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造精功能缺陷的遺傳研究（2/3）

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執行單位：國立成功大學醫學系婦產科

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基因體醫學國家型科技計畫
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計畫名稱 造精功能缺陷的遺傳研究

計畫名稱 Genetic studies of spermatogenic defects

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報告類別：□ 新進研究計畫 □ 修正後計畫書 ☑ 年度成果報告
(New Proposal) (Revised Proposal) (Progress Report)

計畫類別：☑ 個別型計畫 □ 整合型計畫
(Individual Project) (Program Project)

計畫編號：

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1. 中華民國 93 年 5 月 31 日
Specific Aims

The goal of the study is to identify potential candidate gene for human spermatogenic defects. The following approached are applied to identify the potential sterile genes.

1. Y-chromosome deletion mapping analysis to address the role of Y chromosome-linked sterile genes
2. c-DNA microarray analysis of the testicular tissue
3. Quantitative real-time RT-PCR for expression profiles of sterile genes
4. Immunohistochemical staining or RNA-in situ hybridization of sterile genes
5. Haplotype analysis of sterile genes
Progress Summary

1. Y-chromosome deletion mapping analysis to address the roles of Y-chromosome linked sterile genes

The Y chromosome contains fewer than 50 genes or gene families – many expressed exclusively in the testis. Genes located on the Y chromosome are easily studied because they exist in a haploid state. Screening for Y chromosomal deletions has become routine practice. So far, at total of 27 protein-coding genes or gene families on male-specific region of the human Y chromosome (MSY) may be implicated in human spermatogenic defects. We have developed a simplified protocol for screening of Y-chromosomal deletions in infertile men. We screened more than 300 men with severe spermatogenic defects (azoospermia or sperm count less than 5,000,000/ml) and found deletion of Y chromosome occurred in approximately 10% of men with severe spermatogenic defects. We also screened approximately 100 men with mild spermatogenic defects (sperm counts between 5,000,000/ml and 20,000,000/ml). We found only 1% of men with Y chromosome microdeletions. All cases with Y chromosome microdeletions have massive deletion of multiple genes, including at least one of the following four genes: DBY, USP9Y, RBMY1, and DAZ. Based on the preliminary results, we have established a simplified protocol consisting of these four genes (DBY, USP9Y, RBMY1 and DAZ) for rapid screening of Y chromosome microdeletions. Except DBY and USP9Y, there have been no identifiable cases with isolated deletion of other Y-lined sterile genes. At the present time, it is still difficult to characterize the role of Y-linked sterile genes (except DBY and USP9Y) based on deletion mapping analysis alone. The roles of Y-linked gene in human spermatogenic defects will be addressed by another attempt - expression profile of the gene described in part 3 of this section.

2. c-DNA microarray analysis of the testicular tissue

We also sought to identify genes which might be involved in human spermatogenic defects by c-DNA microarray analysis of the testicular tissues. Total RNA was extracted from testicular tissues of infertile men and from normal controls. Subjects were divided into three distinct groups based on histological types of the testes. Group I: Cases without germ cells (Sertoli cell only syndrome); Group II: Cases with maturation of spermatogenic cells arrest at the spermatocyte or spermatid; and Group III: cases with normal spermatogenesis. RNA is extracted from testicular tissues of patients and control subjects with normal spermatogenesis. The same amounts of RNA are reversely transcribed, labeled with different fluorochromes (Cy3 and Cy5), mixed and subjected to microarray experiment. An average of 1-2 µg of total RNA was extracted from each sample. Three-five RNA
samples were pooled together (total amount 5 ~ 10 µg) for each microarray analysis using the Agilent cDNA chip containing approximately 13,000 genes. With this approach, we have identified transcripts which are simultaneously or differentially up-regulated or down-regulated in cases with different types of spermatogenic defects. Cluster analysis showed there were 105 genes which are differentially expressed in cases with Sertoli cell only and maturation arrest. The differentially expressed genes may be involved in either renewal of spermatogenic stem cells or meiosis (or terminal differentiation) of germ cells. Real-time RT-PCR is also ongoing to confirm the findings of microarray analysis. Meanwhile, we found many genes which are significantly down-regulated in the testicular tissue of all patients. Some of these genes may be germ-line specific and play important roles in spermatogenesis. We are now focusing on HLA-linked transcripts and kinase involved in meiotic arrest of male germ cells. For example, our microarray analysis suggests importance of cyclin A1 in human spermatogenesis, a finding consistent with the knock-out mice model. Bioinformatic search is now ongoing to identify genes of special interest.

3. Quantitative real-time RT-PCR for expression profiles of sterile genes
Part I. Manuscript have been accepted or submitted
Considering current DNA-chip can not differentiate between highly homologous members within the same gene family, we also obtained expression profiles of some candidate genes in the testicular tissue only by real time RT-PCR. We found transcripts of potential Y-linked sterile genes - RBMY1 and DAZ - could be used to predict successful sperm retrieval:


We also found expression profiles of DAZ gene family (consisting of DAZ, DAZL and BOULE) well correlates with the histological pattern of patients


Of several transcripts tested, we found transcript of the BOULE gene is most suitable for prediction of sperm retrieval

Transcript of human meiotic regulator BOULE gene: a novel parameter predicting the presence of mature sperm in men with spermatogenic failure (manuscript submitted)
We hypothesize that there may be a threshold of BOULE transcripts required for completion of meiosis and that this level must be exceeded for the presence of intra-testicular sperm. If we used a cut-off value of $10 \times 10^3$ copy/ng RNA to predict the presence of sperm, the sensitivity, specificity, positive predictive value, and negative predictive value were all 100%.

Part II. Manuscript in preparation
We established the expression profiles of an important Y-linked sterile gene (DBY) and its homologues on X chromosome (DBX). We also established the expression profiles of important cell cycle regulators at G2-M transition – CDC25A, CDC25B, CDC25C - in the testicular tissue of azoospermic men. We are now analyzing the results and preparing the manuscript for submission.

4. Immunohistochemical staining or RNA-in situ hybridization of sterile genes
Home-made antibodies
Attempts have been made to generate antibodies for the following important proteins: BOULE, DBY, DBX, USP9Y, USP9X and cyclin A1 using synthetic peptides. Because all of these proteins have highly similar homologues in the human being, neither recombinant proteins nor ribo-probes could be used to identify their expression pattern. Only synthetic peptides can be used to generate gene-specific antibodies. We generated a polyclonal antibody common to DBX and DBY. Immunohistochemical and immunofluorescence staining showed DBX/DBY is a cytoplasmic protein which are expressed throughout the whole process of spermatogenesis. However, we failed to generate antibodies for other proteins.

Commercial probes
By Immunohistochemical and immunofluorescence staining, we found unique expression pattern of CDC25A, which are expressed predominantly in germ cells which have completed meiosis. Immunohistochemical staining failed to identify expression of CDC25B, cyclin A1, CDC25B, and CDC25C in the tissue specimen. All of these genes are potentially important meiotic regulators of germ cells. Immunofluorescence staining is ongoing to characterize the expression pattern of these cell-cycle regulators.

5. Haplotype analysis of sterile genes
To explore the role of DAZL in human spermatogenesis, we screened the polymorphisms out of entire coding sequence of the DAZL gene and identified a SNP (T54A variant) located within RNA-binding motif. We found association of T54A variant with susceptibility to spermatogenic failure. To the best of our
knowledge, T54A variant of DAZL is the first SNP of autosomal genes associated with susceptibility to severe spermatogenic failure. Our finding provides strong evidence for the role of the autosomal DAZL gene in human spermatogenesis.


By single strand conformation polymorphism (SSCP), we have identified additional five SNPs of DAZL gene: 260A→G (exon2), 584+28c→t (intron 5), 386A→G (exon3), 520+34c→a (intron 4), and 796+36g→a (intron 7). We also identified seven mutations which were not present in the population of normal fertile men: 469-5 t>g (intron 4), 427 A->G (exon 3), 2733 c>a (3’-untranslated region), 1422 a>g (3’untranslated region), 1061-12 c<t (intron 10), 1-13 A->G (exon 1), 2940 c>t (3’untranslated region). Based on primer extension analysis, we designed a rapid method for simultaneous genotyping of these SNPs. We predicted haplotype frequencies are calculated by use of Permutation and Model-free analysis (PM-PLUS) and estimating Haplotypes (EH-PLUS). So far, we have identified some haplotypes that are significantly associated with spermatogenic defects.


**Publications**


