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男性生殖障礙患者的 DAZL 單一核酸構形多型性分析及相關研究

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中文摘要

本研究主要探討 DAZL 基因單一核酸構形多型性分析與男性生殖障礙成因的相關性，研究將針對男性造精功能障礙患者進行 *DAZL* SNPs 變異全面性篩檢，並進行 pCEP4-CD5lead-Fc -DAZL-T54A 及 pCEP4-CD5lead-Fc -DAZL-T12A 轉殖質體的構築及細胞表現，分析 T54A 及 T12A 變異點對於 RNA binding 及 *DAZL* 蛋白質表現的影響。本研究以 231 位生殖障礙患者及 191 位具生育力正常人為研究對象進行 260A>G (Thr_{12}→Ala), 386A>G (Thr_{54}→Ala), 520+34c>a, 584+28c>t, and 796+36g>a 等多型性點篩檢及單套型分析統計。其中 386A>G (Thr_{54}→Ala)的發生頻率在患者與正常生育者有顯著差異（$P < 0.0001$）。在連鎖不平衡分析中此五中單一核酸構形多型性點有連鎖不平衡現象。在單套型分析統計方面，在患者群中有三種主要單套型其發生率約為 87.9%，此三種單套型在生育力正常者發生率約為 88%，然個別的單套型發生上有顯著差異，其中五種單套型在患者與正常生育者有顯著差異（$P < 0.05$）。部分的單套型過度表現於患者，推測此與造精功能障礙有關；而部分的單套型正常生育者的表現特別低，因此具有保護之功能。本研究結果推測 DAZL 基因的單套型與男性生殖障礙成因有關，然其是否與人種及地理分佈有關，則需進一步分析。

關鍵字：單一核酸構形多型性、造精功能障礙、單套型、連鎖不平衡分析。

ABSTRACT

To investigate the role of *DAZL* gene in human spermatogenesis, *DAZL* was genotyped in 231 men with spermatogenic defect and in 191 fertile men. Five single nucleotide polymorphisms (SNPs) were analyzed: 260A>G (Thr_{12}→Ala), 386A>G (Thr_{54}→Ala), 520+34c>a, 584+28c>t, and 796+36g>a, the latter three of which were novel. There was a significant difference in the allelic frequencies for the 386A>G polymorphism of *DAZL* gene between infertile men and the control group ($P < 0.0001$). There are three major haplotypes which constitute 87.9% of all the patients' population. Three major haplotypes also constitute 88.0 % of the control population. The linkage disquilibrium map patterns of five SNPs are different for infertile men and control subjects. Of all haplotypes, five showed significant difference in the frequency between infertile men and control subjects ($P < 0.05$). Some haplotypes are over-transmitted in patients with severe spermatogenic failure, while some haplotypes are under-transmitted in the patients. It appears that the over-transmitted haplotyes confers susceptibility to spermatogenic failure, while under-transmitted haplotypes are protective against spermatogenic failure. Results of the present study suggest important roles of the *DAZL* gene in human spermatogenic failure. The functional significance of each haplotype and distribution of haplotpes in different ethnic groups deserve further investigation.

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

INTRODUCTION

Between 2% ad 12% of couples worldwide are affected by reduced by reduced fertility. In roughly half of these cases the defect can be traced to the man (Vassen 1984; World Health Organization Laboratory manual for the Examination of Human Semen and Semen- Cervical Mucus Interaction, 1992). A large proportion of men who are infertile have insufficient sperm production or impaired spermatogenesis (spermatogenic failure). Spermatogenesis is a complex developmental process in which male germ cells progress through mitotic proliferation, two rounds of meiotic divisions, and finally dramatic morphological changes to form mature sperm. The DNA content of the final product is half that of the progenitor cells. This process involves a large portion of the genome of an organism to ensure the quality of final products. It is estimated that mutations in up to 11% of all genes in Drosophila might lead to male sterility (Hackstein et al., 2000). It is likely to be also true for the human being, considering extremely high incidence of infertility in men (De Krester et al., 1997).

Genetic factors have been shown to be associated with impaired production of human sperm. Screening w (AZFs) (Reijo et al., 1996; Vogt et al., 1996; Lahn and Page, 1997; Pryor et al., 1997; Simoni et al., 1998). Other genetic factors involved in spermatogenic failure include HLA-haplotypes, mutations at the mitochondiral DNA polymerase locus, a polymorphism of cytochrome P4501A1 gene, mutations of follicle-stimulating hormone (FSH) receptor gene, FATE gene, and synaptonemal complex protein 3 gene (Trannainen et al., 1997; Fritsche et al., 1998; Tsujimura et al., 1999; Rovio et al., 2001; Olesen et al., 2003; Miyamoto et al., 2003). The association between short CAG repeat expansion in X-linked androgen receptor genes and the risk of impaired spermatogenesis remains uncertain (Patrizio and Leonard, 2001). In a previous study, we have identified a single nucleotide polymorphism (SNP) of the autosomal *DAZL* gene −386 A>G, which confers susceptibility to spermatogenic failure in the Taiwanese population. The goal of the present study was to identify novel SNPs of *DAZL* and to investigate whether specific haplotypes of *DAZL* are associated with male infertility in the Taiwanese population. We identified 9 haplotypes which are over- or under-transmitted in men with severe spermatogenic failure. These findings suggest important roles of *DAZL* in human spermatogenesis.

MATERIALS AND METHODS

Subjects

From January 2001 to June 2004, we studied a total of 281 consecutive, unselected infertile men presenting with severe oligozoospermia or non-obstructive azoospermia. One hundred ninety-one fertile men were enrolled as controls. All study and control subjects belonged to Han Chinese, the major ethnic group in Taiwan (making up more than 95% of the country's population). 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 within 5 years. The experimental design was in accord with the Helsinki Declaration of 1975 on human experimentation, and signed informed consents were obtained for all enrollees. Recruitment of human subjects and evaluation of patients were performed according to our previous studies (Lin et al., 2002a; Teng et al. 2002). In brief, all patients underwent comprehensive surveillance, including a detailed history taking, physical examination, at least 2 semen analyses, endocrinology profiles testing (luteinizing hormone, follicular-stimulating hormone, prolactin, and testosterone), karyotyping, and a molecular test for Y-chromosome microdeletions. Severe oligozoospermia was defined as sperm count less than 5×10^6/ml. Patients with highly suspected non-obstructive azoospermia were advised to undergo bilateral testicular biopsies. Non-obstructive azoospermia was defined as [a] spermatogenic defects in the testicular biopsy (such as hypospermatogenesis, maturation arrest, and Sertoli cell-only syndrome (SCO), or [b] 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 (World Health Organization Laboratory manual for the Examination of Human Semen and Semen- Cervical Mucus Interaction, 1992). Serum levels of FSH, LH, PRL, and T were measured by using commercial radioimmunoassay kits: Coat-A-Count FSH IRMA, Coat-A-Count LH IRMA, Coat-A-Count PRL IRMA, and IMMULITE Total Testosterone (Diagnostic Products Corp., Los Angeles, CA). Chromosome analysis was performed using the GTG method (G-banding by Trypsin-Giemsa technique). Molecular analysis of Y-chromosome microdeletions included a combination of 16 gene-based primers as described in our previous publication (Lin et al., 2002a). Besides infertile patients, all control subjects also underwent Y chromosome deletion test. Subjects with abnormal karyotypes, deletions of the Y chromosome, or other recognizable causes of male infertility were excluded from genotyping for *DAZL*.

Single strand conformation polymorphism (SSCP) and sequence analysis

A total of 112 patients were subjected to SSCP analysis for screening of *DAZL* gene polymorphisms. Genomic DNA was extracted from peripheral blood samples using a Puregene DNA isolation kit (Gentra, Minneapolis, MN, USA). To amplify each exons of *DAZL*, PCRs were performed in 20 μl volumes containing 200 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM

KCl, 0.1% Triton X-100, 200 μM dNTPs, 100 pmol of each primer, and 1 U *Taq* DNA polymerase (Promega, Madison, WI USA). The primers and PCR conditions for different exons of *DAZL* gene have been described in our previous study (Teng et al., 2002). PCR analyses were performed in an automated thermal cycler (OmniGene Thermal Cycler, Hybaid Ltd., Ashford Middlesex, UK). The PCR products 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 ℃ and were then cooled rapidly on ice for 1 min. For each sample, 5 ul mixtures were subjected to SSCP analysis using GeneGel Excel gels as recommended by the manufacturer (Pharmacia Biotech, Uppsala, Sweden). Prior to analysis by SSCP, all PCR products had been sequenced to assure these 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, Applied Biosystems/PE, Foster City, CA, USA).

Genotyping

Primers (sequences available on request) were designed by use of the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Each set of primers was selected to cover the entire coding sequences, exonic/intronic junctions, the 5'- and 3'- un-translated regions. The amplicons were amplified in a multiplex fashion, and each reaction consisting of 50 ng of genomic DNA and 10 pmol of primers in a 20 μl reaction volume containing 4.0 mM MgCl2, 0.2 m*M* deoxynucleotide triphosphates, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The cycling conditions were 95 °C for 5 minutes, followed by 35 cycles at 95 °C for 1 minute, 55 °C for 1 minutes, and 72 °C for 1 minutes and final extension 10 minutes at 72 °C. Each allele was measured by use of primer extension and SNaPshot chemistry (Applied Biosystems). Three μl of multiplex PCR products were treated with 2.5 U of Shrimp alkaline phosphatase (SAP) (Amersham Pharmacia Biotech, Arlington Heights, IL) and 2 U *Exo*I (New England Biolabs, Beverly, MA) in a 10 μl reaction volume containing for 1 hour at 37 °C. SNaPshot multiplex PCR was performed in a 10 μl reaction volume containing 1.25 μl of SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems), 4 μl of SAP/*Exo*I–treated PCR products, and 1 to 3 pmol of each SNaPshot primer. The thermal cycling of SNaPshot reactions was 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 30 seconds. The SNaPshot multiplex reaction was then treated by SAP in an 11 μl reaction volume containing 10 μl of SNaPshot multiplex PCR product and 1 U of SAP. The samples were analyzed using an ABI 3100 genetic analyzer (Applied Biosystems), and the allele determination was carried out with the Genotyper 3.7 program (Applied Biosystems).

Statistical analysis

Tests for association with single markers and haplotypes in our case-control samples were performed by Chi-square test. A *P*-value <0.05 was considered statistically significant. The relative risk of spermatogenic failure was estimated from logistic odds ratios (OR) and 95% CI in multivariate analysis. Maximum likelihood estimates of haplotype frequencies were obtained using the expectation/maximization (EM) algorithm after ruling out deviations from Hardy–Weinberg equilibrium (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Tests for haplotype association with spermatogenic failure were performed using the software EHPLUS (Xie et al. 1993), and statistical significance was estimated using the permutation test PMPLUS (Zhao et al. 2000). Associations between haplotypes and male infertility were analyzed using linear regression models. D' measures of linkage disequilibrium (LD) were calculated by use of the program ldmax within the GOLD software package (http://www.sph.umich.edu/csg/abecasis/GOLD/).

RESULTS

Patients' characteristics

Among 281 patients, 24 showed abnormal karyotypes and 26 were found to have deletion of Y chromosomal genes. All patients with gross karyotypic abnormalities and deletions of Y

chromosomal genes were excluded. Among the 231 patients included in this study, 43 showed severe oligozoospermia, and 188 had non-obstructive azoospermia. None of the control subjects was found to have deletions of Y-chromosomal genes.

Identification of SNPs by SSCP and DNA sequencing analysis

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, 3, 4, 5, and 7 containing DNA fragments. The DNA fragments with aberrant bands were sequenced to identify molecular lesions. Five SNPs －260A>G (Thr_{12}→Ala) in exon 2, and 386A>G (Thr_{54}→Ala) in exon 3, 520+34c>a in intron 4, 584+28c>t in intron 5, and 796+36g>a in intron 7－were identified 112 patents. Of the 5 SNPs, two have been described before － 260A>G in exon 2, and 386A>G in exon 3 (Teng et al., 2002). Three novel SNPs－520+34c>a, 584+28c>t, and 796+36g>a － are shown in Figure 1.

Association of SNPs with susceptibility to spermatogenic failure

We used the five SNPs for genotyping of all enrollees. The allelic frequencies of five SNPs are presented in Table 1. Although the nucleotides in 584+28 and 796+36 are cytosine and guanine, respectively in GenBank (accession number U77471 and U77472, respectively), thymidine and adenine occur more frequently in these two positions for the Taiwanese Han (Table 1). All makers were in Hardy-Weinberg equilibrium ($p < 0.05$). Of five SNPs, only 386 A>G polymorphism is significantly associated with severe spermatogenic failure ($P<0.0001$) with an odds ratio of 11.667 (Table 2).

Linkage disequilibrium between different SNPs

For patients with spermatogenic failure, 260A>G were found to be in complete LD with 584+28c>t and 796+36g>a ($D'=1$). 520+34c>a showed strong LD with 584+28c>t and 796+36g>a ($D' = 0.931$ and 0.945 respectively). 386A>G does not show LD with 260A>G, 520+34c>a, 584+28c>t, and 796+36g>a (Table 3). For control subjects, 260A>G was found to be in complete LD with 386A>G , 584+28c>t and 796+36g>a ($D'=1$). 386A>G showed strong LD with 260A>G, 520+34c>a, 584+28c>t, and 796+36g>a 520+34c>a (($D'>0.926$) (Table 3). The LD map of was apparently different between infertile men and control subjects (data not shown).

Haplotype analysis

The major haplotype for spermatogenic failure is 11111 (accounting for 45.8 % of the population), followed by 11122 (17.8 %), 11211 (15.6 %), and 11222 (8.7%). The frequencies of other haplotype were less than 2% (data not shown). The major haplotypes for the control subjects are 11122 (41.9%) and 11211 (32%) and 11111 (14.1%) (Table 4). The frequencies for other haplotypes were less than 2% (data not shown). Of all haplotypes, seven showed significant difference in the frequency between infertile men and control subjects (Table 4). Haplotypes 11111, 11222, 12122, and 22222 are over-transmitted in patients with severe spermatogenic failure, while haplotypes 11122, 11211, and 11221 are under-transmitted in the patients.

Discussion

In the present study, we confirmed our previous finding that 386A>G allele of *DAZL* confers susceptibility to severe spermatogenic failure in the Taiwanese population. Meanwhile, we identified three novel SNPs which are not associated with spermatogenic failure. Haplotypes including 386A>G do no constitute major haplotypes of *DAZL* because the allelic frequency of 386A>G is rare in both infertile men and control subjects. The major haplotypes for cases with spermatogenic failure are 11111 (45.8 %), 11122 (17.8 %), 11211 (15.6 %), and 11222 (8.7%). These haplotypes account for 87.9% of all the patients' population. The major haplotypes for control subjects are 11122 (41.9%) and 11211 (32%) and 11111 (14.1%), which in combination account for 88.0 % of the control population. It appears that haplotypes 11111, 11222, 12122, and 22222 confer susceptibility to spermatogenic failure, while haplotypes 11122, 11211, and 11221 are

protective against spermatogenic failure.

In the human being, the *DAZ* gene family consists of three members: *BOULE, DAZL* and *DAZ*. *BOULE* is the ancestor gene and is conserved from flies to humans (Xu et al., 2001). In *Drosophila*, *boule* is required for posttranscriptional regulation of gene expression in male germ cells and is considered as a meiotic regulator. Loss-of-function mutations in *boule* results in meiotic arrest of the male fly (Eberhart et al, 1996). In human testis, the *BOULE* protein expression is restricted to stage from leptotene to the stage of late spermatocyte (Xu et al., 2001). There is lack of *BOULE* expression in the spermatocytes of patients with meiotic arrest, suggesting roles of human *BOULE* in spermagoenesis (Luetjens et al., 2004). However, neither mutation nor sequence variant of *BOULE* could be identified in infertile men and controls (Xu et al., 2003; Luetjens et al., 2004).

It was believed that *DAZL* was generated by a transposition of its ancestor gene *BOULE* on chromosome 2 to chromosome 3 (Xu et al, 2001). The protein is expressed in germ cells of diverse species. In *Caenorhabditis elegans*, disruption of *dazl* causes meiotic arrest in oogenesis (Karashima et al., 2000). In *Xenopus, Xdazl* is required for early primordial germ cell differentiation and is indirectly necessary for the migration of primordial germ cells through the endoderm (Houston and King, 1998, 2000). In mice, loss of function of the *DAZL* homologue leads to loss of germ cells in both males and females (Ruggiu et al. 1997). Immunohistochemical studies in human testis reveal that *DAZL* is expressed in many compartments from spermatogonia, meiotic spermatocytes, to mature spermatozoa (Lin et al., 2001; Lin et al., 2002b). We have shown significantly lower transcriptional levels of *DAZL* in men with spermatogenic failure (Lin et al., 2001; Kuo et al., 2004). Results of the present study clearly demonstrate important roles of *DAZL* in human spermatogenesis.

The third member of the *DAZ* gene family is *DAZ* on Y chromosome. It is highly homologous to *DAZL*, with 83% similarity in the coding region of the cDNA (Saxena et al. 1996; Shan et al. 1996; Yen et al. 1996; Chai et al. 1997). In addition to the human being, the *DAZ* orthologs are present only on the Y chromosomes of great apes and Old World monkeys. Other mammals have only the autosomal *boule* and *Dazl* genes (Cooke et al. 1996; Reijo et al. 1996; Eberhart et al. 1996; Houston et al. 1998; Agulnik et al. 1998; Xu et al., 2001). It is believed that the *DAZ* gene arose 40 million years ago, during primate evolution, from the transposition, repeat amplification, and pruning of *DAZL* (Saxena et al. 1996). The *DAZ* protein was found to express from spermatogonia, spermatocyte, round spermatid to mature sperm (Habermann et al., 1998; Reijo et al., 2000). Normal men usually have four copies of *DAZ* (Kurada- Kawaguchi et al., 2001). Complete deletion of *DAZ* gene cluster accounts for ~10% of cases of men with spermatogenic failure (Silber et al., 1998; Ferlin et al., 1999; Krausz et al., 1999; Lin et al., 2002a). However, other AZFs on the AZFc region of Y chromosome are usually deleted along with *DAZ* (Kurada-Kawaguchi et al., 2001). It is therefore very difficult to define the role of *DAZ* gene in human spermatogenesis based on the cases with complete deletions of *DA* gene cluster. There are two types of partial deletion of *DAZ* gene cluster, and both types of deletions had a modest effect on reproductive fitness. The first type － a 1.6-Mb deletion also occurs in about 2% of the United States population (Repping et al., 2003). The second type－ a 1.8 Mb deletion －was described in North Eurasia. Intriguingly, the negative effect of the 1.8 Mb deletion could be counterbalanced by another genetic factor－ possibly a Y-linked factor (Repping et al., 2004). Functions of *DAZ* seem to be affected by as yet un-identified genetic factors considering presence of partial deletions in both fertile men and men with impaired sperm production.

The functions of the *DAZ* gene family are not unknown. All *DAZ* family members encode a highly conserved RNA binding motif (Xu et al., 2001). In *Drosophila*, *Boule* is found to regulate transition from G2 to M phase during meiosis via control of Twine (Cdc25) translation (Maines and Wasserman, 1999). Several messenger RNAs have been shown to be substrate for the *DAZL* protein, including transcripts of Cdc25C, Cdc25A, Pam, GRSF1, and TRF2 (Vemables et al., 2001; Jiao et al., 200). Homology between *BOULE* and *DAZL/DAZ* is limited to the RNA binding motif and the *DAZ* repeat region, which share 78 and 50% similarity in nucleotide sequence. Human *BOULE* and *Xenopus Xdazl* can partially rescue the phenotype of *Drosophila boule* mutant. Likewise, when

human *DAZ* and *DAZL* transgenes was introduced into *Dazl*-null mice, a partial rescue of the mutant phenotype was observed (Houston et al., 1998; Slee *et al*., 1999; Vogel et al., 2002, Xu et al., 2003). These findings suggest a high degree of functional conservation between the *DAZ* family members. Considering partial functional conservation, the *DAZ* family members are likely to share similar sets of interacting proteins and RNA substrates and serve similar functions. On the other hand, the *DAZ* proteins appear to be non-redundant given severe phenotypes (spermatogenic failure) in null mutations of human *DAZ* and mouse *Dazl* genes. Each member therefore may also interact with different proteins or RNA substrates to serve distinct functions.

In the five SNPs of *DAZL*, the 386A>G variant is located within the RNA-binding motif. It is likely that 386A>G substitution significantly reduces binding of substrates which are critical to regulation of cell cycles (Teng et al., 2002). The 260A>G polymorphism is not located in a critical protein domain and is most likely a true variant. Three novel SNPs －520+34c>a, 584+28c>t, and 796+36g>a －are located within introns. 520+34c (a) and 796+36g (a) are located in proximity to the splice donor site, and substitution of cytosine by adenine or guanine by adenine does no seem affect splicing. 584+28c c(t) is located in the pyrimidine tract of intron 5. Substitution of cytosine by thymidine also does no seem to affect splicing. It is not known whether these mutations act as intronic splicing enhancers or silencers or whether they affect stability of mRNA (Nissm-Rafinia et al., 2002). Although neither 584+28c>t nor 796+36g>a is associated with spermatogenic failure, haplotype 11111 has a moderate negative impact on reproductive fitness while haplotype 11122 is protective against spermatigenic failure. The finding suggests a combinatorial effect of 584+28c and 796+36g variants on spermatogenesis. Although haplotype 11111 is deleterious, haplotype 11211 is protective. It is likely that 520+34a allele counteracts the deleterious effect. Alternatively, 520+34a, 584+28c, and 796+36g alleles per se have no effect at al while they are linked to other genetic factors which may modify functions of *DAZL*. Given evidence of partial functional conservation between *DAZ* family members, it would be tempting to hypothesize that *BOULE*, *DAZL* and *DAZ* operate in a complementary or synergistic manner during human spermatogenesis. Therefore, both *BOULE* and *DAZ* are potential candidates of modifier genes for *DAZL*.

It deserve investigation whether 386A>G allele is restricted to certain ethnic groups considering lack of 386A>G allele in the Italian men (Bartoloni et al., 2004). The haplotype 12122 and 22222 include the 386A>G allele and both haplotypes are more prevalent in cases with spermatogenic failure. In a previous study, we have shown absence of expression for the 386A>G allele in the testicular tissue (Teng et al., 1992). It is quite likely that haplotypes 11111, 11222, 12122, and 22222 are associated with suboptimal function of *DAZL* while haplotypes 11122, 11211, and 11221 are associated with optimal function of *DAZL*. In cases with spermatogenic failure, 260A>G was found to be in complete LD with 584+28c>t and 796+36g>a (D′=1). The 520+34c>a allele showed strong LD with 584+28c>t and 796+36g>a (D′> 0.931). However, there is no linkage of 386A>G with other alleles. Paradoxically, 386A>G showed strong LD with other sequence variants in control subjects. It is likely that 386A>G is a deleterious mutation arising recently in certain ethic groups, and development of testicular phenotypes depends on genetic backgrounds of individuals. Of deleterious haplotypes, haplotypes not containing the 386A>G allele (11111 and 11222) are indeed more interesting than those containing the 386A>G allele considering higher prevalence rates in our population. It is noteworthy that all deleterious haplotypes, with or without 386A>G allele, belong to minor haplotypes in the fertile men (in combination making up less than 20% of the population) possibly due to negative selection. The frequencies of haplotypes containing the 386A>G allele are much lower than those not containing the 386A>G allele, suggesting more severe effect on reproductive fitness for those haplotypes.

In the present study, we have described haloptypes of *DAZL* in the Taiwanese Han. Although a large number of association studies have lead to the discovery of genetic factors for many common diseases, there have been few association studies which are focused on sterile genes in the human being (The International HapMap Corsortium, 2003). Y chromosomal haplotypes has been reported to be associated with reproductive fitness in *Drosophila* and men. However, those haplotypes are not related to regions linkage to specific genes (Chippindale and Rice, 2001; Krausz et al., 2001).

For the first time, we show association of haplotypes of an autosomal gene with human spermatogenic failure. The functional significance and distribution of these haplotpes in different ethnic groups deserve further investigation.

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成果自評：

本研究已完成有關的計畫內容項目如下：

1.以多聚合酵素鏈反應完成 231 位無精蟲症及嚴重寡精蟲症患者及 191 位正常生育能力對照組的 *DAZL* 基因表現子 DNA 片段的放大分析。

2.完成 231 位無精蟲症及嚴重寡精蟲症患者及 191 位具正常生育能力者的 DAZL 基因 260A>G (Thr_{12}→Ala), 386A>G (Thr_{54}→Ala), 520+34c>a, 584+28c>t, and 796+36g>a 多型性點篩檢及單套型分析統計。

3. pCEP4-CD5lead-Fc -DAZL-T54A 及 pCEP4-CD5lead-Fc -DAZL-T12A 轉殖質體的構築及細胞表現，然細胞表現為如預期順利，此部分研究有待修正及檢討。

4.DAZL 基因五種單套型在患者與正常生育者表現有顯著差異（$P < 0.05$），部分的單套型過度表現於患者。

5.本研究成果已完成預期部分內容與目標，有關本研究計畫成果將投稿於. Am J Hum Genet（submitted)。

6.未來將可朝 DAZL 基因在不同人種、地理分佈或性別差異等進行分析，將可對 DAZL 基因再生殖上的角色更清楚瞭解。

Table 1 Allelic frequencies of five sequence variants of *DAZL* in infertile men and control subjects

SNP	Allele frequency (%)				Genotype frequency (%)			
	Allele	Control	Spermatogenic failure	P	Genotype	Control	Spermatogenic failure	P
260A>G					AA	93.48	91.94	0.5595
	A	96.74	95.97	0.5671	AG	6.52	8.06	
	G	3.26	4.03		GG	0	0	
386A>G					AA	98.91	88.64	<0.0001
	A	99.46	94.10	<0.0001	AG	1.09	10.79	
	G	0.54	5.90		GG	0	0.57	
520+34c>a					cc	34.94	31.90	0.2616
	c	62.63	58.92	0.3000	ca	55.38	54.05	
	a	37.37	41.08		aa	9.68	14.05	
584+28c>t					tt	27.47	25.54	0.9452
	t	51.92	52.17	0.1679	tc	48.90	53.26	
	c	48.08	47.82		cc	23.63	21.20	
796+36g>a					aa	27.34	27.22	0.9837
	a	50.72	50.63	0.9832	ga	46.76	46.84	
	g	49.28	49.37		gg	25.90	25.94	

NOTE —Allele and genotype frequencies were compared between infertile men and control subjects.

Table 2 The odds ratio for each sequence variant of *DAZL*

Sequence variants	Tests for association			
	Allele (genotypes)	odds ratio	P	C.I.
260A>G	1/1 vs 1/2+2/2	1.256	0.55954	0.583-2.704
	2/2 vs 1/1+1/2	1.146	1.00000	0.023-58.060
386A>G	1/1 vs 1/2+2/2	11.667	0.00005	2.685-50.700
	2/2 vs 1/1+1/2	0.317	0.30588	0.013-7.835
520+34c>a	1/1 vs 1/2+2/2	1.147	0.53291	0.745-1.767
	2/2 vs 1/1+1/2	0.655	0.19234	0.346-1.241
584+28c>t	1/1 vs 1/2+2/2	1.104	0.67588	0.694-1.757
	2/2 vs 1/1+1/2	1.150	0.57709	0.703-1.881
796+36g>a	1/1 vs 1/2+2/2	1.006	0.98106	0.603-1.679
	2/2 vs 1/1+1/2	0.997	0.99216	0.593-1.678

NOTE — Allele and genotype frequencies were compared between infertile men and control subjects. The common allele is referred to as allele 1 and the rare allele as allele 2. C.I.= 95% confidence interval.

Table 3. Pairwise linkage disequilibrium (D′) in infertile men and control subjects

	260A>G	**386A>G**	**520+34c>a**	**584+28c>t**	**796+36g>a**
260A>G	—	0.108	0.288	1.000*	1.000*
386A>G	1.000	—	0.208	0.050	0.290
520+34c>a	0.583	0.999	—	0.931*	0.945*
584+28c>t	1.000*	0.926	0.846*	—	0.918*
796+36g>a	1.000	1.000	0.978*	0.792*	—

NOTE— Estimates in the upper right are for infertile men and in the lower left are for control subjects.

* Indicates $P < 0.05$, which was based on the permutation tests of exact test statistics.

Table 4. Haplotypes which show significantly different frequencies between infertile men and control subjects

Haplotype					Haplotype frequency (%)		Spermatogenic failure vs controls		
260A>G	386A>G	520+34c>a	584+28c>t	796+36g>a	Spermatogenic failure	Controls	P value	Odds ratio	C.I.
1	1	1	1	1	45.8	14.1	<0.0001	5.1510	3.6620 - 7.2450
1	1	1	2	2	17.8	41.9	<0.0001	0.2994	0.2188 - 0.4097
1	1	2	1	1	15.6	32.0	<0.0001	0.3934	0.2826 - 0.5478
1	1	2	2	2	8.65	1.09	<0.0001	8.9570	3.1740 - 25.280
2	2	2	2	2	4.72	1.58	0.0100	3.1330	1.2570 - 7.8100

NOTE — The common allele is referred to as allele 1 and the rare allele as allele 2. C.I.= 95% confidence interval. The P values were based on the permutation tests of exact test statistics.

Legends

Figure 1 DNA sequence analysis of the PCR products containing aberrant SSCP conformers. (a) The **520+34c>a is in** the intron 4 of *DAZL* harbors a c→a transversion. (b) The **584+28c>t** is in the intron 5 of *DAZL* harbors an c→t transition. (c) The **796+36g>a** is in the intron 7 of *DAZL* harbors an g→a transition.