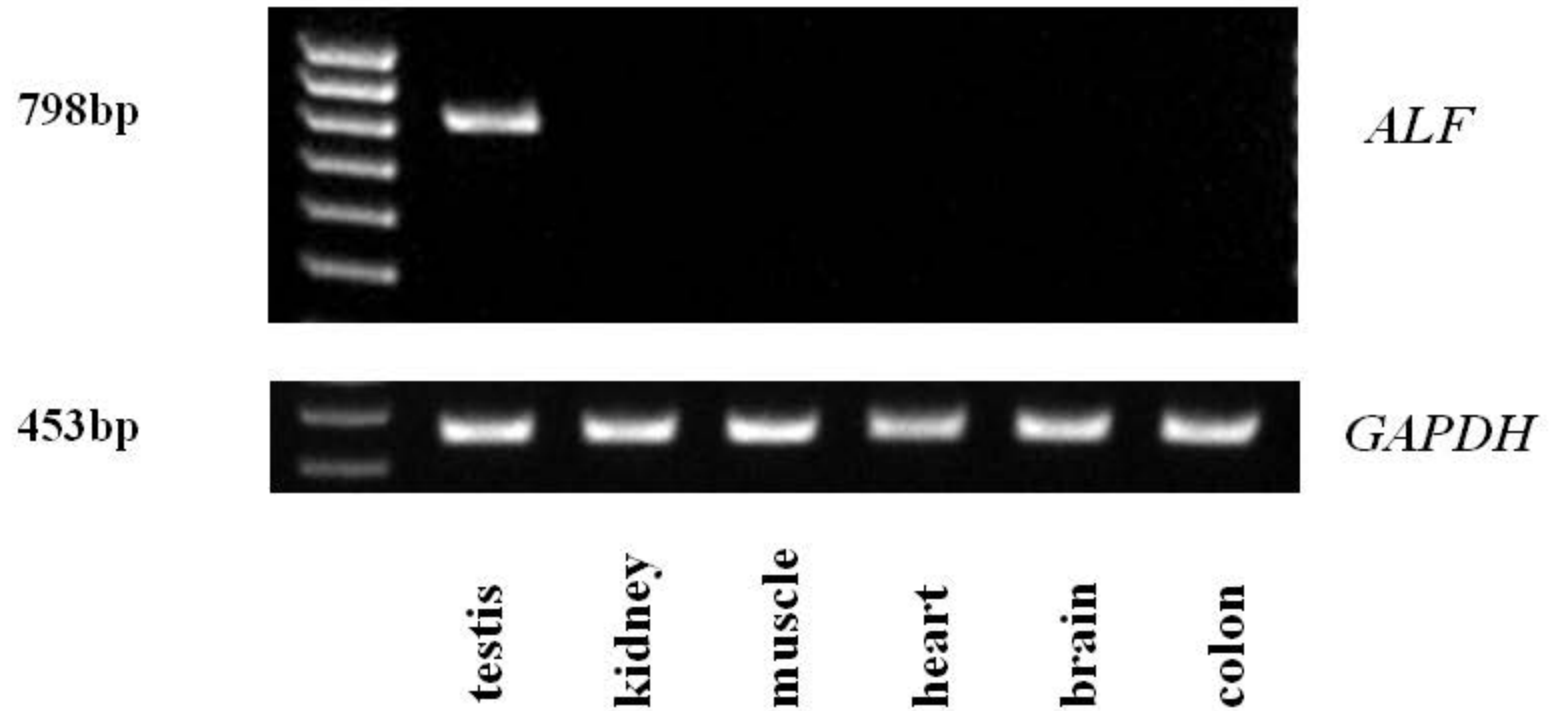


Fig 1

Location: 2p16.3

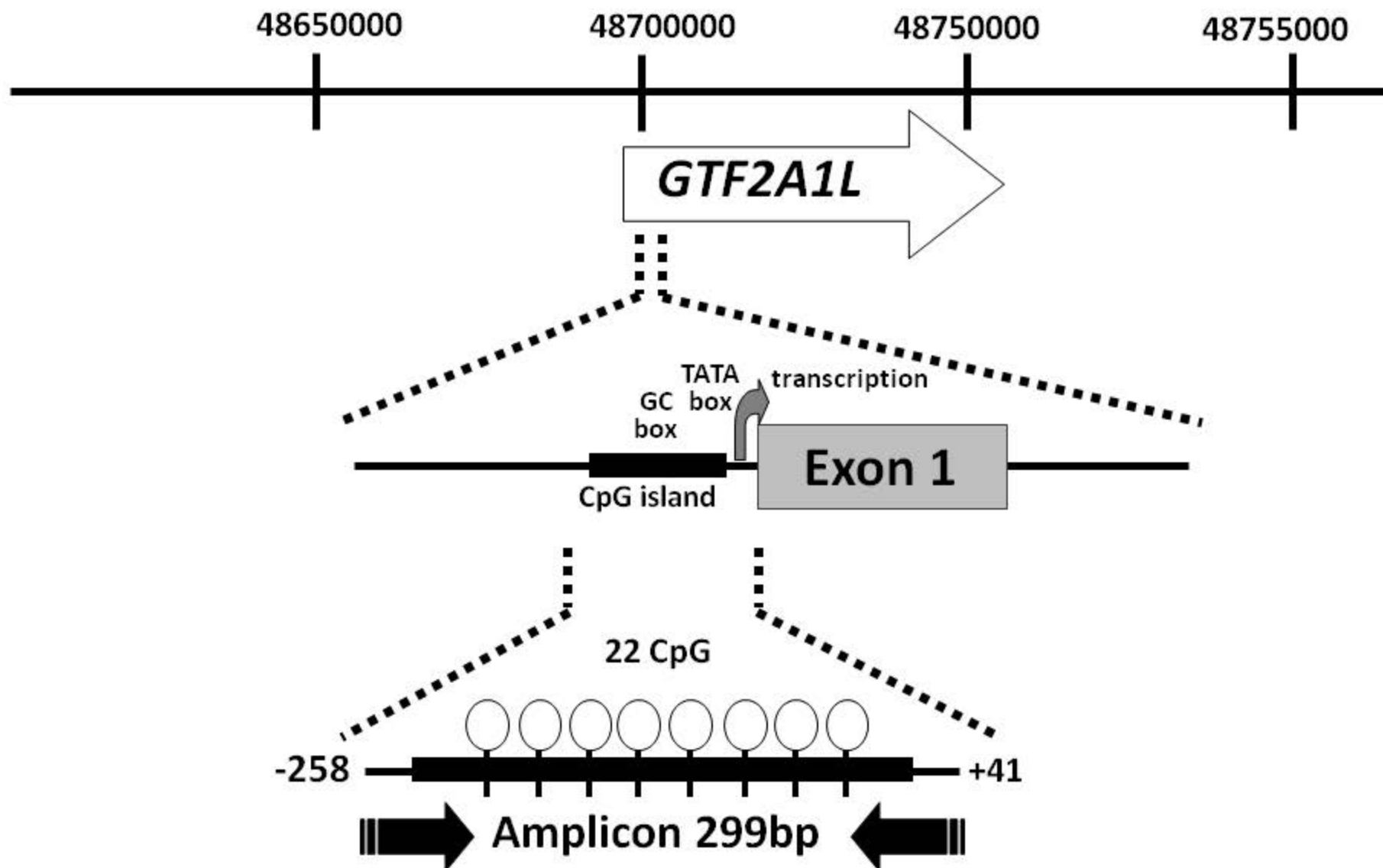


Fig 2

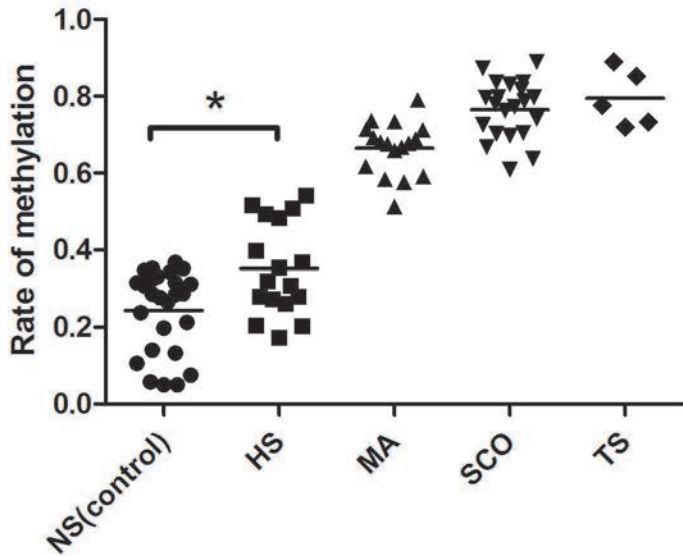


Fig2

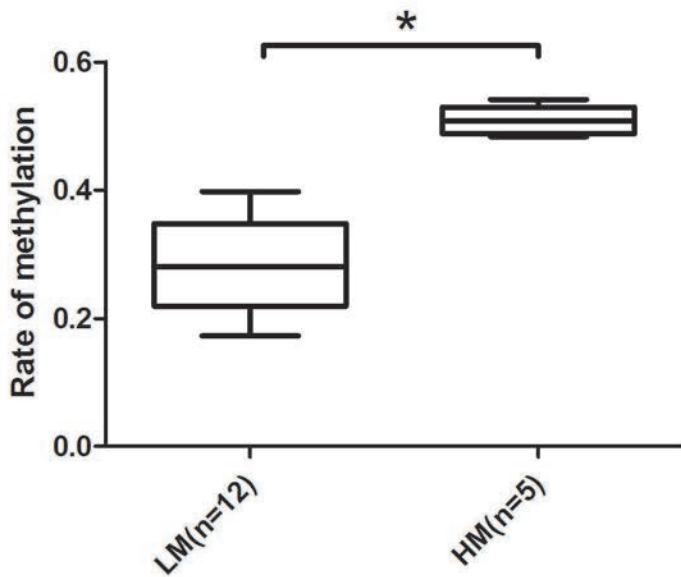


Fig 4a

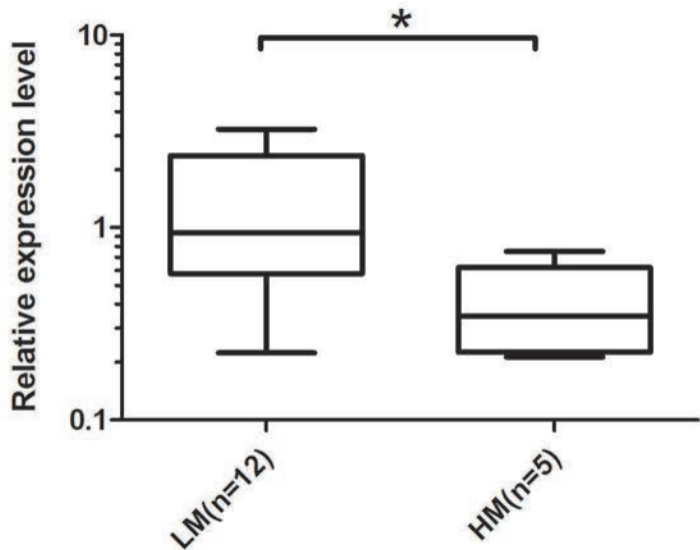


Fig 4b

Table 1 Clinical features of HS patients with high methylation

Age(years)	FSH(mU/mL)	LH(mU/mL)	Testosterone(ng/mL)	TESE	Sperm Retrieval	ICSI	Fertilization	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

HS=hypospermatogenesis; TESE=testicular sperm extraction including microdissection; ICSI=intracytoplasmic sperm injection; N/P=not performed

Table 2 Clinical features of HS patients with low methylation

Age(years)	FSH(mU/mL)	LH(mU/mL)	Testosterone(ng/mL)	TESE	Sperm Retrieval	ICSI	Fertilization	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

HS=hypospermatogenesis; TESE=testicular sperm extraction including microdissection; ICSI=intracytoplasmic sperm injection; N/P=not performed