Characterization of a Novel Human Testis-Specific Gene: Testis Developmental Related Gene 1 (TDRG1)

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Spermatogenesis is a highly coordinated physiological process that requires the correct expression and functions of thousands of developmentally regulated genes. The regulation of spermatogenesis is not well defined, since majority of the related genes have neither been identified nor fully characterized. Hence, it is meaningful to identify and characterize these genes to reveal the mechanism underlying spermatogenesis. In this study, using digital differential display, we identified a novel human testis-specific gene, testis developmental related gene 1 (TDRG1, GenBank DQ168992), via electronic subtraction of human testis UniGene databases from those of non-reproductive tissues. The transcript of the TDRG1 gene has an open-reading frame that encodes 100 amino acids. We next prepared the anti-TDRG1 monoclonal antibody 10B6 and confirmed that it specifically recognizes an 11-kDa protein in the tissue extracts from an adult human testicular sample (age 31 years) by Western blot analysis. RT-PCR coupled with immunohistochemistry of human tissues demonstrated that TDRG1 is exclusively expressed in the testis but not in any other non-reproductive tissues. TDRG1 is mainly located in spermatogenic cells in seminiferous tubules of adult testis. Furthermore, TDRG1 shows the highest expression level in human post-puberty testis, with the expression levels decreasing afterwards with aging. Importantly, TDRG1 mRNA is undetectable in the fetal tests, as judged by RT-PCR. In conclusion, TDRG1 is a developmentally regulated testicular-specific gene. We suggest that TDRG1, a newly identified testis-specific gene, may play important roles in human spermatogenesis.

Keyword: cloning; expression; gene; spermatogenesis; testis

Spermatogenesis occurs in successive mitotic, meiotic and postmeiotic phases in the seminiferous epithelium of the testis, and it is a highly developmentally controlled process that relies on the spatiotemporal expression of specific genes directing a number of distinct regulatory pathways (Sassone-Corsi 1997). The expression of a set of germ cell-specific genes in pachytene spermatocytes usually triggers the expression of post-meiotic transcription for many downstream genes that are required for morphological and biochemical reconstructions in spermatids (Sassone-Corsi 2002). These germ cell-specific transcripts mainly fall into three categories: (1) Homologs of genes expressed in somatic cells with different spatiotemporal expression patterns or alternative conformations/functions; (2) Unique genes that are expressed only in male germ cells without homologs in other cells; (3) Alternative transcripts in testicular tissue that are different in size and/or overall sequences of the same gene transcripts in somatic cells (Eddy 2002). Thus, identification and functional characterization of these germ cell-specific genes are crucial for revealing the mechanism underlying spermatogenesis.

A large number of genes, such as many germ-cell unique genes (Hong et al. 2005), general transcriptional factors (Kolthur-Seetharam et al. 2008), kinases/enzymes (Esposito et al. 2004; Miki et al. 2004), hormones (Kendall et al. 1995) and membrane proteins (Griswold 1995), have been proposed to be involved in different stages of spermatogenesis from mitosis to spermiogenesis in various species. Their expression levels are developmentally regulated and stage specific, which determines the unique developmental process of the germline. However, little is known about genes influencing spermatogenesis in humans. Only a limited number of genes have been fully characterized, and thus many genes related to spermatogenesis need further functional determination or remain to be identified.

In this study, we take the advantage of a computerized program, digital differential display (DDD) (Scheurl et al. 2000; Olesen et al. 2001; Yan et al. 2002), to compare the
differences of gene expression profiles among cDNA librar-
ies from various tissues. We screened and cloned a novel
human testis-specific gene: testis developmental related
gene 1 (TDRG1). We have demonstrated that TDRG1 is a
human testis-specific gene and may play important roles in
normal human spermatogenesis.

Materials and Methods

Clinical Biopsy Samples

This study was approved by the National Scientific Council of
China and the Institutional Review Board of XiangYa Medical
School, Central South University. All human embryonic specimens
were collected from spontaneous or medically indicated abortions.
Specimens from various tissues of mature healthy human were
obtained at autopsies from individuals who died from motor vehicle
collisions. Consent has been obtained from all patients or their fami-
lies. All procedures are in accordance with the China legislation and
supervised by the Ethical Committee of Central South University.

DDD

DDD, a bioinformatics tool available at UniGene division at the
NCBI server (http://www.ncbi.nlm.nih.gov/UniGene/ddd.cgi?ORG= Hs), allows us to compare the differences in frequencies of cDNA and
expressed sequence tags (EST) among libraries from various tissues.
The DDD analysis was carried out based on the procedure described
in the handbook at the NCBI server. Briefly, in order to identify the
genomes that may contribute to unique characteristics of testis tissue,
electronic subtraction was performed between 9 human testis cDNA
libraries (pool A), and 76 cDNA libraries (pool B) from other human
tissues and cell lines including heart, spleen, liver, lung, brain, skin,
kidney, intestine, ovary, prostate, etc via Unigene database at the
NCBI server. The candidate transcripts with abundant expression profiles
only in testis but not in any other tissues were selected for
further analysis.

Amplification and determination of TDRG1 gene

Based on putative TDRG1 gene transcripts (BC071820.1, BC033995.2 and BC042123.1), two specific primers were designed to amplify the
hypothetical full-length cDNA of TDRG1. We initially amplified putative full length TDRG1 gene from Marathon-Ready Testis cDNA library (Clontech, CA, USA)
using above specific primers. The PCR amplification was performed at
95°C for 1 min, followed by 40 cycles of 95°C for 1 s, 57°C for 1 s, and
72°C for 45 s, with a final extension at 72°C for 5 min. The cycle
threshold (Ct) number at which fluorescence crossed a prescribed
background level is used for relative quantification (Tan et al. 2003).
The relative ratio of target mRNA against internal control is deter-
moved by the equation of 2-[(target gene Ct - internal control Ct)].
The data was then normalized to TDRG1 mRNA level in puberty group
that was set as 100%. The relative RT-PCR was conducted by using
SMART cDNA PCR kit (Clontech, CA, USA), the same primers and
amplification program stated above. The PCR products were ana-
yzed on 2% agarose gels.

Northern Blot Analysis

The expression profiles of TDRG1 gene among various tissues
were determined by Northern blot using the amplified 320-bp TDRG1
fragment as a probe. The β-actin gene was used as an internal control
to assess the equal loading quantity for each sample. The total RNA
isolated from each tissue was heated at 65°C for 5 min and separated
on a 1.2% agarose gel containing 1.8% formaldehyde. The total
RNAs were transferred a Hybond-XL membrane (Amersham Bioscience). Total RNA (20 µg) from each tissue was loaded for each
lane of Northern blot. Labeling of the probes, hybridization and signal
detection were performed using DIG-High Prime DNA Labeling
and Detection Starter Kit II based on manufacturer’s manual (Roche, Mannheim, Germany).

Preparation of Monoclonal Antibody against TDRG1 and Western
Blot Analysis

TDRG1 open reading frame (ORF) with 6×His tag at 3’ end
was cloned into pET21 prokaryotic expression vector (Novagen, CA,
USA). The recombinant vector was transformed into BL21 (DE3)
and TDRG1 expression was induced by IPTG. Recombinant TDRG1
protein was purified by TALON column (Clontech, CA, USA) based
on the protocol described by others (Nie et al. 2006). The purified
TDRG1 was used for immunization of BALB/C mouse to produce the
monoclonal antibody according to the protocol described by
Schook et al. (1987).

Human testis specimens obtained at biopsies were homogenized
in lysis buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na3VO4,
and Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany)]. The tissue lysates were boiled for 5 min in loading buffer
and subject to load on 10% SDS-PAGE. The proteins were trans-
ferred to PVDF membrane (BioRad, CA, USA). The membrane was
was obtained and subsequently cloned into a T-vector. From the testicular cDNA library. A 1.1-kb PCR product specific primers to amplify the predicted full-length cDNA sequence of TDRG1 shown with abundant expression profiles in testicular tissue with the DDD analysis between the testicular EST library and the cDNA libraries from other non-reproductive tissues. Among them, 168 ESTs are known genes that fall into the following categories: enzymes, ribosome proteins, receptor/membrane proteins, ligands and secreted proteins. The remaining 195 ESTs may represent novel genes with >10-fold higher expression levels in testis than in non-reproductive tissues. The EST Hs. 180197 shows the highest possibility to be a new testicular specific expression gene. After analyzing three partially overlapped sequences BC071820.1, BC033995.2 and BC042123.1 in this EST cluster, we obtained a 1.1-kb theoretical full-length cDNA sequence and predicted this is a novel gene. We designed two specific primers to amplify the predicted full-length cDNA from the testicular cDNA library. A 1.1-kb PCR product was obtained and subsequently cloned into a T-vector. After sequencing, we confirmed it is a new full-length cDNA, matching the same sequences we predicted. The transcript of new gene has an ORF of 303 bp (504-806 bp) that encodes 100 amino acids. It is located at 6p21.1-p21.2 spanning 1.18 kb with 2 exons and 1 intron. There are five predicted phosphorylation sites in this protein: T16, S58, S60, S91, and S96. This protein is not homologous to any other known protein. We named this new gene as Testis Developmental Related Gene 1 (TDRG1), with a GenBank registration number as DQ168992. The cDNA and protein sequences of TDRG1 are shown in Fig. 1.

**TDRG1 is a tissue-specifically expressed gene in testis**

In order to examine whether TDRG1 is uniquely expressed in the testicular tissue, we performed semi-quantitative RT-PCR and Northern Blot analysis to analyze the TDRG1 mRNA levels from various tissues. We collected various tissues from human aging from 25 to 32 for semi-quantitative RT-PCR, Northern Blot analysis and immunostaining experiments. We tested 3 samples from different people for each organ. QRT-PCR results showed TDRG1 mRNA was only detected in testicular tissue but not in other tissues such as heart, liver, brain, epididymis, lung, kidney and spleen (Fig. 2A). The full-length transcript of TDRG1 was examined by Northern blot analysis from various tissues using a DIG-labeled TDRG1 specific probe. Consistent with QRT-PCR results, a 1.19-kb transcript was only detected in testicular tissue but not in any other tissues such as brain, heart, liver, lung, spleen, kidney and skeletal muscles (Fig. 2B). Thus, the TDRG1 gene is specifically expressed in testis.

We next examined the TDRG1 protein expression pattern in the testicular tissue. We raised a monoclonal antibody against TDRG1 using recombinant TDRG1 protein expressed and purified from E. coli. After immunization of BALB/C mouse and screening hybridoma cells, we obtained two hybridoma lines 10B6 and 5C5. The subtype for both hybridoma lines of anti-TDRG1 monoclonal antibodies was IgG1. The highest dilution that could recognize the purified TDRG1 protein for both lines antibodies was 1:1.6 × 106 using ELISA method (data not shown). We here used clone 10B6 for the later detection of TDRG1 protein levels in various tissues in this study.

An 11-kDa protein was detected from an adult human biopsy testicular tissue (age 31 years) on Western Blot with the monoclonal antibody 10B6 (Fig. 2C), which was consistent with the theoretical TDRG1 protein size. We repeated the experiments with 3 human biopsy testicular tissues by Western blot analysis. Immunohistochemistry was used to examine cellular locations of TDRG1 protein in human biopsy testicular tissues. The TDRG1 protein was detected in the primary and secondary spermatocytes in seminiferous tubules and localized in cytoplasm, but not in spermatids and germ cells in the adult normal human testis (Fig. 3A). About 27.0% of spermatogenic cells are TDRG1 positive (n = 185 out of 686 cells). TDRG1 expression was not
Fig. 1. cDNA and protein sequences of TDRG1.
Fig. 2. TDRG1 mRNA and protein are specifically expressed in human testis but not in non-reproductive tissues.
A. TDRG1 mRNA is detected from testis but not any other tissue by RT-PCR analysis. GAPDH mRNA is amplified from each tissue as internal control.
B. Analysis of TDRG1 mRNA levels in various human tissues by Northern blot using a DIG-labeled TDRG1 specific probe. Full length TDRG1 mRNA is detected in testis only but not in any other tissue. β-actin mRNA was detected using DIG-labeled β-actin specific probe as an internal control.
C. TDRG1 protein was detected by the specific monoclonal antibody 10B6 from normal human testicular tissue (31 years old male testis sample).

Fig. 3. TDRG1 protein is expressed in human testicular spermatogenic cells, but not in non-reproductive tissues. Immunohistochemistry was performed to detect TDRG1 protein levels in the frozen sections from various human tissues using a specific monoclonal antibody 10B6.
A. TDRG1 is expressed in the cytoplasm of spermatocytes in seminiferous tubules, but not in sertoli and germ cells, in the normal human testis (age 31 years).
B-D. TDRG1 expression is undetectable in human heart (B), kidney (C) and spleen (D). The brown cells are TDRG1-positive cells that are indicated by arrows. Scale bar: 40 μm.
detected in heart, kidney, and spleen (Fig. 3B-D). These results confirmed that the TDRG1 protein is exclusively expressed in human testis.

**TDRG1 expression pattern is associated with testis aging stages**

The TDRG1 expression levels during different testis aging stages were investigated by real time RT-PCR. We extracted total RNAs from human biopsy testicular tissues from 5 different ages: fetal period (4, 6, 7, 8 and 9 months, \( n = 6 \)), post-puberty stage (15 years old male, \( n = 1 \)), adult (24, 31, 33 and 35 years old males, \( n = 4 \)) and elderly group (74, 79, 65 and 69 years old males, \( n = 4 \)). The TDRG1 mRNA was barely detected in the testicular tissues during fetal period (4.0 ± 0.1%, **\( P < 0.05 \)) and decrease in elderly group (56.0 ± 3.6%, *\( P < 0.05 \)) comparing with those in adult male testis (100.0 ± 4.8%). Values are presented as mean ± s.d.

**Discussion**

In the present study, we identified human TDRG1, a
testicular specific gene, and characterized its expression pattern in various human tissues and different ages of human testicular biopsy samples. The expression of TDRG1 is found only in testicular tissue, not in other human organs, which confirms it is a unique gene in human germ cells. The germ cell-specific and developmental regulated genes underlying the intrinsic genetic network are the primary regulators of spermatogenesis. Their developmentally regulated and stage-specific expression patterns determine the unique developmental sequence of spermatogenesis, such as from mitotic, meiotic to postmeiotic phase (Eddy 2002; Sassone-Corsi 2002). Testicular unique genes are mostly expressed during the postmeiotic phase, when the spermatooza are formed with the specialized morphological transformation (Eddy 2002). These genes play significant roles in remodeling of the nucleus, condensation of the chromatin and assembling of some specialized structures during spermatogenesis. For example, unique genes encode SCP1 and COR1 form the major components for synaptonemal complex (Meuwissen et al. 1992; Dobson et al. 1994), one of unique structures present during the meiotic phase. As one of the unique germ cell genes, TDRG1 is expressed in spermatogenic cells, but not in Sertoli cells, with the highest expression level during human puberty stage, and decreasing levels of expression with aging. Our data suggests that TDRG1 expresses in certain types of germ cells during spermatogenesis and may play a role in normal human sperm formation and fertilization.

There are a large number of genes involved in spermatogenesis. The functions of many of these genes still remained to be defined and characterized. It is believed that many genes related to human spermatogenesis remain unknown thus to be discovered. It is of great significance to identify these germ-cell specific genes and characterize their roles in spermatogenesis, which will provide essential information to reveal the causative genes for human infertility. The completion of high throughput genomics projects and construction of many ESTs databases, such as UniGene, has greatly inspired the actions of identification of cell- and tissue-specific transcriptome genes for spermatogenesis. UniGene is featured with tissue types of gene expression profiles and gene-oriented clusters that each one likely represents a single gene. Combined with other powerful bioinformatics tools, such as DDD, UniGene has been successfully screened in discovering many germ cell- and testis-specific novel genes that provide fundamental insights into physiological process of spermatogenesis (Olesen et al. 2001; Fu et al. 2003; Li and Lu 2004; Yang et al. 2005). In the present study, we performed electronic subtraction between 9 human testis cDNA libraries and 76 cDNA libraries from other human tissues and cell lines including heart, spleen, liver, lung, skin, kidney, intestine, ovary, prostate, etc via UniGene databases at NCBI servers, and initially obtained 195 ESTs that may represent novel genes with significant higher expression levels in testis than in any other nonreproductive tissues. After further characterization, we identified TDRG1 as one of the testis-unique genes.

Collectively, we discovered a novel human testicular-specific gene, TDRG1. TDGR1 is exclusively expressed in testicular tissue, in spermatogenic cells. TDRG1 shows the highest expression level in human post-puberty testis biopsy tissue, with expression levels decreasing afterwards with aging, but not expressed in fetal period of human testis. Our study suggests TDRG1 is a developmentally related testicular-specific gene. We predict that TDRG1 is involved in normal human spermatogenesis and fertilization. The characterization of the function of TDRG1 is our next focus.

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Conflict of Interest

The authors declare (1) no conflict of interest that could be perceived as prejudicing the impartiality of the research reported; or (2) no any financial or other potential conflict of interest.

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