

## Molecular Cloning of *TSARG6* Gene Related to Apoptosis in Human Spermatogenic Cells

Gang LIU, Guang-Xiu LU\*, and Xiao-Wei XING

( Human Reproductive and Stem cell Engineering Institute, Central South University, Changsha 410078, China )

**Abstract** Beginning from a mouse EST (GenBank accession No. BE644537) which was significantly up-regulated in cryptorchidism and represented a novel gene, we cloned a new gene (GenBank accession No. AY138810) which is related to apoptosis in human spermatogenic cells by means of GeneScan program and PCR technology. The gene whose full cDNA length is 1875 bp containing 8 exons and 7 introns is located in human chromosome 11q13.3. Its protein containing 316 amino acid residues is a new member of HSP40 protein family because the sequence contains the highly conserved J domain which is present in all DnaJ-like proteins and is considered to have a critical role in DnaJ-DnaK protein-protein interactions. *TSARG6* protein displays a 45% identity in a 348-amino acid overlap with DJB5\_HUMAN protein. The result of RT-PCR and Northern blot analysis showed that *TSARG6* is specifically expressed in adult testis and the transcript is 1.8 kb. Based upon all these observations, it is considered that we cloned a new gene which probably inhibited human testis spermatogenesis apoptosis.

**Key words** gene cloning; PCR; GeneScan; cryptorchid testis; heat shock protein (HSP); apoptosis

It was observed that the amount of mature sperms in human testis was 20%–75% less than that was expected though the testis is a kind of tissue with high proliferation ability. The explanation was that apoptosis in testis resulted in spontaneous degeneration of spermatogenic cells. The apoptosis could be induced by many signals including temperature. It was proved by many experiments that spermatogenesis was very susceptible to temperature, especially primary spermatocyte and round sperm [1]. The proportion of apoptosis sperm was only 0.1% in normal man while 20% in patients with cryptorchidism [2]. So the disturbance of spermatogenic cells apoptosis which involved multi-gene is an important factor to improve male infertility. Cloning of new spermatogenic cell-specific gene related to apoptosis is of momentous physiological and pathological significance to illustrate the apoptosis mechanism and the biology process of spermatogenic cells.

In previous study, Jiang *et al.* [3] cloned 24 ESTs of

mouse testis spermatogenic cell apoptosis-related gene by creating mouse cryptorchidism model and making use of suppression subtractive hybridization. Beginning with the EST BE644537, one of the ESTs mentioned above, we cloned a novel human gene's full-length cDNA sequence, *TSARG6*, from a human testis cDNA library by means of GeneScan software and PCR technology.

### Materials and Methods

#### Materials

50×Advantage 2 DNA polymerase, human testis Marathon-Ready™ cDNA library, Northern blot membrane (MTN™) and Express Hyb™ hybridization solution were purchased from Corporation Clontech. Primers were synthesized by Corporation BioAisa. Reagents for electrophoresis and culture medium, pUCm-T vector and dNTP were purchased from Biological Engineering Corporation. Sequencing was performed by Corporation Unigene.

#### Molecular cloning of full-length cDNA

The outside primers LA1, 5'-TGCTAGAGTCTGAG-

Received: September 22, 2003 Accepted: November 22, 2003

This work was supported by a grant from the Major State Basic Research Development of China (No. G1999055901)

\*Corresponding author: Tel, 86-731-4373187; Fax, 86-731-4497661; E-mail, lgxdirector@sina.com

GACTATCCAG-3', LA3, 5'-AGCCTCCTCTCACCCCT-GCT CCA-3', and inside primers LB1, 5'-CGGAATTC-CCATGGGCCAGGATTAT-3', LB2, 5'-CCGTCGACT-CACCCCTGCTCCAG-3' were designed according to the sequence mentioned above. LB1/LB2 sequence was inserted with *EcoRI* and *Sall* sites for protein expression. LA1/LA3 was used in PCR assay with Advantage 2 DNA polymerase (Clontech) and Marathon-Ready™ cDNA of human testis (Clontech) as template. PCR was performed as follows: initial denaturation at 95 °C for 1.5 min, and 35 cycles of 94 °C for 10 s, 58 °C for 30 s, and 72 °C for 2 min, then 72 °C for 5 min, hold at 4 °C. Then LB1/LB2 was used in PCR assay with the PCR product mentioned above as template (PCR amplification procedure is the same as mentioned above except that annealing temperature is 53 °C). This PCR fragment was cloned into pUCm-T vectors and sequenced.

### Bioinformatics analysis of TSARG6

Translation program in ExpASY was performed to identify ORF. Comparison to human genome draft sequence in GenBank databases was proceeded to locate the new gene in human chromosome. ProtParam tool was utilized to identity physico-chemical parameters of new protein sequence. Tmpred was used to predict the transmembrane regions and protein orientation. SignalP V1.1 was performed to predict signal peptide cleavage sites [4]. PSORT WWW Server was utilized to predict protein subcellular localization. SMART was used to analyze motif. BLAST program in NCBI and CLUSTAL program were performed to analyze similarity of nucleotides and proteins.

### Identification of TSARG6 mRNA in human fetal tissues by RT-PCR

Total RNAs in human multi-tissue (fetal lung, heart, small intestine, skeletal muscle, spleen, liver, kidney, testis and epididymidis, and adult lung, brain, skeletal muscle, kidney, liver, ovary and testis) were isolated by RNA isolation kit (Promega). cDNA was synthesized according to the instruction of kit and was used as template in following PCR reaction. PCR amplification cycles were the same as above. RT-PCR product was separated in 2.0% agarose gel. *GAPDH* was amplified as control.

### Identification of TSARG6 mRNA in human sperm by RT-PCR

Total RNAs isolated from human sperm were used to perform RT-PCR. Because of poor RNAs in human sperm, GQ1/GQ2 (GQ1, 5'-TCTGCTTCTTCTGGGGTGTGAG-3'; GQ2, 5'-GAGGTAGATTTGAACCTT-GGGGG-3')

was designed to amplify a fragment about 500 bp in 3' end of *TSARG6*. RT-PCR was performed as follows: 48 °C for 45 min, then initial denaturation at 94 °C for 2 min, and 40 cycles of 94 °C for 30 s, 58 °C for 1 min, and 68 °C for 3 min, then 68 °C for 7 min, hold at 4 °C. Product was separated in 6% PAGE gel. The correct fragment was cut by sharp knife and dissolved in 30 µl water to be used as template in following PCR reaction. GQ1/GQ2 were used as primers again. PCR was performed as follows: initial denaturation at 95 °C for 90 s, and 35 cycles of 94 °C for 10 s, 58 °C for 30 s, and 72 °C for 2 min, then 72 °C for 5 min, hold at 4 °C. This PCR fragment was cloned into pUCm-T vectors and sequenced. *GAPDH* was amplified as control.

### Northern blot

cDNA probe with [ $\alpha$ -<sup>32</sup>P]-dCTP was labeled by PCR reaction. PCR condition was the same as that of RT-PCR. Probe was purified through Sephadex G-50 column. After pre-hybridization at 65 °C for 1 h, membrane was hybridized with *TSARG6* cDNA at 65 °C overnight followed by washing three times with 2×SSC/0.1% SDS at 65 °C for 10 min and then twice with 0.1×SSC/0.5% SDS at 62 °C for 15 min. Then membrane was exposed to X-ray film at -70 °C for 3 d. The film was developed and fixed.

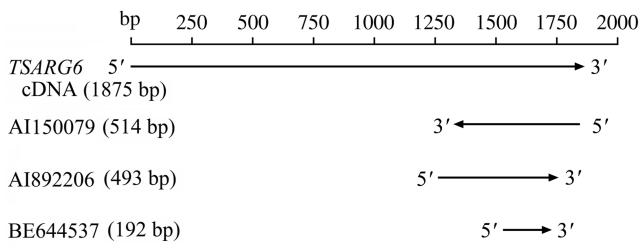
## Results

### E-cloning of human novel gene cDNA from a mouse EST

Electronic hybridization in mouse EST database was performed by using BE644537 as an electronic probe to get a longer mouse EST AI892206 (493 bp) with high sequence similarity. Homology comparison was proceeded between AI892206 and human EST database. Then the human homology EST AI150079 (514 bp) was compared with genome draft sequence to obtain the human genome sequence containing AI150079. An ORF (open reading frame) sequence containing AI150079 was obtained by analysis of GeneScan software. Comparison result between the sequence and nr database showed that it represented a new gene which was denominated *TSARG6* (GenBank accession No. AY138810) (Fig. 1).

### Molecular cloning of full-length cDNA

The nest PCR was performed using primers LA1/LA3 and LB1/LB2. PCR product (994 bp) was sequenced and the result is identical with predicted sequence. This gene



**Fig. 1** The map of *TSARG6* cDNA and its homolog ESTs

whose full cDNA length is 1875 bp contained 8 exons and 7 introns and encoded a 316-aa protein (Fig. 2). There were a start codon ATG from nucleotide position 752 to 754 and a stop codon TGA from nucleotide position 1700 to 1702. GCCATGGA sequence which was in accordance with Kozak Rule was found in the start region of ORF and potential polyadenylation signal (AATAAA) was found at 3' end. The boundary of exon and intron is coincident with gt-ag rule (Table 1).

```

1 taatgtaagatgtaataataggggaatgtgaatgcagtggctcacacctgtaatcccagc 61
62 actttgggaggccaagccgagcccaagagttcaagaccagctctgggcaacatggtgaaac 121
122 cccatctctacaaaacatacaaaaattagccagcatggtagtgacagcctgtgtctcag 181
182 ctacttgaaggtgaggtggaagaatcccttgagcctgggaggttgagactgcagtgag 241
242 gtgtgacctgccactgccaccagcctgggtgacagagtgaagaccctgtctcaaaaac 301
302 atgataatgataataataataataaaaataggagaatgtggatgtgaggtatatgggaact 361
362 ctctgtactgtcttctcaacttttctaaactctaaaactgttctaaaataaatgtat 421
422 taaaaaattagccaaatgcatttcttctaaaaccttttattaaaactaattactgtct 481
482 ctttaaaaacaaaaacaaaataaaaacagagccctgtgagcttcaatttccaggtgag 541
542 gaccttccacagggtcgcagaatcagccccagctctccccagctcttccactgactcct 601
602 ctctgtggcagagctgaaattgttttagggaagtgggactacaactccagagtgccact 661
662 gtgcggttgtcaggagcaaccaaggaagccaactaacagccttgctagagtctgaggact 721
                                     *
722 atccagggacctgactgccagctagccagccatgggccaggattattactctgtgctcggg 781
                                     *           M G Q D Y Y S V L G
782 atcactcgcaattcagaggatgccagatcaagcagcgtaccgcagactcgccttaag 841
11 I T R N S E D A Q I K Q A Y R R L A L K 30
842 caccaccggttgaagtcaaatgagccgtcttcagcagagattttcaggcaaatagcagag 901
31 H H P L K S N E P S S A E I F R Q I A E 50
902 gcctacgacgtgctgagtgaccccatgaagagagcctctacgacaagtttgagaagag 961
51 A Y D V L S D P M K R G I Y D K F G E E 70
962 ggctgaaggggtgggattcctttggagtttgatcccagaccatggacaactggttac 1021
71 G L K G G I P L E F G S Q T P W T T G Y 90
1022 gtcttccacggcaaacctgaaaaggtgttccacagattcctttgggtggaacaacccttc 1081
91 V F H G K P E K V F H E F F G G N N P F 110
1082 agtgagtttttgatgcagaaggaagtgaggtagatttgaactttgggggctccagggc 1141
111 S E F F D A E G S E V D L N F G G L Q G 130
1142 cgaggggtcaagaagcaggacccccgaagtcgaacgggatctctacctgtccctggaggac 1201
131 R G V K K Q D P Q V E R D L Y L S L E D 150
1202 ttattctttggctgcacaaaaaaattaagatctccagaaggggtgctgaacgaggatggg 1261
151 L F F G C T K K I K I S R R V L N E D G 170
1262 tactctccaccatcaaggacaagatcctgaccattgatgtgaagcccggttgaggcag 1321
171 Y S S T I K D K I L T I D V K P G W R Q 190
1322 ggcacagcgateacctttgagaaggaaggggaccagggcccaacatcatcccagcagac 1381
191 G T R I T F E K E G D Q G P N I I P A D 210
1382 atcatttcatcgtaaaggagaagctacaccctcgcttccgcaggagaaatgacaacctc 1441
211 I I F I V K E K L H P R F R R E N D N L 230
1442 ttcttcgtgaacccatccctcttggcaaggctctcacctgctgcaactgtggaggtgagg 1501
231 F F V N P I P L G K A L T C C T V E V R 250
1502 accctagatgaccgtctgctcaacatccccatcaatgacatcatccaccccaatacttc 1561
251 T L D D R L L N I P I N D I I H P K Y F 270
1562 aagaaggtgccaggggaggggatgccattgccggaggaccccactaagaaaggggatctc 1621
271 K K V P G E G M P L P E D P T K K G D L 290
1622 ttcatctcttcgacatccagttccccaccgctcacacccagaagaagcagatgctg 1681
291 F I F F D I Q F P T R L T P Q K K Q M L 310
1682 cgccaggcattgctgacatgactgtggtgggctggagcaggggtgagaggaggctagccg 1741
311 R Q A L L T * 316
1742 ggccctacccccacctaccgcccacagcctcagggtgtgcaggggagcctgctgcacag 1801
1802 atatgatacaaggggtgggatggcgcagggttaaaactgacataataaagatctatttct 1861
1862 gtccctcagctaca 1875
    
```

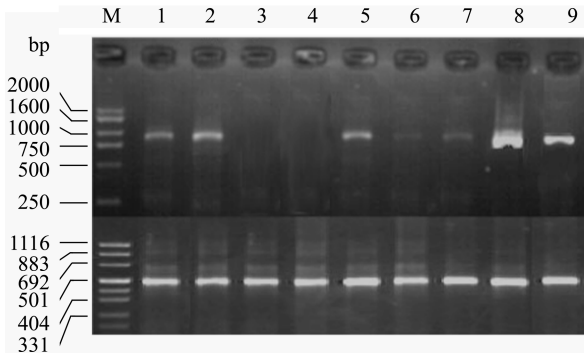
**Fig. 2** *TSARG6* cDNA and predicted protein sequence of *TSARG6*

Primer is marked in bold and italic. Polyadenylation signal is underlined. Stop codon is indicated by asterisk (\*).



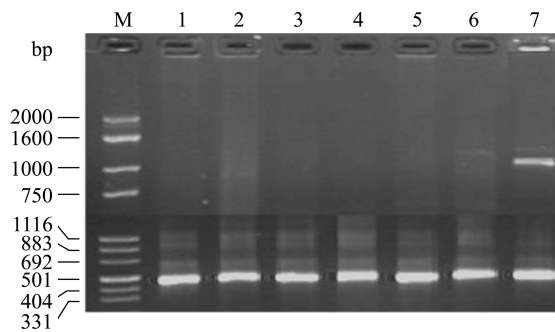
**Tissue distribution of *TSARG6* gene in human fetal and adult**

The result showed that *TSARG6* gene was expressed strongly in fetal testis and epididymidis, weakly in fetal kidney, liver, spleen, heart and lung (Fig. 5). *GAPDH* was expressed in all kinds of tissues. The result in Fig. 6 showed that *TSARG6* gene was expressed specifically in adult testis, and *GAPDH* was expressed in all kinds of tissues.



**Fig. 5 The agarose gel electrophoresis analysis of *TSARG6* gene expression in various fetus tissues by RT-PCR**

Above: amplification of *TSARG6* in fetal multiple tissues; Below: amplification of gene *GAPDH* in fetal multiple tissues. 1, lung; 2, heart; 3, small intestine; 4, skeletal muscle; 5, spleen; 6, liver; 7, kidney; 8, testis; 9, epididymidis; M, DGL2000 (above), pUC Mix8 (below).



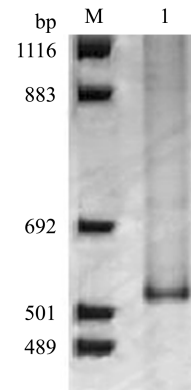
**Fig. 6 The agarose gel electrophoresis analysis of *TSARG6* gene expression in the various adult tissues by RT-PCR**

Above: amplification of *TSARG6* in adult multiple tissues; Below: amplification of gene *GAPDH* in adult multiple tissues. 1, lung; 2, brain; 3, skeletal muscle; 4, kidney; 5, liver; 6, ovary; 7, testis; M, DGL2000 (above), pUC Mix8 (below).

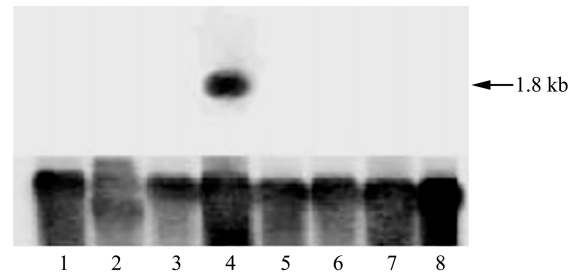
The result in Fig. 7 showed that *TSARG6* gene was expressed in human sperm.

**Northern blot result**

The *TSARG6* transcript appeared as a band of 1.8 kb mRNA only in human testis, and  $\beta$ -actin gene expressed in all tissues (Fig. 8).



**Fig. 7 The PAGE gel electrophoresis analysis of *TSARG6* gene**  
1, human sperm; M, pUC Mix8.



**Fig. 8 Northern blot analysis of *TSARG6* in 8 kinds of tissues of human**

Above: *TSARG6* probe; Below:  $\beta$ -actin gene probe. 1, spleen; 2, thymus; 3, prostate; 4, testis; 5, ovary; 6, small intestine; 7, colon (no mucosa); 8, peripheral blood leukocyte.

**Discussion**

Heat shock proteins (HSP), especially HSP70, are required for spermatogenesis and also protect cells from environmental hazards such as heat, radiation, and chemicals [5–7]. Apoptosis in spermatocytes was temporally correlated with the expression of stress-inducible Hsp70-1 and Hsp70-3 proteins in spermatocytes [8]. HSP70-2 is required for synaptonemal complex desynapsis, and its absence severely impairs the transition of spermatogenic cells through the late meiotic stages and results in apoptosis beginning with the first wave of germ cell development in juvenile mice [9]. The anti-apoptosis mechanism of HSP70 which needs the assistance of HSP40 is related to factors as follows. (1) Inhibit the activation of P53 [10]. Research data showed that *p53* expressed highly in spermatocyte from leptotene to pachytene stage and is related to apoptosis of spermatogenic cell induced by heat pressure [11–13]. (2) Interrupt the FAS pathway which is the key factor to activate the apoptosis of

spermatogenic cell at initiation stage of apoptosis [14–16]. (3) Assist apoptosis inhibitor Bcl-2 and inhibit apoptosis inducer Bax at apoptosis effector stage [17–18]. (4) Inhibit the effect of protease caspase at apoptosis execution stage. (5) Protect the mitochondrion.

Beginning from a mouse EST (GenBank accession No. BE644537) which was significantly changed in cryptorchidism and represented a novel gene, we get a new gene *TSARG6* (GenBank accession No. AY138810) by means of GeneScan program and PCR technology. The result of RT-PCR in adult tissues and Northern blot that *TSARG6* gene expressed specifically in adult testis, even in sperm, and the transcript appeared as a sole band indicated that *TSARG6* is a spermatogenic cell related gene. The result of RT-PCR in fetal tissues that *TSARG6* gene expressed strongly in fetal testis and epididymidis and weakly in some other tissues illustrated that the gene concerned about cell proliferation and development. Result of bioinformatics analysis that the protein was a new member of HSP40 family clarified that the protein may assist HSP70 to perform anti-apoptosis functions by binding to HSP70 with DnaJ domain. Based upon all these observations, it is considered that we cloned a new gene which probably inhibited human testis spermatogenesis apoptosis. Now we engage in the work of protein expression and antibody purification. We believe that it is of positive significance to clarify the control mechanism of apoptosis signal in spermatogenic cells and prevent and cure the infertility if the biological function of *TSARG6* can be understood more completely.

## References

- 1 Yin Y, Hawkins KL, de Wolf WC, Morgentaler A. Heat stress causes testicular germ cell apoptosis in adult mice. *J Androl*, 1997, 18: 159–165
- 2 Liu SF, Li LY, Fu JJ, Liu G, Xing XW, Lu GX. Rapid identification of human testis spermatocyte apoptosis-related gene, *TSARG2*, by nested PCR and draft human genome searching. *Acta Biochim Biophys Sin*, 2002, 34: 378–382
- 3 Jiang H, Li LY, Lu GX. Molecular cloning of genes related to apoptosis in spermatogenic cells of mouse. *Acta Biochim Biophys Sin*, 2001, 33: 421–425
- 4 Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng*, 1997, 10: 1–6
- 5 Hunter ES 3rd, Dix DJ. Heat shock proteins Hsp70-1 and Hsp70-3 are necessary and sufficient to prevent arsenite-induced dysmorphology in mouse embryos. *Mol Reprod Dev* 2001, 59(3): 285–293
- 6 Rockett JC, Mapp FL, Garges JB, Luft JC, Mori C, Dix DJ. Effects of hyperthermia on spermatogenesis, apoptosis, gene expression, and fertility in adult male mice. *Biol Reprod*, 2001, 65(1): 229–239
- 7 Gabai VL, Yaglom JA, Volloch V, Meriin AB, Force T, Koutroumanis M, Massie B *et al*. Hsp72-mediated suppression of c-Jun N-terminal kinase is implicated in development of tolerance to caspase-independent cell death. *Mol Cell Biol*, 2000, 20(18): 6826–6836
- 8 Rockett JC, Mapp FL, Garges JB, Luft JC, Mori C, Dix DJ. Effects of hyperthermia on spermatogenesis, apoptosis, gene expression, and fertility in adult male mice. *Biol Reprod*, 2001, 65(1): 229–239
- 9 Dix DJ, Allen JW, Collins BW, Poorman-Allen P, Mori C, Blizzard DR, Brown PR *et al*. HSP70-2 is required for desynapsis of synaptonemal complexes during meiotic prophase in juvenile and adult mouse spermatocytes. *Development*, 1997, 124(22): 4595–603
- 10 Li H. The anti-apoptosis mechanism of HSPs. *Clinical Biochemistry and Lab Technology*. 2000, 21(3): 118–120
- 11 Almon E, Goldfinger N, Kapon A, Schwartz D, Levine AJ, Rotter V. Testicular tissue-specific expression of the P53 suppressor gene. *Dev Biol*, 1993, 156: 107–116
- 12 Socher SA, Yin Y, de Wolf WC, Morgentaler A. Temperature-mediated germ cell loss in the testis is associated with altered expression of the cell-cycle regulator p53. *J Urol*, 1997, 157: 1986–1989
- 13 Yin Y, de Wolf WC, Morgentaler A. Experimental cryptorchidism induces testicular germ cell apoptosis by p53-dependent and -independent pathways in mice. *Biol Reprod*, 1998, 58: 492–496
- 14 Ricci JE, Maulon L, Battaglione-Hofman V, Bertolotto C, Luciano F, Mari B, Hofman P *et al*. A Jurkat T cell variant resistant to death receptor-induced apoptosis. Correlation with heat shock protein (Hsp) 27 and 70 levels. *Eur Cytokine Netw*. 2001, 12(1): 126–134
- 15 Lee J, Richburg JH, Younkin SC, Boekelheide K. The Fas system is a key regulator of germ cell apoptosis in the testis. *Endocrinology*, 1997, 138: 2081–2088
- 16 Lee J, Richburg JH, Shipp EB, Meistrich ML, Boekelheide K. The Fas system, a regulator of testicular germ cell apoptosis, is differentially up-regulated in Sertoli cell *versus* germ cell injury of the testis. *Endocrinology*, 1999, 140: 852–858
- 17 Yamamoto CM, Sinha Hikim AP, Huynh PN, Shapiro B, Lue Y, Salameh WA, Wang C *et al*. Redistribution of Bax is an early step in an apoptosis pathway leading to germ cell death in rats, triggered by mild testicular hyperthermia. *Biol Reprod*, 2000, 63: 1683–1690
- 18 Furuchi T, Masuko K, Nishimune Y, Obinata M, Matsui Y. Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia. *Development*, 1996, 122: 1703–1709

Edited by  
Yong-Lian ZHANG