附件：封面格式

行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※※※
※ ※
※造精功能障礙患者DAZL基因突變篩檢及SNP分析研究※
※ ※
※※※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：∨個別型計畫　□整合型計畫

計畫編號：NSC90－2314－B－041－002－

執行期間：90年8月1日至91年7月31日

計畫主持人：鄧燕妮

共同主持人：郭保麟、林永明

計畫參與人員：鄧燕妮、郭保麟、林永明

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：嘉南藥理科技大學

中　華　民　國　91　年　10　月　14　日

摘要

本研究以163位不明原因男性不孕症患者及116位具生育力正常人為研究對象，萃取血液中基因組DNA，以PCR將*DAZLA*基因表現子及啟動子區域分別放大，進行SSCP分析，對有異常構型的樣本進行DNA定序，選擇適當的限制酵素進行RFLP篩檢。PCR-SSCP篩檢出五個變異點，其中T12A、A54T會造成DAZLA蛋白質中的胺基酸改變，分別以*Dde* I、*Alu* I對上述兩個族群進行RFLP篩檢並進行統計分析。T12A在不孕症與正常人族群的存在比例在整體統計中無顯著差異。T54A的存在比例在不明原因男性不孕症與具生育力正常族群中有顯著差異（$p < 0.01$），T54A與*DAZ*基因缺失之間是否有互補或加成關係仍有待研究。T54A存在於DAZLA蛋白的RBM中，根據DAZLA蛋白質結構推測，此蛋白會與RNA結合，可能調控RNA的轉譯、代謝，故推測T54A的多型性可能會影響DAZLA蛋白與RNA分子的結合力，進而可能導致男性造精功能差異。

關鍵字：單一核酸構形多型性、DAZL基因、造精功能障礙。

ABSTRACT

Single-strand conformation polymorphism (SSCP) analysis of exon-containing genomic DNA segments of the *DAZL* gene was performed in 163 infertile Taiwanese men presenting with severe oligozoospermia and non-obstructive azoospermia. An A→G transition at nucleotide 386 (386 A→G) in exon 3 was identified. The mutation is located within the RNA recognition motif (RRM, aa 32-117) domain of the *DAZL* protein and will lead to Thr54→Ala change (T54A) of *DAZL* protein. Analysis of cDNA from testicular tissue of infertile carriers showed absence of expression for the T54A allele, implying that T54A polymorphism is hardly, if ever, expressed at the RNA level. The frequencies of T54A allele in patients and control group were 7.39% and 0.86%, respectively (P = 0.0003). The phenotypes varied significantly in cases with heterozygous T54A polymorphism, ranging from hypospermatogenesis and maturation arrest to Sertoli cell-only syndrome. A combination of *DAZ* gene deletion and T54A polymorphism did not worsen the phenotype. Our findings provide strong evidence for the role of the autosomal *DAZL* gene in human spermatogenesis.

Key Words: Single nucleotide polymorphism, *DAZL* gene, spermatogenic failure

INTRODUCTION

Approximately 5% of otherwise healthy men suffer from involuntary childlessness for which no clinical explanation can be given . In roughly half of these cases the defect can be traced to the man. In recent years, there has been an intensive search for genetic causes of male infertility, of which spermatogenic failure is the most common form. Screening with markers on the long arm of the human Y chromosome has detected Yq microdeletions in 5-15% of males with spermatogenic failure. Among cases with Yq microdeletions, deletion involving the *DAZ* (Deleted in AZoospermia) gene family represents the most frequent finding. The *DAZ* gene has an autosomal homologue, *DAZL* (*DAZ*-Like), on chromosome 3p24. It is highly homologous to the *DAZ* gene, with 83% similarity in the coding region of the cDNA. Both genes encode RNA-binding proteins. It is believed that the *DAZ* gene arose 40 million years ago, during primate evolution, from the transposition, repeat amplification, and pruning of an ancestral autosomal gene *DAZL*.

We found that *DAZL* protein is expressed in different types of male germ cells and that the concentrations of *DAZL* transcripts were lower in men with spermatogenic failure. To date, however, there are no reported instances of *DAZL* gene mutations in infertile men. In the present study, we have identified a single nucleotide polymorphism (SNP) in exon 3 of the *DAZL* gene. This SNP is more prevalent in the group of patients with severe oligozoospermia and non-obstructive azoospermia. To the best of our knowledge, this is the first report on the association of SNP of an autosomal gene with susceptibility to severe spermatogenic failure.

RESULTS

SSCP and DNA sequencing analysis

The alteration in conformation of PCR product was detected as a change in electrophoretic mobility in polyacrylamide gels. Of 11 exons examined, abnormal SSCP patterns were observed with exon 2 and exon 3. The exons with aberrant bands were sequenced to identify molecular lesions. Sequence analyses revealed an A→G transition at nucleotide 260 (260 A→G) in exon 2, and an A→G transition at nucleotide 386 (386 A→G) in exon 3 .

T12A allele frequencies

Among 142 patients presenting with spermatogenic failure, 10 showed heterozygous T12A. The allelic frequency was 3.52 %. The frequency of the T12A allele in the normal group was 2.59 % (6/232). There was no difference in allelic frequency in T12A polymorphism between patients with spermatogenic failure and control subjects (P =0.542).

T54A allele frequencies

Among 142 patients presenting with spermatogenic failure, 21 showed heterozygous T54A with an allele frequency of 7.39 % (21/284). The frequency of the T54A allele was 0.86 % (2/232) in the control group. There was a significant difference in the allelic frequency of T54A polymorphism between patients with spermatogenic failure and control subjects (P = 0.0003).

Sequencing analysis and mutation detection with testicular cDNA

Sequence analysis of testicular cDNA from 9 patients who were heterozygous for the T54A polymorphism in their genomic DNA (No. 3, 10, 32, 35, 38, 40, 85, 97, 161) showed the wild type allele only and there was absence of expected nucleotide substitution (386 A→G) in exon 3. The T54A polymorphism are hardly, if ever, expressed at the RNA level.

Phenotypes and Y-deletion status in infertile patients with T54A polymorphism

A summary of 21 infertile patients with T54A polymorphisms, Y-chromosome microdeletion status, and their corresponding testicular phenotypes are shown in Table 2 and Figure 5. Of the three patients (nos. 85, 97, 165) with both T54A polymorphism and *DAZ* gene deletions, only 2 (nos.85, 97) showed testicular histopathology (maturation arrest and hypospermatogenesis, respectively). It seems that a combination of *DAZ* gene deletion and T54A polymorphism did not worsen the phenotypic expression. The infertile men with heterozygous T54A variant tended to have a higher FSH, LH , prolactin levels, and lower testosterone level as compared with normal ranges of the Han Chinese. However, the hormonal profiles did not differ significantly between infertile men with or without T54A variant.

DISCUSSION

T54A polymorphism in exon 3 is more prevalent in patients with spermatogenic failure. This polymorphism is located within the RNA recognition motif domain of the *DAZL* protein and will create a protein with substitution of threonine by alanine in the 54th amino acid. Threonine in the 54th amino acid is conserved for human *DAZL*, *DAZ* and mouse *Dazl* . It has been shown that RNA recognition motif domain of both *DAZ* and *DAZL* genes is associated with germ-cell specific regulation of mRNA translation through binding to poly (A) RNA . Threonine in the 54th amino acid is conserved for both *DAZ* and *DAZL* genes and might be critical for the RNA-binding function. We showed that RNA level is not detectable for the T54A allele in the testicular tissue of infertile carriers. Presumably, T54A polymorphism creates a transcript with impaired stability, which in turn affects stability, editing, alternative polyA site selection, proper localization, or translational activation/repression of target RNAs. Further studies are required to investigate the mechanisms involving stability of the T54A transcript and the effect of this variant on proteins or RNAs interacting with the *DAZL* protein.

The frequency of T54A was 0.86% and 7.39% in control subjects and infertile men, respectively. The expected frequency of T54A homozygote would be 1 in 13521 and 1 in 1 in 183 in the control subjects and infertile men, respectively. It would be enlightening to observe the phenotype of subjects homozygous for the T54A polymorphism. It is possible, however, that men homozygous for the T54A variant may not be compatible with reproduction considering absence of specific variant in the heterozygous carriers.

In the present study, we screened the polymorphisms out of the entire coding sequence of the *DAZL* gene and identified a SNP (T54A variant) located within the RNA recognition motif domain. We found an association between the T54A variant and a susceptibility to spermatogenic failure. To the best of our knowledge, the T54A variant of *DAZL* is the first SNP of autosomal genes associated with a susceptibility to severe spermatogenic failure. Our finding provides strong evidence for the role of the autosomal *DAZL* gene in human spermatogenesis.

成果自評：

本研究已完成有關的計畫內容項目如下：

1. 完成 163 位無精蟲症及嚴重寡精蟲症患者及 116 位具生育能力的正常人血液樣品或睪丸組織的收集，並完成基因組 DNA 的萃取。
2. 以多聚合酵素鏈反應完成 163 位患者及 116 位正常生育能力對照組的 *DAZLA* 基因啟動子及 11 個表現子 DNA 片段的放大。
3. 以單股核酸構形多型性(SSCP)方法完成 163 位患者及 116 位正常生育能力對照組的 *DAZLA* 基因啟動子及 11 個表現子 DNA 片段多型性分析。
4. 針對特定的變異或多型性點設計 RFLP 及 ASO 進一步分析，並對所有樣品群做進一步篩檢
5. T12A 在不孕症與正常人族群的存在比例在整體統計中無顯著差異。T54A 的存在比例在不明原因男性不孕症與具生育力正常族群中有顯著差異（$p < 0.01$），T54A 與 *DAZ* 基因缺失之間是否有互補或加成關係仍有待研究。T54A 存在於 DAZLA 蛋白的 RBM 中，根據 DAZLA 蛋白質結構推測，此蛋白會與 RNA 結合，可能調控 RNA 的轉譯、代謝，故推測 T54A 的

多型性可能會影響 DAZLA 蛋白與 RNA 分子的結合力，進而可能導致男性造精功能差異。
6.本研究成果符合當初預期完成的內容與目標，並能得到國際重要期刊編審者的認同，對於男性不孕症發生的成因探討具有其重要的意義。有關本研究計畫成果已發表於 *The Journal of Clinical Endocrinology & Metabolism*（in press）（如附件）

參考文獻

Teng YN, Lin YM, Lin YH, Tsao SY, Hsu CC, Lin SJ, Tsai WC, Kuo PL 2002 Association of a Single Nucleotide Polymorphism of the *DAZL* Gene with Susceptibility to Spermatogenic Failure. The Journal of Clinical Endocrinology & Metabolism（in press）

balt1/eg-jcem/eg-jcem/eg1102/eg8942-02a | hurtr | S=9 | 9/17/02 | 10:21 | 4/Color Figure(s): 2,4 | Art: 1019235 | Input-RWS

0013-7227/02/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 87(11):0000–0000
Copyright © 2002 by The Endocrine Society
doi: 10.1210/jc.2002-020016

Association of a Single-Nucleotide Polymorphism of the Deleted-in-Azoospermia-Like Gene with Susceptibility to Spermatogenic Failure

YEN-NI TENG, YUNG-MING LIN, YING-HUNG LIN, SHU-YI TSAO, CHAO-CHIN HSU, SHIO-JEAN LIN, WAN-CHING TSAI, AND PAO-LIN KUO

Department of Early Childhood Education and Nursery (Y.-N.T.), Chia Nan University of Pharmacy and Science, Tainan 717; Departments of Urology (Y.-M.L., W.-C.T.), Medicine (S.-Y.T.), Pediatrics (S.-J.L.), and Obstetrics and Gynecology (P.-L.K.) and Institute of Molecular Medicine (Y.-H.L.), National Cheng Kung University College of Medicine, Tainan 704; and Taiwan United Birth Promoting Experts (C.-C.H.), Tainan 700, Taiwan

AQ: A

Single-strand conformation polymorphism analysis of exon-containing genomic DNA segments of the deleted-in-azoospermia-like (*DAZL*) gene was performed in 160 infertile Taiwanese men presenting with severe oligozoospermia and nonobstructive azoospermia. An A→G transition at nucleotide 386 in exon 3 was identified. The mutation is located within the RNA-recognition motif (aa 32–117) domain of the *DAZL* protein and will lead to Thr54→Ala change (T54A) of *DAZL* protein. Analysis of cDNA from testicular tissue of infertile carriers showed absence of expression for the T54A allele, implying that the allele carrying T54A polymorphism is hardly, if ever, expressed. The frequencies of T54A allele in patients and the control group were 7.39% and 0.86%, respectively (*P* = 0.0003). The phenotypes varied significantly in cases with heterozygous T54A polymorphism, ranging from hypospermatogenesis and maturation arrest to Sertoli cell-only syndrome. A combination of *DAZ* gene deletion and T54A polymorphism did not worsen the phenotype. Our findings provide strong evidence for the role of the autosomal *DAZL* gene in human spermatogenesis. (*J Clin Endocrinol Metab* 87: 0000–0000, 2002)

APPROXIMATELY 5% OF otherwise healthy men suffer from involuntary childlessness for which no clinical explanation can be given (1). In roughly half of these cases, the defect can be traced to the man. In recent years, there has been an intensive search for genetic causes of male infertility, of which spermatogenic failure is the most common form. Screening with markers on the long arm of the human Y chromosome has detected Yq microdeletions in 5–15% of males with spermatogenic failure. Among cases with Yq microdeletions, deletion involving the *DAZ* (deleted in azoospermia) gene family represents the most frequent finding (2–6). The *DAZ* gene has an autosomal homolog, *DAZL* (*DAZ*-like), on chromosome 3p24. It is highly homologous to the *DAZ* gene, with 83% similarity in the coding region of the cDNA. Both genes encode RNA-binding proteins (7–10). It is believed that the *DAZ* gene arose 40 million years ago, during primate evolution, from the transposition, repeat amplification, and pruning of an ancestral autosomal gene *DAZL* (7).

In addition to their presence in human beings, *DAZ* orthologs are present only on the Y chromosomes of great apes and Old World monkeys. Other mammals have only the autosomal *DAZL* gene (11–15). In many species, *DAZL* homologs are essential for the differentiation of germ cells. For example, the loss of *Boule* results in a meiotic arrest and azoospermia in *Drosophila* (16). A loss of germ cells and the absence of gamete production were observed in *Dazl* knockout mice. The spermatogenic defects of *Boule* flies could be partially rescued by the *Xenopus* Xdazl gene (14). Similarly, sterility of *Dazl* knockout *mice* was also partially rescued by the human *DAZ* gene (17). These facts indicate functional conservation of *DAZ*, *Dazl*, and *Xdazl*.

AQ: B

We have previously determined the expression patterns and transcript concentrations of *DAZL* in the human testes. We found that *DAZL* protein is expressed in different types of male germ cells and that the concentrations of *DAZL* transcripts were lower in men with spermatogenic failure (18). These findings suggest the important roles of the *DAZL* gene in human spermatogenesis. Another approach to investigate the role of *DAZL* is to detect mutations or polymorphisms in infertile men. To date, however, there are no reported instances of *DAZL* gene mutations in infertile men. In the present study, we have identified a single-nucleotide polymorphism (SNP) in exon 3 of the *DAZL* gene. This SNP is more prevalent in the group of patients with severe oligozoospermia and nonobstructive azoospermia. To the best of our knowledge, this is the first report on the association of SNP of an autosomal gene with susceptibility to severe spermatogenic failure.

Subjects and Methods

Subjects

From January 1997 to June 2000, we studied a total of 163 consecutive, unselected infertile men presenting with severe oligozoospermia or nonobstructive azoospermia. One hundred sixteen fertile men were enrolled as controls. All study and control subjects belonged to Han

Abbreviations: AZF, Azoospermia factor; DAZ, deleted in azoospermia; DAZL, DAZ-like; PRL, prolactin; IRMA, immunoradiometric assay; SCO, Sertoli cell-only syndrome; SNP, single-nucleotide polymorphism; SSCP, single-strand conformation polymorphism; STS, sequence-tagged-site; T12A, Thr_{12}→Ala change; T54A, Thr_{54}→Ala change.

baltl/eg-jcem/eg-jcem/eg1102/eg8942-02a | hurtr | S=9 | 9/17/02 | 10:23 | 4/Color Figure(s): 2,4 | Art: 1019235 | Input-RWS

Chinese, the major ethnic group in Taiwan (making up more than 95% of the country's population). They are distributed around 5 counties in Southern Taiwan: Yun-Lin, Chia-Yi, Tainan, Kaoshiung, and Ping-Tung. The control subjects were recruited from husbands of women who received regular prenatal care at the University Hospital. All of the control subjects had fathered at least 2 children without assisted reproductive technologies. The experimental design was in accord with the Helsinki Declaration of 1975 on human experimentation, and signed informed consent was obtained for all enrollees. All patients underwent comprehensive surveillance, including a detailed history taking, physical examination, at least 2 semen analyses, endocrinology profiles testing [LH, FSH, prolactin (PRL), and testosterone], karyotyping, and a molecular test for Y-chromosome microdeletions. Severe oligozoospermia was defined as sperm count less than 5×10^6/ml. In-patients with highly suspected nonobstructive azoospermia were advised to undergo bilateral testicular biopsies. Nonobstructive azoospermia was defined as: 1) spermatogenic defects in the testicular biopsy [such as hypospermatogenesis, maturation arrest, and Sertoli cell-only syndrome (SCO)]; or 2) elevated serum FSH level, total testicular volume less than 30 ml, and none of the other diagnoses applicable. Semen analysis was performed according to the standard methods outlined by the World Health Organization (1). Serum levels of FSH, LH, PRL, and testosterone were measured by using commercial RIA kits: Coat-A-Count FSH immunoradiometric assay (IRMA), Coat-A-Count LH IRMA, Coat-A-Count PRL IRMA, and IMMULITE Total Testosterone (Diagnostic Products, Los Angeles, CA). The intraassay and interassay precision coefficients of variation were 2.4% and 4% for FSH, 1.2% and 2.2% for LH, 1.9% and 2.4% for PRL, and 6.7% and 7.7% for testosterone, respectively. Chromosome analysis was performed using the G-banding by trypsin-Giemsa technique. Molecular analysis of Y-chromosome microdeletions included a combination of 24 sequence-tagged-site (STS)-based markers mapped to intervals 5 and 6 of Yq11 and 16 gene-based primers as described in our previous publications (19–21). All infertile patients and control subjects underwent a Y-chromosome deletion test.

AQ: C

Mutation screening by single-strand conformation polymorphism (SSCP)

Subjects with abnormal karyotypes and other recognizable causes of male infertility were excluded from screening. Genomic DNA was extracted from peripheral blood samples using a Puregene DNA isolation kit (Gentra, Minneapolis, MN). PCRs were performed in 20-μl vols containing 200 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 200 μM deoxynucleotide triphosphates, 100 pmol of each primer, and 1 U *Taq* DNA polymerase (Promega Corp., Madison, WI). The primers and PCR conditions for different exons of *DAZL* gene are listed in Table 1. PCR analyses were performed in an automated thermal cycler (OmniGene Thermal Cycler; Hybaid Ltd., Ashford Middlesex, UK). PCR products of exons 5–6 were restricted into 295- and 230-bp fragments by *StuI* for SSCP analyses (Table 1). The PCR products, with or without treatment by restriction enzymes, were mixed with an equal volume of formamide buffer (95% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol). The mixtures were denatured for 5 min at 95 C and were then cooled rapidly on ice for 1 min. For each sample, 5-μl mixtures were subjected to SSCP analysis using GeneGel Excel gels as recommended by the manufacturer (Pharmacia Biotech, Uppsala, Sweden). Before analysis by SSCP, all PCR products had been sequenced to assure that there was no cross-amplification with *DAZ*. After SSCP analysis, the PCR products with aberrant band-shift were subjected to sequence analysis to identify mutations or polymorphisms. Sequence analysis was performed with an automatic sequencer (ABI 377; PE Applied Biosystems, Foster City, CA).

T1

Mutation detection by restriction enzymes in genomic DNA

A mutation in exon 2 of the *DAZL* gene was digested with *DdeI* (BM Biochemica, Mannheim, Germany) and separated on a 2.5% agarose gel. A mutation in exon 3 was digested with *AluI* (BM Biochemica) and separated on a 12% polyacrylamide gel.

Confirmation of DAZL variants with testicular cDNA

The testicular tissue from patients was stored in liquid nitrogen using 2-methylbutane as a cryoprotectant until use. Before the isolation of total cellular RNA, each specimen was sliced into 10-μ-thick pieces. RT-PCR was performed according to methods described previously (18). In brief, total cellular RNA was extracted using standard methods (High Pure RNA Tissue Kit; Roche Molecular Biochemicals, Indianapolis, IN) and quantified by total absorbance at 260 nm. For the synthesis of cDNA, 12-μl aliquots of master mixture containing 2 μl RNA, 1 μl oligo(dT)$_{12-18}$ primer (500 ng/μl; Life Technologies, Inc., Grand Island, NY), and 9 μl DEPC-treated water were heated to 70 C for 10 min and put on ice. RT-PCRs were performed in 20-μl aliquots containing master mixture, 4 μl 5× first-strand synthesis buffer, 0.1 M dithiothreitol, 10 mM of each

TABLE 1. Primer sets used for PCR

Amp.[a] frag.	Primer pairs	Primer sequence[b]	Anneal.[c] temp. C	Cond.[d] (mmol/$MgCl_2$)	Product size (bp)
Exon 1	DAZL1-F	CCACGCGCCCCGATACCCGGC	73	1.2	303
	DAZL1-R	GTGAGTTGAGGGAGAGGCCCGAG			
Exon 2	DAZL2-F	CCTGTGTATCTAATTATGATG	56	1.5	264
	DAZL2-R	CCTTAAGTTTGTAACAGGGCC			
Exon 3	DAZL3-F	GAATGCTGAATTTTTACTCTTGAAG	62	1.5	181
	DAZL3-R	CTCTATACGTGGCTAGAGTTC			
Exon 4	DAZL4-F	GTCATGATCACTTCCGTATATAG	60	1.5	210
	DAZL4-R	ACTGAGTATATCACTTGACAC			
Exons 5–6	DAZL56-F	GGGTAAGGTAGCTTCATGATG	58	1.5	525
	DAZL56-R	GTAATTCCACAGAAGGTACGAT			(295 & 230)[e]
Exon 7	DAZL7-F	GAATTCTGCATTGTTGTATATAG	60	1.5	291
	DAZL7-R	AAATGACAAACCATTCAGACAA			
Exon 8	DAZL8-F	AGGAGCCAGCATGATAAGTAC	56	2.5	230
	DAZL8-R	CGATCAAGAAATATAGTTAACCC			
Exon 9	DAZL9-F	CTGTATATCTGTTTAATTACAC	60	1.5	243
	DAZL9-R	GCTGAAGGATGATTGCTTCTC			
Exon 10	DAZL10-F	GAGTGAGTGAAAGAGTGGTC	62	1.5	202
	DAZL10-R	CTTTAAAGCTAACAAGTGTGC			
Exon 11	DAZL11-F	AGTAAAAGGACTCTTTCCGTC	60	1.5	254
	DAZL11-R	TCTAATGAAGAACAGTTTAGG			

[a] Amp. frag., Amplified fragment.
[b] The nucleotide sequences in Chai et al. (10).
[c] Anneal. temp. C, Annealing temperature in degrees Centigrade.
[d] Cond. (mmol/$MgCl_2$), Conditions.
[e] The PCR product was digested with restriction enzyme *StuI*. The numbers are the digested fragment lengths.

balt1/eg-jcem/eg-jcem/eg1102/eg8942-02a | hurtr | S=9 | 9/17/02 | 10:21 | 4/Color Figure(s): 2,4 | Art: 1019235 | Input-RWS

deoxynucleotide triphosphate, and 200 U Superscript II RNase H$^-$ reverse transcriptase (Life Technologies, Inc.). The reverse transcriptase temperature profile was 42 C for 1 h, 75 C for 15 min, and final cooling to 4 C. The cDNA was aliquoted and stored at -20 C until use. Primers for the first round of PCR were 5′-CACGCCTCAGTCCGCCTGCGC-3′ (nucleotides 1–21, sense) and 5′-TCAGACCAACAAAATTCTGAC-3′ (nucleotides 1562–1582, antisense) (9) for amplification of the whole coding region of *DAZL* transcript. The PCR reactions were carried out at 94 C for 1 min, 55 C for 1 min, and 72 C for 1.5 min for 40 cycles, with a final extension at 72 C for 10 min. The PCR products were subjected to sequence analysis to identify mutations or polymorphisms. Sequence analysis was performed as previous described. For confirmation of T54A (Thr_{54}→Ala change) variant by the restriction enzyme, a second round of PCR was performed using products of the first round PCR as templates. Primers for nested PCR were 5′-TTCATCTTTGGCTCCTTT-GAC-3′ (nucleotides 91–111, sense) and 5′-CATATCTAGCAAAGAG-GCTTC-3′ (nucleotides 396–416, antisense) for *DAZL* 5′ end fragment. The PCR reactions were carried out at 94 C for 1 min, 62 C for 1 min, and 72 C for 1 min for 40 cycles, with a final extension at 72 C for 10 min. The PCR products containing *DAZL* 5′ end cDNA fragment were digested with *AluI* (BM Biochemica) and separated on an 8% polyacrylamide gel.

AQ: D

Statistical analysis

The allelic frequency was determined as the number of chromosomes harboring polymorphisms divided by the total number of chromosomes analyzed. Data were analyzed for statistical significance by a χ^2 test. A *P* value < 0.05 was considered statistically significant.

Results

Patient characteristics

Among the 163 patients, 21 showed abnormal karyotypes: 17 with *47,XXY*, 1 with pericentric inversion of chromosome 11: *46,XY, inv (11)(p12q23.3)*, and 3 with gross structural rearrangements of the Y chromosome. All patients with gross karyotypic abnormalities were excluded. Among the 142 patients included in this study, 47 showed severe oligozoospermia, and 95 showed nonobstructive azoospermia.

AQ: E

SSCP and DNA sequencing analysis

Except for fragments containing exons 5 and 6, PCR products had been digested by restriction enzymes for SSCP analyses. The alteration in conformation was detected as a change in electrophoretic mobility in polyacrylamide gels. Of 11 exons examined, abnormal SSCP patterns were observed with exon 2 and exon 3 (Fig. 1). The exons with aberrant bands were sequenced to identify molecular lesions. Sequence analyses revealed an A→G transition at nucleotide 260 in exon 2, and an A→G transition at nucleotide 386 in exon 3 (Fig. 2).

F1 F2

Mutation detection by restriction enzymes in genomic DNA

The mutation in exon 2 leads to a Thr_{12}→Ala change (T12A) of the *DAZL* protein. The T12A variant creates a *DdeI* restriction site (CTNAG). On digestion, gel electrophoresis showed 67- and 197-bp fragments for the variant, instead of the 264-bp fragments for the wild-type allele (Fig. 3A). The mutation in exon 3 leads to a T54A of *DAZL* protein. The T54A variant creates an *AluI* restriction site (AGCT). After restriction enzyme digestion, gel electrophoresis showed 53-, 13-, and 115-bp fragments for the variant, instead of the 66- and 115-bp fragments for the wild-type allele (Fig. 3B).

F3

T12A allele frequencies

Among 142 patients presenting with spermatogenic failure, 10 showed heterozygous T12A. The allelic frequency was 3.52% (10 of 284). The frequency of the T12A allele in the normal group was 2.59% (6 of 232). There was no difference in allelic frequency in T12A polymorphism between patients with spermatogenic failure and control subjects (*P* = 0.542).

T54A allele frequencies

Among 142 patients presenting with spermatogenic failure, 21 showed heterozygous T54A with an allele frequency of 7.39% (21 of 284). The frequency of the T54A allele was 0.86% (2 of 232) in the control group. There was a significant difference in the allelic frequency of T54A polymorphism between patients with spermatogenic failure and control subjects (*P* = 0.0003).

Lack of association between T12A and T54A alleles

Among 10 patients with T12A polymorphism, 3 patients (nos. 129, 161, and 171) also had T54A polymorphism.

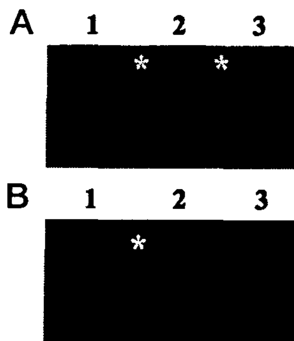

FIG. 1. SSCP analyses of PCR products spanning (A) the exon 2 and (B) exon 3 of *DAZL*. *Stars*, Bands representing the major conformers of mutant alleles.

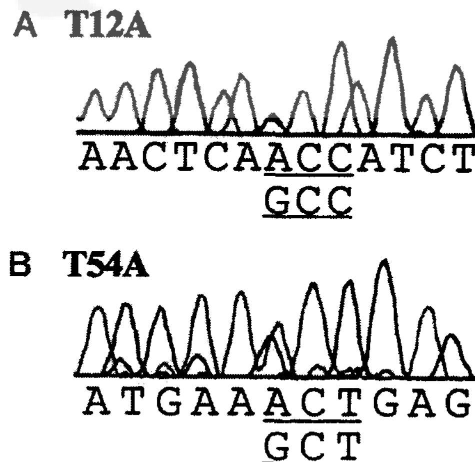

FIG. 2. DNA sequence analysis of the PCR products containing aberrant SSCP conformers. A, The exon 2 of *DAZL* harbors a A→G transition, which results in a threonine (ACC) to alanine (GCC) substitution at codon 12 (T12A); B, the exon 3 of *DAZL* harbors an A→G transition, which results in a threonine (ACT) to alanine (GCT) substitution at codon 54 (T54A).

THE ENDOCRINE SOCIETY

| balt1/eg-jcem/eg-jcem/eg1102/eg8942-02a | hurtr | S=9 | 9/17/02 | 10:24 | 4/Color Figure(s): 2,4 | Art: 1019235 | Input-RWS |

Among 6 control subjects with T12A polymorphism, no one had T54A polymorphism (P = 0.1366). Among 21 patients with T54A polymorphism, only 3 (nos. 129, 161, and 171) were found to have T12A polymorphism (Table 2). Among 2 control subjects with T54A polymorphism, no one had T12A polymorphism (P = 0.5665). Therefore, the T12A allele is not linked with T54A allele.

T2

Sequencing analysis and mutation detection with testicular cDNA

Sequence analysis of testicular cDNA from nine patients who were heterozygous for the T54A polymorphism in their genomic DNA (nos. 3, 10, 32, 35, 38, 40, 85, 97, and 161) showed the wild-type allele only, and there was absence of expected nucleotide substitution (386 A→G transition at nucleotide 386) in exon 3 (Fig. 4A). There were no other mutations or polymorphisms detected in the coding region of *DAZL* using testicular cDNA. After restriction enzyme digestion by *AluI* for the *DAZL* 5′ end cDNA fragment, the wild-type allele is expected to show 206- and 121-bp fragments instead of the 206-, 91-, and 30-bp fragments for the mutant allele. There are no 91- and 30-bp fragments in all of the patients' samples (Fig. 4B), implying that T54A allele is hardly, if ever, expressed at the RNA level.

F4

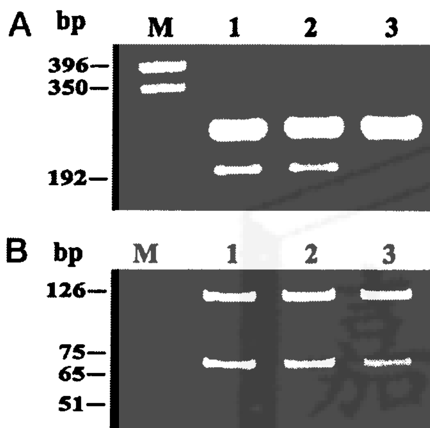

FIG. 3. Restriction enzyme analyses of the mutant alleles. A, 264-bp PCR products of exon 2 from patients (lanes 1 and 2) and control subjects (lanes 3) were digested with *DdeI* and resolved on 2.5% agarose gel. B, 181-bp PCR products of exon 3 from a patient (lane 1) and control subjects (lanes 2 and 3) were digested with *AluI* and resolved on 12% polyacrylamide gel. M, molecular weight marker.

AQ: F

Phenotypes and Y deletion status in infertile patients with T54A polymorphism

A summary of 21 infertile patients with T54A polymorphisms, Y-chromosome microdeletion status, and their corresponding testicular phenotypes are shown in Table 2 and Fig. 5. Briefly, none of control subjects carried Y-chromosomal deletions. Of the 21 patients with T54A polymorphism, testicular histopathologies were found in 9 patients. Their testicular phenotypes varied from hypospermatogenesis (2 patients) and maturation arrest (2 patients) to SCO (5 patients). No specific T54A variant and phenotype correlation could be addressed. Four patients showed microdeletions of one or more Y-specific genes: 3 (nos. 85, 97, and 165) had deletions confined to the azoospermia factor (AZF)c region, and 1 (no. 10) had deletion of the AZFa region. Of the 3 patients (nos. 85, 97, and 165) with both T54A polymorphism and *DAZ* gene deletions, only 2 (nos. 85 and 97) showed testicular histopathology (maturation arrest and hypospermatogenesis, respectively). It seems that a combination of *DAZ* gene deletion and T54A polymorphism did not worsen the phenotypic expression. Some of infertile men heterozygous for the T54A allele had higher FSH, LH, or PRL

F5

TABLE 2. Clinical features and T12A polymorphism in 21 men with T54A polymorphism

Patient number	Age (years)	Sperm count ($\times 10^6$/ml)	FSH[a] (mIU/ml)	LH[a] (mIU/ml)	TT[a] (ng/dl)	PRL[a] (ng/ml)	Biopsy[b]	T12A[c]
3	39	Azoospermia	14.4	2.67	3.00	7.70	MA	−/−
10	30	Azoospermia	24.6	8.9	3.6	26.8	SCO	−/−
12	35	Azoospermia	4.5	4	2.7	12	NA	−/−
18	46	Azoospermia	2.10	1.11	7.9	11.8	NA	−/−
32	39	Azoospermia	4.00	0.74	6.90	4.00	HS	−/−
33	39	Azoospermia	7.20	1.38	4.30	4.40	NA	−/−
35	31	Azoospermia	9.20	2.00	3.50	10.2	SCO	−/−
38	28	Azoospermia	15.6	4.10	1.80	85.4	SCO	−/−
40	37	Azoospermia	22.7	6.70	1.60	32.0	SCO	−/−
50	31	Azoospermia	27.70	11.88	2.20	14.0	NA	−/−
64	42	2.6	22.1	5.3	3.1	15.2	NA	−/−
81	38	Azoospermia	34.1	4.46	0.96	30.1	NA	−/−
85	26	Azoospermia	10.1	1.8	3.8	7.3	MA	−/−
97	35	Azoospermia	13.7	3.9	6.6	8.8	HS	−/−
129	44	Azoospermia	38.9	13.26	2.3	25.7	NA	+/−
131	29	1.2	24.7	15.3	3.1	8.6	NA	−/−
147	36	4.9	7.4	6.5	3.8	7.4	NA	−/−
161	30	Azoospermia	41.1	12.61	5.2	7.8	SCO	+/−
165	27	3.2	8.5	5.2	3.7	9.2	NA	−/−
166	41	0.7	16.3	5.2	4.7	8.3	NA	−/−
171	34	3.3	11.3	2.5	4.1	8.2	NA	+/−

[a] Normal ranges of hormone profiles in the Han Chinese are: FSH = 1–14 mIU/ml, LH = 1.5–9.2 mIU/ml, TT = 2.7–10.7 ng/dl, PRL = 3.1–16.5 ng/ml.
[b] HS, Hypospermatogenesis; MA, maturation arrest; NA, not available; TT, testosterone.
[c] + and −, Presence and absence of T12A allele, respectively.

balt1/eg-jcem/eg-jcem/eg1102/eg8942-02a | hurtr | S=9 | 9/17/02 | 10:21 | 4/Color Figure(s): 2,4 | Art: 1019235 | Input-RWS

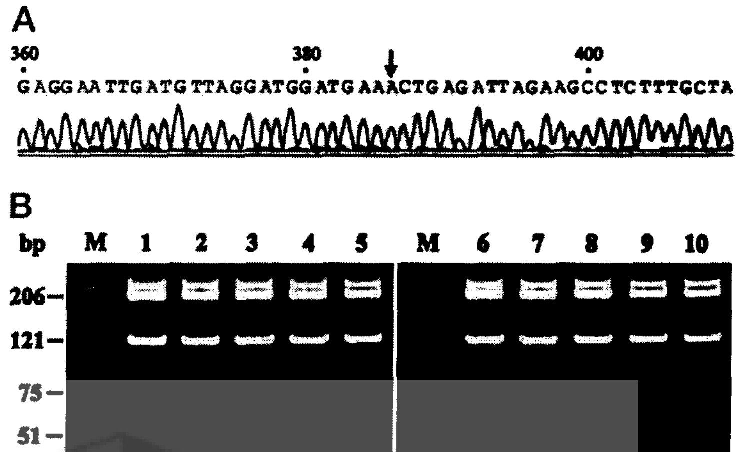

FIG. 4. *DAZL* cDNA sequencing and mutation detection of the mutant allele by enzymatic digestion. A, DNA sequence analysis of *DAZL* cDNA fragments. The cDNA of *DAZL* didn't harbor the A→G transition. The 386th nucleotide is marked by the *arrow*. B, The *DAZL* 5′-end cDNA fragments (lanes 1–9 representing cases 3, 10, 32, 35, 38, 40, 85, 97, and 161, respectively; and lane 10 representing a normal subject) were digested by *AluI* and resolved on an 8% polyacrylamide gel. The mutant allele is expected to show 201-, 91-, and 30-bp fragments. There are no 91- and 30-bp fragments in all of the nine testicular specimens of infertile carriers. M, molecular weight marker.

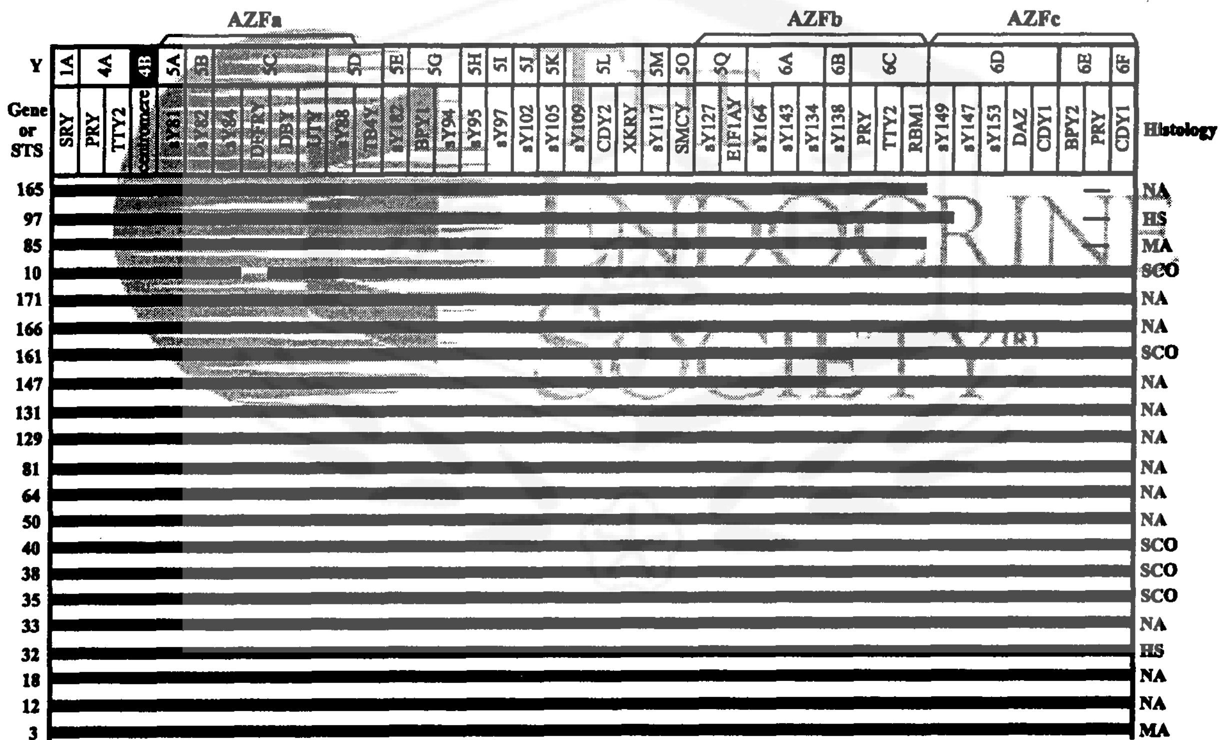

FIG. 5. The Y-chromosomal map of patients with T54A polymorphism and spermatogenic failure. Presence of a gene or STS is indicated by a *vertical black bar*, and absence of a gene or STS is indicated by the absence of any symbol. A *thin vertical line* represents positive PCR results and should be considered presence of a specific gene on the Y chromosome. Testicular histology abbreviations: HS, hypospermatogenesis; MA, maturation arrest; NA, not available.

levels, and a lower level of testosterone (Table 2). However, the hormonal profiles did not differ significantly between infertile men, with or without T54A variant (data now shown).

Discussion

Yen *et al.* (9) have described three other putative polymorphisms of the *DAZL* gene, of which the frequencies and significance in infertile men were not addressed. In the present study, we screened the entire coding sequences of *DAZL* gene, and two SNPs were identified. The frequencies of the two alleles were calculated for the Taiwanese population. The T12A polymorphism is most likely a true variant, given a high prevalence rate in normal fertile men. In contrast, T54A polymorphism in exon 3 is more prevalent in patients with spermatogenic failure. This polymorphism is located within the RNA recognition motif domain of the *DAZL* protein and will create a protein with substitution of threonine by alanine in the 54th amino acid.

balt1/eg-jcem/eg-jcem/eg1102/eg8942-02a | hurtr | S=9 | 9/17/02 | 10:21 | 4/Color Figure(s): 2,4 | Art: 1019235 | Input-RWS

Comparative analysis of 86-residue RNA recognition motif domain of human *DAZL*, human *DAZ*, and mouse *Dazl* reveals that human *DAZL* and mouse *Dazl* differ by only one amino acid substitution, whereas both differ from human Y-encoded *DAZ* at nine residues (7). Threonine in the 54th amino acid is conserved for human *DAZL*, *DAZ*, and mouse *Dazl* (Fig. 6) (8). It has been shown that RNA recognition motif domain of both *DAZ* and *DAZL* genes is associated with germ-cell-specific regulation of mRNA translation through binding to poly (A) RNA (22). The 54th amino acid of DAZL and DAZ is not in the RNP-1 and RNP-2 regions (8), but it is in the highly conserved region of DAZ family proteins, which may be the major determinants of RNA-binding specificity (23). Threonine in the 54th amino acid is conserved for both *DAZ* and *DAZL* genes and might be critical for the RNA-binding function (Fig. 6). We showed that the specific transcript is not detectable for the T54A allele in all infertile carriers with testicular tissues available. Presumably, T54A polymorphism creates a transcript with impaired stability, which, in turn, affects stability, editing, alternative polyA site selection, proper localization, or translational activation/repression of target RNAs (24, 25). Additional studies are required to investigate the mechanisms involving stability of the T54A transcript and the effect of this variant on proteins or RNAs interacting with *DAZL*.

The functions of the *DAZ* gene family are not unknown. From the protein expression pattern, it seems clear that *DAZ*/*DAZL* is involved in mitosis and meiosis of human germ cells (18, 26, 27). Except for Old World monkeys and human beings, a single copy of the *DAZL* gene is sufficient to maintain spermatogenesis in all species, such as *Dazl* in the mouse and *Boule* in *Drosophila*. The reasons for the additional requirement of a Y-chromosome *DAZ* gene family in humans and Old World monkeys are still unclear. It has been suggested that the *DAZ* gene cluster may give a quantitative dosage effect to provide a reproductive advantage during evolution (28). In support of that view, *DAZ* and *DAZL* genes are functionally complementary or synergistic to each other. However, results of the present study and previous investigations seem to prefer a certain degree of redundancy for the *DAZ* family. Deletions of Yq involving the *DAZ* gene are associated with a variable phenotypic expression that can include normal fertility (4–6, 19, 21, 29). In the present study,

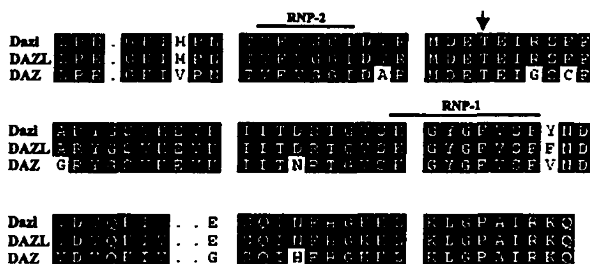

FIG. 6. Comparison of the putative translation products encoded by mouse *Dazl*, *DAZL*, and *DAZ* within their RNA-recognition motif (aa 32–117) domains. The 54th aa is marked by the *arrow*. The highly conserved protein boxes of the RNA-recognition motif domain [RNP-1 (an octapeptide) and RNP-2 (a hexapeptide)] are marked. Protein sequences were aligned by the software PRETTYBOX, highlighting identical amino acids by *black boxes*.

the phenotypes varied significantly in cases with heterozygous T54A polymorphism alone. Even more intriguingly, patients with a combination of *DAZ* gene deletion and T54A polymorphism, such as patient no. 97, may still retain some degree of spermatogenesis. Apparently, other genes involved in spermatogenesis also contribute to the phenotypic expression.

Lilford *et al.* (30) suggested that up to 60% of undiagnosed male infertility arises from autosomal recessive mutations. *DAZL* seems to be an attractive candidate for autosomal recessive infertility. In the mice, the dosage effect of *Dazl* gene mutation on the testicular phenotype is obvious. Male mice lacking one allele of *Dzal* gene showed reduced sperm counts and a high level of abnormal sperm. Male mice with *Dazl* being completely knocked out showed almost an absence of germ cells (16). In our study, men heterozygous for the T54A polymorphism are susceptible to the development of spermatogenic failure, a finding consistent with the dosage effect observed in the transgenic mice model. The frequency of T54A was 0.86% and 7.39% in control subjects and infertile men, respectively. The expected frequency of T54A homozygote would be 1 in 13,521 and 1 in 183 in the control subjects and infertile men, respectively. It would be enlightening to observe the phenotype of subjects homozygous for the T54A polymorphism. It is possible that men homozygous for the T54A variant may not be compatible with reproduction, considering the absence of a specific variant in the infertile carriers.

Krausz et al. (31) have identified a Y-chromosome haplogroup associated with reduced sperm counts. During primate evolution, the *DAZ* gene cluster arose by transposing the autosomal gene on chromosome 3 to the Y, followed by amplification of the transposed unit (7, 32). Therefore, it is tempting to hypothesize that some haplotypes around the *DAZL* locus may predispose to spermatogenic defects or deletion formation of Y chromosome. In the present study, however, we only screened coding sequences of the *DAZL* gene. No attempts have been made to search haplotypes around the *DAZL* locus. Nor did we try to show the association between *DAZL* variants with deletion of *DAZ* gene cluster attributable to the small sample size of cases (only four) with Y deletions. Additional studies are required to confirm or deny the hypothesis.

Other genetic factors have been shown to be associated with impaired production of human sperm. These include HLA-haplotypes, mutations at the mitochondrial DNA polymerase locus, and a polymorphism of cytochrome P4501A1 (33–35). The association between short CAG repeat expansion in X-linked androgen receptor genes and the risk of impaired spermatogenesis remains uncertain (36). Indeed, the Online Mendelian Inheritance in Man database (http://www.ncbi.nlm.nih.gov/omin/) lists about 50 monogenic disorders associated with male infertility, all of which are always associated with a complex phenotypic expression other than male infertility. In the present study, we screened the polymorphisms out of the entire coding sequence of the *DAZL* gene and identified a SNP (T54A variant) located within the RNA recognition motif domain. We found an association between the T54A variant and a susceptibility to spermatogenic failure. To the best of our knowledge, the

| balt1/eg-jcem/eg-jcem/eg1102/eg8942-02a | hurtr | S=9 | 9/17/02 | 10:21 | 4/Color Figure(s): 2,4 | Art: 1019235 | Input-lmm(v) |

T54A variant of *DAZL* is the first SNP of autosomal genes associated with a susceptibility to severe spermatogenic failure. Our finding provides strong evidence for the role of the autosomal *DAZL* gene in human spermatogenesis.

Acknowledgments

We thank Bill Franke for revising the manuscript.

Received January 10, 2002. Accepted July 23, 2002.

Address all correspondence and requests for reprints to: Pao-Lin Kuo, M.D., Division of Genetics, Department of Obstetrics and Gynecology, National Cheng Kung University Hospital, 138 Sheng-Li Road, Tainan, Taiwan 704. E-mail: paolink@mail.ncku.edu.tw.

This work was supported by grants from the National Science Council of the Republic of China (NSC-90-2314-B-041-002, NSC 90-2314-B-006-164, and NSC-90-2314-B-006-168).

References

1. **World Health Organization** 1992 Laboratory manual for the examination of human semen and semen-cervical mucus interaction, ed 3. Cambridge: Cambridge University Press; 3–21
2. **Reijo R, Alagappan RK, Patrizio P, Page DC** 1996 Severe oligospermia resulting from deletions of azoospermia factor gene on Y chromosome. Lancet 347:1290–1293
3. **Vogt PH, Edelmann A, Kirsch S, Henegariu O, Hirschmann P, Kiesewetter F, Kohn FM, Schill WB, Farah S, Ramos C, Hartmann M, Hartschuh W, Meschede D, Behre HM, Castel A, Nieschlag E, Weidner W, Grone HJ, Jung A, Engel W, Haidl G** 1996 Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. Hum Mol Genet 5:933–943
4. **Pryor JL, Kent-First M, Muallem A, Van Bergen AH, Nolten WE, Meisner L, Roberts KP** 1997 Microdeletions in the Y chromosome of infertile men. N Engl J Med 336:534–539
5. **Simoni M, Kamischke A, Nieschlag E** 1998 Current status of the molecular diagnosis of Y-chromosomal microdeletions in the work-up of male infertility. Initiative for international quality control. Hum Reprod 13:1764–1768
6. **McElreavey K, Krausz C** 1999 Sex chromosome genetics '99. Male infertility and the Y chromosome. Am J Hum Genet 64:928–933
7. **Saxena R, Brown LG, Hawkins T, Alagappan RK, Skaletsky H, Reeve MP, Reijo R, Rozen S, Dinulos MB, Disteche CM, Page DC** 1996 The *DAZ* gene cluster on the human Y chromosome arose from an autosomal gene that was transported, repeatedly amplified and pruned. Nat Genet 14:292–299
8. **Shan Z, Hirschmann P, Seebacher T, Edelmann A, Jauch A, Morell J, Urbitsch P, Vogt PH** 1996 A *SPGY* copy homologous to the mouse gene *Dazla* and the *Drosophila* gene *boule* is autosomal and expressed only in the human male gonad. Hum Mol Genet 5:2005–2011
9. **Yen PH, Chai NN, Salido EC** 1996 The human autosomal gene *DAZLA*: testis specificity and a candidate for male infertility. Hum Mol Genet 5:2013–2017
10. **Chai NN, Phillips A, Fernandez A, Yen PH** 1997 A putative human male infertility gene *DAZLA*: genomic structure and methylation status. Mol Hum Reprod 3:705–708
11. **Cooke HJ, Lee M, Kerr S, Ruggiu M** 1996 A murine homologue of the human *DAZ* gene is autosomal and expressed only in the male and female gonads. Hum Mol Genet 5:513–516
12. **Reijo R, Seligman J, Dinulos MB, Jaffe T, Brown LG, Disteche CM, Page DC** 1996 Mouse autosomal homolog of *DAZ*, a candidate male sterility gene in humans, is expressed in male germ cells before and after puberty. Genomics 35:346–352
13. **Eberhart CG, Maines JZ, Wasserman SA** 1996 Meiotic cell cycle requirement for a fly homologue of human deleted in azoospermia. Nature 381:783–785
14. **Houston DW, Zhang J, Maines JZ, Wasserman SA, King ML** 1998 A *Xenopus DAZ*-like gene encodes an RNA component of germ plasm and is a functional homologue of *Drosophlila boule*. Development 125:171–180
15. **Agulnik AI, Zharkikh A, Boettger-Tong H, Bourgeron T, McElreavey K, Bishop CE** 1998 Evolution of the DAZ gene family suggests that Y-linked DAZ plays little, or a limited, role in spermatogenesis but underlines a recent African origin for human populations. Hum Mol Genet 7:1371–1377
16. **Ruggiu M, Speed R, Taggart M, McKay SJ, Kilanowski F, Saunders P, Dorin J, Cooke HJ** 1997 The mouse *Dazla* gene encodes cytoplasmic protein essential for gametogenesis. Nature 389:73–77
17. **Slee R, Grimes B, Speed RM, Taggart M, Maguire SM, Ross A, McGill NI, Saunders PT, Cooke HJ** 1999 A human *DAZ* transgene confers partial rescue of the mouse *Dazl* null phenotype. Proc Natl Acad Sci USA 96:8040–8045
18. **Lin YM, Chen CW, Sun HS, Tsai SJ, Hsu CC, Teng YN, Lin JS, Kuo PL** 2001 Expression patterns and transcript concentrations of the autosomal *DAZL* gene in the testes of azoospermic men. Mol Hum Reprod 7:1015–1022
19. **Lin YM, Chen CW, Sun HS, Hsu CC, Chen JM, Lin SJ, Lin JS, Kuo PL** 2000 Y-chromosome microdeletion and its effect on reproductive decisions in Taiwanese patients presenting with nonobstructive azoospermia. Urology 56:1041–1046
20. **Lin YM, Teng YN, Lee PC, Lin YH, Hsu CC, Lin JS, Kuo PL** 2001 AZFa candidate gene deletions in Taiwanese patients with spermatogenic failure. J Formos Med Assoc 100:592–597
21. **Lin YM, Lin YH, Teng YN, Hsu CC, Lin JSN, Kuo PL** 2002 Gene-based screening for Y-chromosome deletions in Taiwanese men presenting with spermatogenic failure. Fertil Steril 5:897–903
22. **Tsui S, Dai T, Warren ST, Salido EC, Yen PH** 2000 Association of the mouse infertility factor DAZL1 with actively translating polyribosomes. Biol Reprod 62:1655–1660
23. **Burd CG, Dreyfuss G** 1994 Conserved structures and diversity of functions of RNA-binding proteins. Science 265:615–621
24. **Siomi H, Dreyfuss G** 1997 RNA-binding proteins as regulators of gene expression. Curr Opin Genet Dev 7:345–353
25. **Grant SR** 1999 Dissecting the mechanisms of posttranscriptional gene silencing: divide and conquer. Cell 96:303–306
26. **Reijo R, Lee TY, Salo P, Alagappan R, Brown LG, Rosenberg M, Rozen S, Jaffe T, Straus D, Hovatta O** 1995 Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. Nat Genet 10:383–393
27. **Reijo RA, Dorfman DM, Slee R, Renshaw AA, Loughlin KR, Cooke H, Page DC** 2000 *DAZ* family proteins exit throughout male germ cell development and transit from nucleus to cytoplasm at meiosis in humans and mice. Biol Reprod 63:1490–1496
28. **Gromoll J, Weinbauer GF, Skaletsky H, Schlatt S, Rocchietti-March M, Page DC, Nieschlag E** 1999 The Old World monkey (deleted in azoospermia) gene yields insight into the evolution of the *DAZ* gene cluster on the human Y chromosome. Hum Mol Genet 8:2017–2024
29. **Chang PL, Sauer MV, Brown S** 1999 Y chromosome microdeletion in a father and his four infertile sons. Hum Reprod 14:2689–2694
30. **Lilford R, Jones AM, Bishop DT, Thornton J, Mueller R** 1994 Case-control study of whether subfertility in men is familial. Br Med J 309:570–573
31. **Krausz C, Quintana-Murci L, Rajpert-De Meyts E, Jorgensen N, Jobling MA, Rosser ZH, Skakkebaek NE, McElreavey K** 2001 Identification of a Y chromosome haplogroup associated with reduced sperm counts. Hum Mol Genet 10:1873–1987
32. **Kuroda-Kawaguchi T, Skaletsky H, Brown LG, Minx PJ, Cordum HS, Waterston RH, Wilson RK, Silber S, Oates R, Rozen S, Page DC** 2001 The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. Nat Genet 29:279–286
33. **Tsujimura A, Takahara S, Kitamura M, Miura H, Koga M, Sada M, Tsuji T, Matsumiya K, Okuyama A** 1999 HLA-DR antigen and HLA-DRB1 genotyping genotyping with non-obstructive azoospermia in Japan. J Androl 20:545–550
34. **Rovio AT, Marchington DR, Donat S, Schuppe HC, Abel J, Fritsche E, Elliott DJ, Laippala P, Ahola AL, McNay D, Harrison RF, Hughes B, Barrett T, Bailey DM, Mehmet D, Jequier AM, Hargreave TB, Kao SH, Cummins JM, Barton DE, Cooke HJ, Wei YH, Wichmann L, Poulton J, Jacobs HT** 2001 Mutations at the mitochondrial DNA polymerase (POLG) locus associated with male infertility. Nat Genet 29:261–262
35. **Fritsche E, Schuppe HC, Dohr O, Ruzicka T, Gleichmann E, Abel J** 1998 Increased frequencies of cytochrome P4501A1 polymorphisms in infertile men. Andrologia 30:125–128
36. **Patrizio P, Leonard DG** 2001 Expansion of the CAG trinucleotide repeats in the androgen receptor gene and male infertility: a controversial association. J Androl 22:748–749