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*·Original Article·*

# A differentially methylated region of the *DAZ1* gene in spermatic and somatic cells

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**Abstract**

**Aim:** To investigate the methylation status of the deleted in azoospermia 1 (*DAZ1*) gene promoter region in different cell types. **Methods:** Using CpG island Searcher software, a CpG island was found in the promoter region of the *DAZ1* gene. The methylation status of this region was analyzed in sperm and leukocytes by bisulfited sequencing. **Results:** The methylation status of the CpG island in the *DAZ1* gene promoter region differed in leukocytes and sperm: it was methylated in leukocytes, but unmethylated in sperm. **Conclusion:** A differentially methylated region of the *DAZ1* gene exists in spermatic and somatic cells, suggesting that methylation of this region may regulate *DAZ1* gene expression in different tissues. (*Asian J Androl 2006 Jan; 8: 61-67* )

**Keywords**: *DAZ1* gene; DNA methylation; CpG island; methylated region; spermatogenesis

## 1 Introduction

In mammals, DNA can be methylated after replication; this modification always occurs at the 5' position of cytosine in the sequence 5'-CpG-3'. Tissue-specific genes always have a condensed CpG region at the promoter, called the "CpG island". Through modification by methylation and demethylation, the gene may be silenced or expressed in different cells. Many biological processes are associated with DNA methylation changes, including genomic imprinting, cell differentiation, X chromosome inactivation and chromatin remodeling [1]. The deleted in azoospermia (*DAZ*) gene, which is specifically expressed in the testis, has four homologous genes (*DAZ1*, *DAZ2*, *DAZ3* and *DAZ4*) and they are located in the azoospermia factor (*AZF*c) region of the Y chromosome. It is one of the major candidate genes for spermatogenesis. Should it be deleted or its expression altered, the consequence is male infertility [2]. The aim of the present study was to compare the methylation status of *DAZ1* gene in spermatic and somatic cells.

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## 2 Materials and methods

### *2.1 CpG island screening and primer design*

The *DAZ1* gene sequence was downloaded from NCBI (location: Yq11.223; gene ID: 1617). We selected the region from –2.5 kb to 0.5 kb of the transcriptional point (original accession number: NC 000024; region: 24701305..24704304), and then screened this region using CpG island Searcher software (http://cpgislands.usc.edu/). The limit values were: length> 200 bp, G+C con-

tent > 50 %, observed/expected CpG ratio (ObsCpG/ExpCpG) > 0.6, and gap between adjacent islands > 100 bp. The primer pairs for bisulfite sequencing PCR (BSP) were selected using Methyprimer software (http://www.ucsf.edu/urogene/methprimer/index1.html). The sequence of *DAZ-TF* was: 5'-TAT GTA TAT TTA TTT TAA GGG TGT T-3'; *DAZ-TR*: 5'-ATT TCA CCC ACC ACT TCT AAA TCT A -3' (*DAZ-T*: the bisulfite treated DNA). The primer pairs to be amplified from this region were selected with the assistance of software packages in Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) for identification of the CpG island region. The sequence of *DAZ-WF* was: 5'-CCG GAA GCC ATT TTG AAA C -3'; *DAZ-WR*: 5'-GAC AGG CTC AAG GAG GAA CA -3' (*DAZ-W*: the CpG island of DNA gene from genomic DNA).

*2.2 Genomic DNA extraction, amplification and sequencing in spermatic and leukocyte cells*

Semen was collected from four men who had fathered children normally by masturbation following 7 days of sexual abstinence. The sperm concentration was > 50 × $10^6$/mL. After routine analysis, the semen was washed twice with phosphate buffered saline (PBS), and the sperm concentration was adjusted to 50 × $10^6$/mL and stored at –20 ºC. Then 2mL blood samples were collected and stored at –20 ºC. Leukocyte or spermatic genomic DNA was isolated using a genomic DNA purification kit (Shenergy Biocolor Cat. #K201, Shanghai, China) according to the user's manual. Polymerase chain reaction (PCR) was performed with the Shenergy Biocolor Universal Taq PCR Master Mix (Shanghai, China). With a total volume of 50 µL, the PCR mix contained Taq DNA polymerase 2.5 U, dNTP 0.2 mmol/L each, $MgSO_4$ 2 mmol/L, KCl 10 mmol/L, $(NH_4)_2SO_4$ 8 mmol/L, Tris-Cl 10 mmol/L (pH = 9.0), forward and reverse primer 200 nmol/L each, and genomic DNA 100 ng–200 ng. Reaction was performed with a Biometra T gradient thermo block (Goettingen, Lower Saxony, Germany). Cycling conditions were: 96 ºC denaturing for 5 min, then 35 cycles of 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s, and a final extension of 72 ºC for 7 min, paused at 4 ºC. The PCR products were separated by 1.5 % agarose gel electrophoreses with 1 × Tris-acetate-EDTA (TAE) and 0.5 µg/mL ethidium bromide, and visualized under UV illumination. The PCR products around the 500 bp band were sent to the Shenergy Biocolor Company (Shanghai, China) for sequencing.

*2.3 Bisulfate-sequencing PCR (BSP)*

About 4 µg of sperm or leukocyte genomic DNA in 50 µL Tris-EDTA (TE) and 0.2 mol/L NaOH (by adding 5.5 µL 2 mol/L NaOH) was denatured for 15 min at 37 ºC. Then 30 µL of 10 mmol/L hydroquinone (Sigma, St. Louis, USA) and 520 µL of 3 mol/L sodium bisulfate (Sigma, St. Louis, USA) at pH 5.0 were added and mixed, and 200 µL of mineral oil was added to cover the solution. Incubation was carried out for 16 h at 52 ºC for bisulfate modification. The modified genomic DNA was purified using the wizard DNA purification resin according to the manufacturer's instructions (Promega Cat. #A7280, Madison, Wisconsin, USA), eluted into 50 µL water, and modification was completed by NaOH treatment (final concentration 0.3 mol/L) for 10 min at room temperature. This was followed by ethanol precipitation (achieved by adding 33 µL $NH_4Ac$, 270 µL cold ethanol, mixing, precipitating at –20 ºC for 30 min, and centrifuging at 13 000 × *g* at 4 ºC for 10 min). DNA was resuspended in milli-Q water and used immediately or stored at –70 ºC. In this procedure, the unmethylated cytosine in genomic DNA is converted to uracil and the methylated cytosine is unchanged.

With the same PCR mix, touchdown PCR was used to amplify this region. The cycling conditions were: denaturing at 96 ºC for 5 min, then 10 touchdown cycles of 94 ºC for 30 s, 60 ºC –55 ºC for 30 s and 72 ºC for 30 s, 25 cycles of 94 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 30 s, and a final extension of 72 ºC for 7 min, paused at 4 ºC. The PCR products were separated by 1.2 % agarose gel electrophoresis with 1 × TAE and 0.5 µg/mL ethidium bromide, and visualized under UV illumination. Products from around the 400 bp band were sent to the Shenergy Biocolor Company (Shanghai, China) for sequencing.

## 3 Results

*3.1 CpG island screening*

Around the *DAZ1* gene promoter region, we found a CpG condensed region (GC = 66.8 %, ObsCpG/ExpCpG = 0.652, Length = 355 bp) with CpG island searcher software (http://cpgislands.usc.edu/). The region extended from –240 bp to + 39 bp of the *DAZ1* gene, including the transcriptional point (Figure 1A). The primers and the PCR product were shown in Figure 1B.

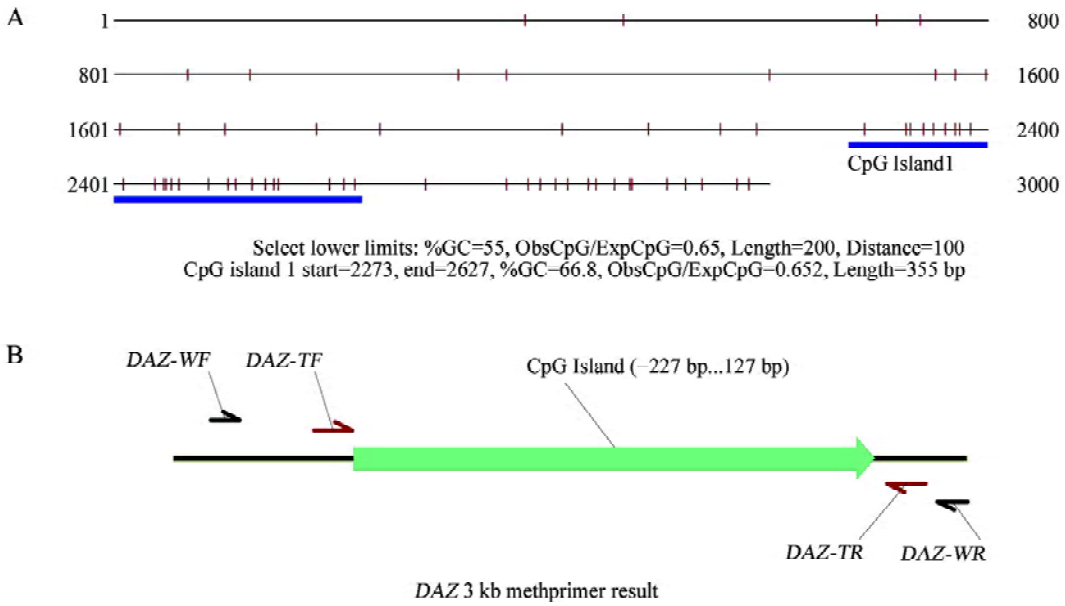



Figure 1. (A): *DAZ1* gene promoter region and the CpG island. (B): Primers designed for CpG island identification and bisulfated sequencing.

*3.2 DAZ-W PCR amplification and sequencing*

We extracted genomic DNA from the sperm and leucocytes of four men who had fathered children normally and amplified the CpG island region with *DAZ-W* primers. All DNA samples of spermatic and male somatic cells gave a *DAZ-W* PCR product of about 500 bp; female genomic DNA gave no products for *DAZ-W* primers, as shown in Figure 2. After sequencing, we aligned the product sequence with the sequence from NCBI with DNAMAN(Version 5.2.2, Lynnon Biosoft, Quebec, Canada). We found that our sequence was identical to that of NCBI.

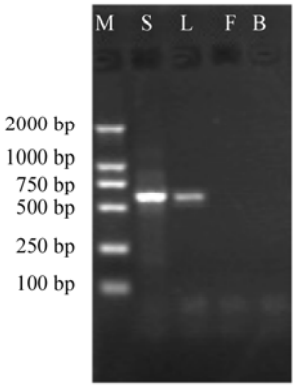


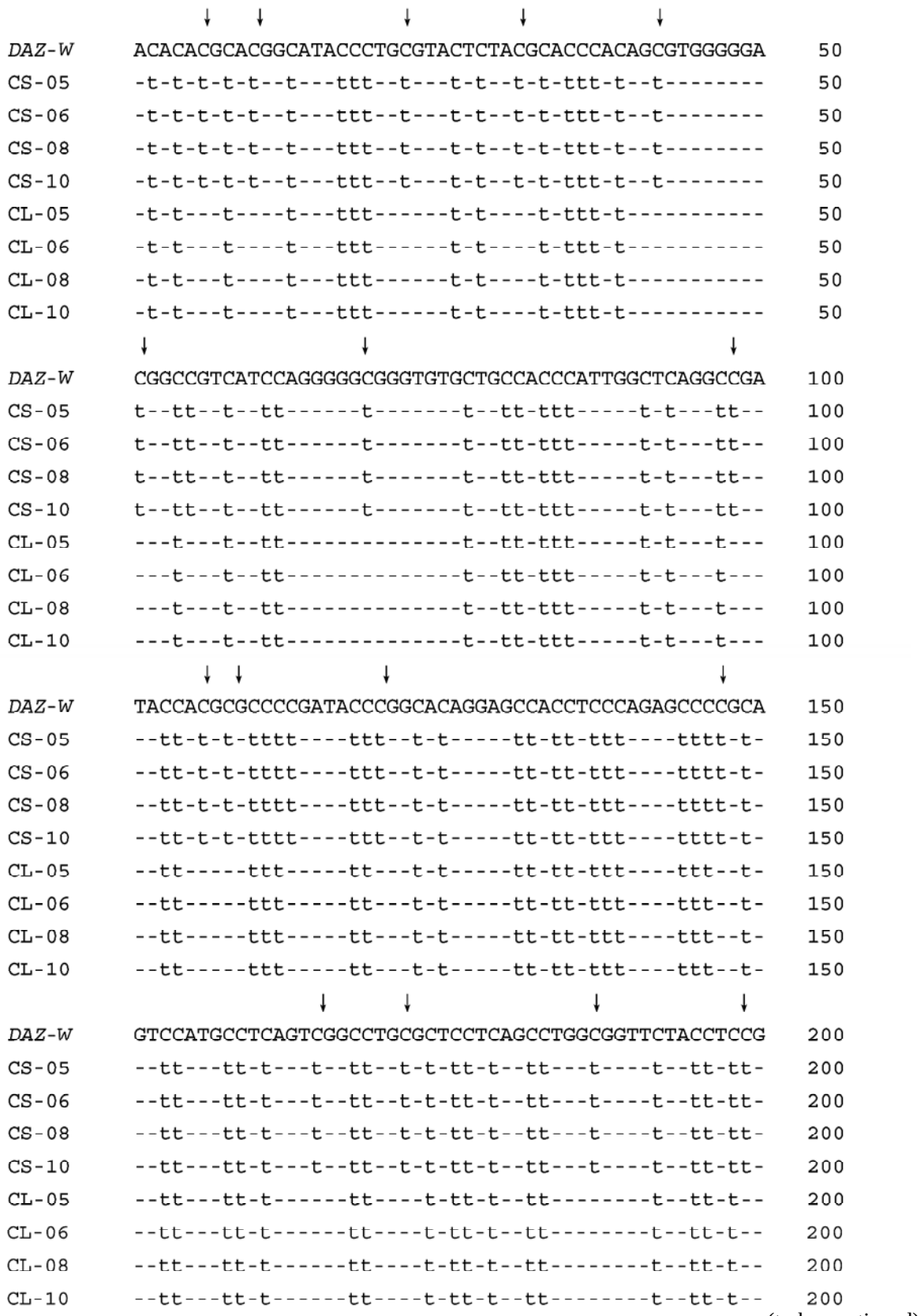

Figure 2. *DAZ-W* PCR. M: DL2000 marker; S: spermatic DNA; L: male leukocyte DNA; F: female leukocyte DNA; B: blank.

*3.3 BSP analysis*

After bisulfate treatment (all unmethylated cytosine was converted to uracil, shown as thymine in the sequencing results, while methylated cytosine was unchanged by this procedure, see black arrow in Figure 3), we amplified the CpG island region using touchdown PCR and sequenced it. Alignment results showed that all the cytosines in the CpG island were converted to thymines in the sperm sample, while they were unchanged in the leukocyte sample. That is, the entire "CpG island" cytosines were methylated in somatic cells, and unmethylated in sperm (Figures 4–6).

## 4 Discussion

DNA methylation is one of the several post-synthetic modifications that normal DNA undergoes after each replication. In mammals, this modification is carried out by the enzyme DNA methyltransferase, which acts on the DNA sequence 5'-CpG-3'. DNA methylation or demethylation can change the interaction between protein and DNA, and affect gene transcription or change chromatin structure. A gene may be silenced or expressed in different cells or tissues by changing DNA methylation and demethylation. DNA methylation can subsequently impart an inactivating marker on DNA that can be propagated semi-conservatively through many cell divisions.




(to be continued)

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                    ↓   ↓
DAZ-W      AGGGTTCGCCCGCCCTTGGTTTTCCTTACACCTTAGCCTTTGGCTCCTTT   250
CS-05      ------t-ttt-ttt--------tt---t-tt----tt-----t-tt---   250
CS-06      ------t-ttt-ttt--------tt---t-tt----tt-----t-tt---   250
CS-08      ------t-ttt-ttt--------tt---t-tt----tt-----t-tt---   250
CS-10      ------t-ttt-ttt--------tt---t-tt----tt-----t-tt---   250
CL-05      --------tt--ttt--------tt---t-tt----tt-----t-tt---   250
CL-06      --------tt--ttt--------tt---t-tt----tt-----t-tt---   250
CL-08      --------tt--ttt--------tt---t-tt----tt-----t-tt---   250
CL-10      --------tt--ttt--------tt---t-tt----tt-----t-tt---   250
                     ↓            ↓         ↓
DAZ-W      GACCACTCGAAGCCCCACAGCGTGTTCCAGCGGA                   284
CS-05      --tt-t-t----tttt-t--t-----tt--t---                   284
CS-06      --tt-t-t----tttt-t--t-----tt--t---                   284
CS-08      --tt-t-t----tttt-t--t-----tt--t---                   284
CS-10      --tt-t-t----tttt-t--t-----tt--t---                   284
CL-05      --tt-t------tttt-t--------tt------                   284
CL-06      --tt-t------tttt-t--------tt------                   284
CL-08      --tt-t------tttt-t--------tt------                   284
CL-10      --tt-t------tttt-t--------tt------                   284
```

Figure 3. *DAZ-W* and *DAZ-T* sequence alignment. *DAZ-W,* genomic sequence of *DAZ*; CL05..CL10, BSP sequence from normal male leucocyte DNA; CS05..CS10, BSP sequence from normal sperm DNA. The differentially methylation CpG site was pointed as black arrow.

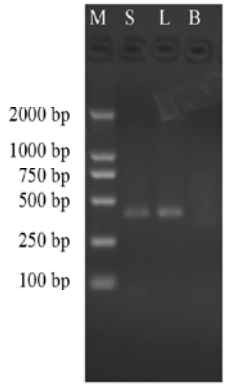


Figure 4. *DAZ-T* PCR results. M: DL2000 Marker; S: Sperm DNA; L: Male leukocyte DNA; B: Blank.

The *DAZ* gene is one of the most important candidate genes for spermatogenesis [3]. Spermatogenesis has a long and complex developmental process. Can mutation or altered expression of genes result in disruption of spermatogenesis? The *DAZ* gene is specifically expressed in the testis, and the transference of the human *DAZ* gene to *DAZ*-like (*DAZL*) null mice can partially rescue the mutant phenotype, suggesting that *DAZ* has a role in spermatogenesis [4]. This gene has 11 exons: exons 2–5 encode a RNA-binding domain, while each of the seven 24-aa repeat sequences of exon 7 in *DAZ* genes encodes a single 24-aa repeat sequence [5]. Four *DAZ* gene are clustered in the *AZF*c region. DAZ and other proteins such as PUM2 (human homolog of Pumilio) can form stable complexes; they are expressed in germ cells, and are required for RNA binding, protein-protein interaction and rescue of the Pumilio mutation in flies. This implies that they are conserved cellular machinery that may be required for germ cell development [6].

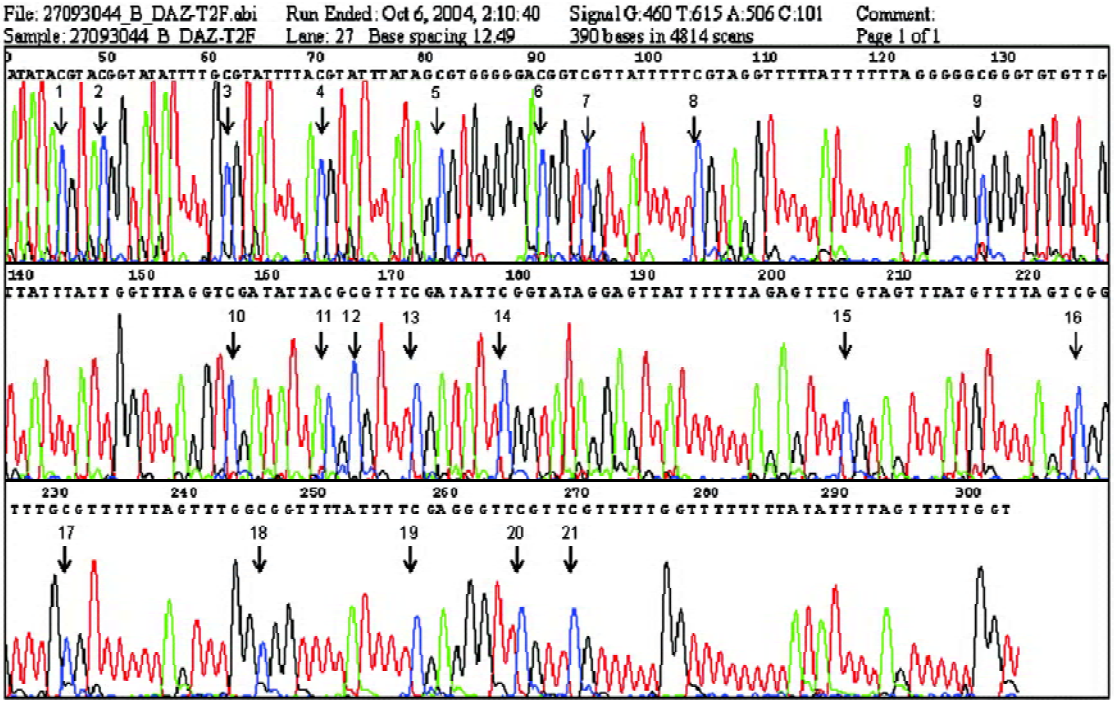


Figure 5. Leukocyte BSP results: None of the cytosines of the CpG site in this region were converted to thymines, while the cytosines outside the CpG cite were converted to thymines. That is, all the cytosines at the CpG site were methylated. The differentially methylation CpG site was pointed as black arrow.

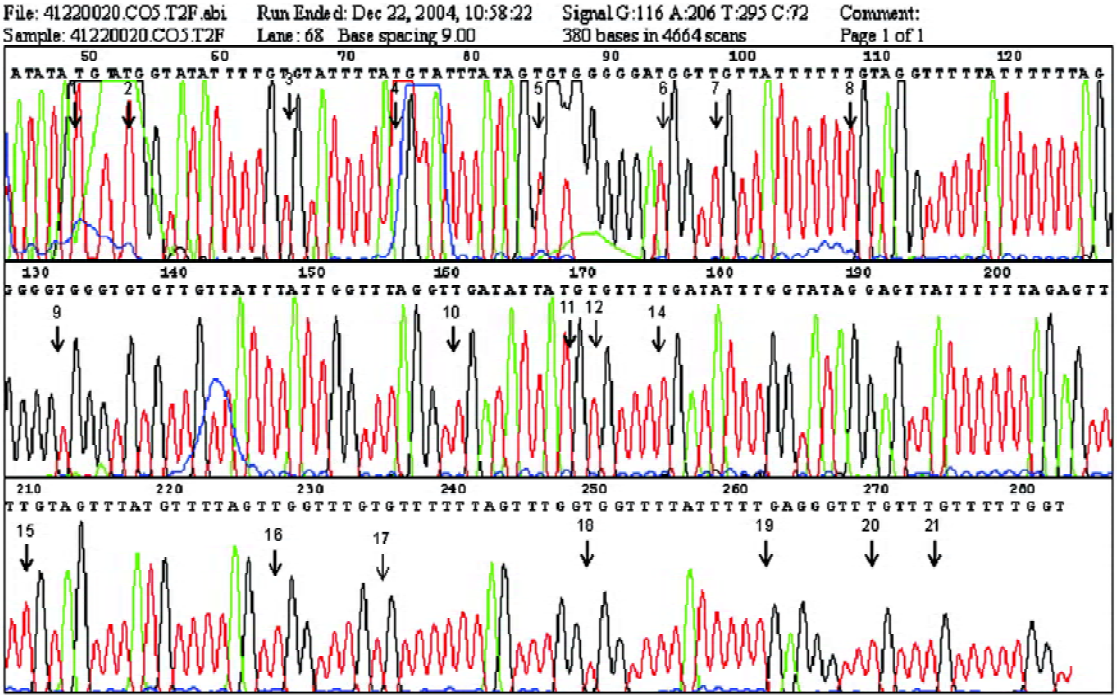


Figure 6. Sperm *DAZ-T* BSP results: All the cytosines in this region were converted to thymines. That is, none of the cytosines at the CpG site in this region were methylated. The differentially methylation CpG site was pointed as black arrow.

Many reports have shown that the *DAZ* gene may be regulated by modifying methylation. By analyzing the expression of genes specific to the Y chromosome in 5-aza-2'-deoxycytidine-treated cell lines, Dasari *et al*. [7] found that many of these genes, such as *DAZ, SRY, RBMY1A, RBMY1H, RBMII, BPY1, PRY* and *TSPY*, are regulated by DNA methylation in prostate cancer cells. Using a methylation-specific restriction enzyme, Chai *et al*. [8] found that both *DAZ* and *DAZLA* genes are methylated in somatic cells and demethylated in embryonic cells [8]. Using the CpG island searcher software (http://cpgislands.usc.edu/), we screened the 3-kb region around the *DAZ1* gene transcriptional point, and found a CpG island. We analyzed this region in spermatic and somatic cells by bisulfate sequencing. Our results showed that in normal men, this region was methylated in somatic cells and unmethylated in spermatic cells. Our results and others, showed that the opposite methylation status in spermatic and somatic cells may reflect the regulation of this gene's expression. Through bisulphate sequencing of *H19* and *MEST* in spermatozoa, studies have shown that the *H19* gene methylation status was changed in patients with moderate and severe oligozoospermia compared with normal individuals. This implied that abnormal genomic imprinting may be associated with hypospermatogenesis [9]. In-depth research on the relationship between methylation status in differentially methylated region (DMR) and male infertility is needed.

Around the promoter region of *DAZ1*, we found a CpG island and demonstrated that it is a DMR in spermatic and somatic cells. Altering DNA methylation in CpG island is a core mechanism for regulating gene expression. This mechanism is strongly affected by environmental factors, such as diet, drugs and intracytoplasmic sperm injection (ICSI) procedures [10]. The relationship between the methylation status of this DMR and male infertility remains to be clarified.

**Acknowledgment**

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**References**

1 Cisneros FJ. DNA methylation and male infertility. Front Biosci 2004; 9: 1189–200.
2 Vogt PH. Molecular genetic of human male infertility: from genes to new therapeutic perspectives. Curr Pharm Des 2004; 10: 471–500.
3 Chiang HS, Yeh SD, Wu CC, Huang BC, Tsai HJ, Fang CL. Clinical and pathological correlation of the microdeletion of Y chromosome for the 30 patients with azoospermia and severe oligoasthenospermia. Asian J Androl. 2004; 6: 369–75.
4 Vogel T, Speed RM, Ross A, Cooke HJ. Partial rescue of the *DA21* knockout mouse by the human *DAZL* gene. Mol Hum Reprod 2002; 8: 797–804.
5 Habermann B, Mi HF, Edelmann A, Bohring C, Backert IT, Kiesewetter F, *et al*. *DAZ* (Deleted in Azoospermia) genes encode proteins located in human late spermatids and in sperm tails. Hum Reprod 1998; 13: 363–9.
6 Moore FL, Jaruzelska J, Fox MS, Urano J, Firpo MT, Turek PJ, *et al*. Human Pumilio-2 is expressed in embryonic stem cells and germ cells and interacts with *DAZ* (Deleted in Azoospermia) and *DAZ*-like proteins. Proc Natl Acad Sci USA 2003; 100: 538–43.
7 Dasari VK, Deng D, Perinchery G, Yeh CC, Dahiya R. DNA methylation regulates the expression of Y chromosome specific genes in prostate cancer. J Urol 2002; 167: 335–8.
8 Chai NN, Phillips A, Fernandez A, Yen PH. A putative human male infertility gene *DAZL*A: genomic structure and methylation status. Mol Hum Reprod 1997; 8: 705–8.
9 Marques CJ, Carvalho F, Sousa M, Barros A. Genomic imprinting in disruptive spermatogenesis. Lancet 2004; 363: 1700–2.
10 Cox GF, Burger J, Lip V, Mau UA, Sperling K, Wu BL, *et al*. Intracytoplasmic sperm injection may increase the risk of imprinting defects. Am J Hum Genet 2002; 71: 162–4.