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Cloning and characterization of a novel spermiogenesis-related gene, T6441, in rat testis

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1. ABSTRACT

We report in the present study the cloning and characterization of a novel gene, named T6441, initially derived by the suppressive subtracted hybridization (SSH) cDNA library. The full-length T6441cDNA was 664 bp long, containing a complete open-reading frame for a protein of 149 amino acids (aa). The protein bears no homology to any reported genes. It is predicted that the molecular mass was about 16.7kDa. Northern blot analysis showed that the T6441 gene had about 4 transcripts in adult rat testis and was temporally regulated in a stage-dependent manner in the testis. *In situ* hybridization showed that T6441 mRNA was specifically localized in spermatids, and its expression level varied in the cells at different stages of the testicular development, with the highest level at steps 7-14. RT-PCR results showed that the T6441 mRNA was transcribed in most of the tested tissues with its strongest signal in the testis. Recombinant T6441 protein was prepared, purified, and was used to raise rabbit. Western blot analysis using the antiserum revealed four possible testicular specific proteins with their molecular weights being about 22, 25, 50 and 55kDa respectively. The T6441 protein was expressed mainly in the cytoplasm of spermatids with the maximal levels at steps 12-19. At step 19 spermatid, the T6441 was mainly localized in the residual bodies. The cytoplasm localization of T6441 protein was supported by transient over expression of GFP-fusion protein in Hela cells. Interestingly, the expression of T6441 caused death of transfected cells within 48 h. Our preliminary experimental results suggest that the T6441 gene may play a role in cytoplasm movement and removal during spermiogenesis.

2. INTRODUCTION

Spermiogenesis a complicated process of germ cell differentiation, involving programmatic expression of diverse cell-type and developmental-stage specific genes, and is a process in which round spermatids undergo a series of metamorphism and ultimately form an elongated, hydrodynamic shape (1). The changes include acrosome formation, nuclear compaction, development of flagellum, reorganization of cytoplasm and cell organelles, and spermiation (2). All the processes, such as meiosis, genetic recombination, haploid germ cell chromatin remodeling and condensation, acrosome and flagellum formation, are highly ordered and regulated (3-6). Characterization of novel genes expressed in testis may provide new insights into the mechanism of spermatogenesis and spermiogenesis.

To elucidate the detailed mechanism of spermatogenesis and spermiogenesis, characterization of the cell type specific genes, especially germ cell-specific genes, becomes more and more important. However, the available knowledge in the literature regarding genes participating in spermatogenesis and spermiogenesis is still poor.

To isolate developmentally expressed cDNAs related to spermatogenesis, we have constructed a SSH library by subtracting mRNAs of 20-day-old rat testis from that of 41-day-old rat testis, and identified various novel cDNA sequences from the library (data not shown). In this paper, we reported the cloning and characterization of a novel gene named T6441 from this subtracted cDNA library.

3. MATERIALS AND METHODS

3.1. Experimental animals

Sprage-Dawley (SD) rats were obtained from the Experiment Animal Center, Chinese Academy of Sciences and maintained under 16-h light, 8-h dark schedule with food and water *ad libitum*. The rats were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All the protocols for the animal treatment had been approved by the Institute Committee on Animal Care and Use.

3.2. Rapid amplification of 5' and 3'-cDNA ends

Total RNA from adult SD rat tissues was isolated using TRIzol (Invitrogen) as described by the manufacturer. 5' and 3'-RACE was performed using SMART RACE cDNA Amplification Kit (BD Clontech). Briefly, 5' and 3'-RACE ready cDNA was synthesized using 1μg total RNA from testicular tissue of the adult rats by reverse transcriptase according to the manufacturer's protocol (BD Clontech). Universal primer mix provided in the kit and gene-specific primers based on the sequence of a cDNA fragment isolated from a SSH library were used for the 5' and 3'-RACE experiments. The PCR products were cloned into pGEM-T Vector (Promega). The assembled full-length cDNA of T6441 was searched against NCBI Blast server:(http://www.ncbi.nlm.nih.gov/BLAST/). Its putative protein product and the secondary structure were analyzed using DNAstar and DNAman program.

3.3. Recombinant protein expression and antibody production

The peptide used for raising antibody was derived from the full length of T6441. The primer pairs for cloning are: forward/*Nde*I (5'-GGA ATC ATA TGG TTC TCC AAG ATG A-3') and reverse/*Bam*HI (5'-GGT GGA TCC CTT CCG TGG CCT GC-3'). After being double digested with *Nde*I and *Bam*HI, the PCR product was cloned into pET21b (+) vector in frame with the C-terminal His6-tagged fusion protein and the construct was verified by DNA sequencing. The recombinant construct was transformed into the Rosetta (DE3) strain (Novagen) of *Escherichia coli*. A single colony bearing T6441 insert was inoculated into Luria-Bertaine broth medium and induced with 1.0 mM isopropyl-thio-ß-D-galactopyranoside (IPTG) at 0.4 optical density (O.D. at A_{600}), and cells were harvested 3h later. Tagged recombinant protein was purified using His-binding resin column HiTrap Chelating HP according to the manufacturer's protocol (Amersham Biosciences).

Male rabbits with body weight about 2 kilograms were immunized s.c.with 200 μg of affinity-purified recombinant T6441 protein emulsified in Freunds complete adjuvant (FCA), and were boosted four times at intervals of 2 weeks with 200 μg of protein emulsified in Freunds incomplete adjuvant (FIA). Ten days after the last booster, the animals were bled and the serum was collected. Also the serum prior to immunization was collected, and all the serum was stored at -20°C for later use.

3.4. RT-PCR and northern blot analysis

Total RNAs from adult rat tissues including brain, lung, heart, stomach, liver, spleen, intestine, kidney, skeletal muscle, prostate, epididymis, testis and ovary were extracted for RT-PCR analysis. Total RNAs (2μg) from each tissue were used as templates for reverse transcription using Superscript III (Invitrogen). The primers used to amplify a 467 bp fragment were 5'- TGA GGC TCG AGA TGG TTC TCC AAG AT-3' forward) and 5'-GGT GGA TCC CTT CCG TGG CCT GC-3' (reverse). Glyceraldehyde-3-phosphate dehydrogenase (G_3pdh) was amplified as an internal control.

20 μg of total RNA for each fraction was subjected to electrophoresis in 1% denatured agarose gel, and vacuum transferred to nylon membrane (Hybond N+; Amersham). The membranes were prehybridized in prehybridization solution (5×SSC, 50% deionized formamide, 2% dextran sulfate, 0.1% sod. lauryl creatine and 0.02% SDS) at 68□ for 2 h.

We used the same primer pairs as in RT-PCR to synthisize the probe. Briefly, purified PCR product of 467bp was ligated to pGEM-T vector (Promega). After the recombinant being amplified using T7 and SP6 primers, the PCR product was utilized to be transcribed by T7 and SP6 RNA polymerase (Progema) respectively with DIG RNA Labeling Mix (Roche) according to the manufacturer's protocol. cRNA probes were denaturalized in prehybridization solution at 68°C for 10min. Hybridization was carried out overnight at 60□ with an optimized concentration (25 ng/ml) of cRNA probe in prehybridization solution. The membranes were washed in 2 x SSC/0.1% SDS at room temperature, twice for 15 min each, then in 0.1 x SSC/0.1% SDS at 68□, twice for 15 min each. After washing in buffer I (100mM Tris-Cl, 150mM NaCl, pH7.5) for 5min, blocked in buffer II (1% blocking reagents in buffer I) for 1h. Incubated with 1:5000 anti-digoxigenin-AP fab fragments (Roche) in buffer II for 1h. After washing twice in buffer I and balanced in buffer III (100mM Tris-Cl, pH9.5; 100mM NaCl, 50mM $MgCl_2$) for 5min, the membrane was incubated in CDP-star illumination substrate according to protocol (New England Biolabs), then exposed on x-ray film.

3.5. *In situ* hybridization

The digoxigenin-labelled specific cRNA probe was the same as in northern blot described above. *In situ* hybridization was performed as previously reported (7), with slight modifications. Briefly, sections (5μm) of Bouin's fixative-fixed, paraffin-embedded adult rat testicular tissue were de-paraffinized, rehydrated and digested with proteinase K (4μg/ml) for 15 min at 37°C and washed in PBS. Sections were then post-fixed in 4% paraformaldehyde in PBS for 5 min and washed in PBS. Before hybridization, the sections were incubated in 5×SSC for 15 min, and prehybridized with 50% formamide/ 2×SSC for 2 h at 50°C. Then hybridized overnight with digoxigenin-labeled rat T6441 cRNA probe in hybridization buffer (10mmol/L Tris-HCl, PH7.5, 2×SSC, 50% deionized formamide, 1× Demhardt's, 2.5mmol/L DTT, 5% dextran sulfate, 50μg/ml yeast tRNA, and 0.5% SDS) at 55°C. After hybridization, the sections were thoroughly washed twice each in 2×, 1×, and 0.1×SSC at 42°C, each for 15min. Following washing with buffer I (0.1mol/l Tris, 150mmol/l NaCl, pH 7.5) for 10 min at room temperature, blocking solution was applied

containing 1% blocking reagent in buffer I for 1 h. Anti-DIG-alkaline phosphatase diluted in blocking solution (1:5000) was then applied for 2 h at room temperature. Finally the color reaction was developed with BCIP/NBT solution for 2h in the dark at room temperature. The sections were hybridized with sense probes as negative controls.

3.6. Western blot analysis

Immunoblot was done according to the method of Laemmli (8). The tissues were homogenized and the supernatant was separated by 15% PAGE (50μg total protein/lane). After transferred to nitrocellulose membranes, the membranes were blocked with 5% nonfat milk/PBS, followed by incubation at room temperature for 1 h with the primary antibodies (1:2000) in 5% milk/PBS. After three 5 min washes at room temperature, the membranes were incubated with HRP-conjugated secondary antibody (goat anti-rabbit IgG, 1:6000) in 5% milk/PBS. After three washes in PBS with tween-20, then followed by 5 min of incubation with SuperSignal West Pico substrate (Pierce), the membranes were exposed on x-ray film. For negative controls, the primary antibodies were replaced with the preimmune rabbit serum.

3.7. Immunohistochemistry

Immunohistochemistry was done as previously described (7) with slight modifications. 5 μm paraffin sections of rat testis tissue were deparaffinized and rehydrated, then incubated in 3% H_2O_2/distilled water (v/v) for 5-10 min to quench endogenous peroxidase or endogenous biotin activity. Antigen retrieval was performed by incubating the sections in 0.01M sodium citrate buffer (pH6.0) at 96°C for 15 min. Then, the sections were incubated with 10% horse serum in PBS for 15 min at room temperature to block nonspecific binding, followed by incubation with the rabbit anti-T6441 antibody (diluted 1:300 in 10% horse serum-PBS) for 1 h at room temperature. After washing in PBS, the sections were incubated with the biotin-labeled secondary antibody (RT, 30min) and then were incubated with the horseradish peroxidase-conjugated streptavidin (RT, 30min) or alkaline phosphatase-conjugated streptavidin (RT, 30min). Following extensive washing with PBS, the sections were developed with DAB or with alkaline phosphate red substrate (Vector Laboratories), then counterstained with hematoxylin, dehydrated and mounted. The sections were incubated with the preimmune rabbit serum serving as negative control. Preabsorbed antibodies with the recombinant T6441 protein were used as negative control.

3.8. T6441-GFP fusion plasmid transfection and confocal fluorescence

The whole coding sequence of T6441 cDNA was was PCR cloned directionally into *XhoI-BamHI* sites of EGFP-N1 (BD Clontech) in frame with the N-terminus of EGFP-N1 and was verified by sequencing. Forward/*XhoI* (5'- TGA GGC TCG AGA TGG TTC TCC AAG AT-3') and reverse/*Bam*HI (5'- GTC GGA TCC TTC CGT GGC CTG CC-3'). Hela cells were transfected with the lipofectamine 2000 essentially as described as the manufacture's manuals (Invitrogen). Briefly, to the 24-well plate, 4-8 x 10^5 cells were added in 500 μl of the growth medium without antibiotics. Dilute 1μg DNA and 2μl lipofectamine 2000 seperately in 50μl serum-free DMEM. Incubated for 5 minutes at room temperature. Combined the diluted DNA with diluted Lipofectamine 2000 (total volume:100 μl). Mixed gently and incubated for 20 minutes at room temperature. Added 100μl of complexes to each well containing cells and medium for 5 h prior to reintroduction to the serum-containing medium. After 16 h, the medium was added 400μg/ml G418 for cell selection. Cultured the cells at 37°C in a CO_2 incubator for 18-48 hours prior to testing for transient expression. After washing in PBS, the coverslips were mounted and visualized immediately under laser scanning microscopy (Zeiss). The empty vector of EGFP-N1 was transfected as positve control.

3.9. Microscopic assessment

Experiments for the T6441 RT-PCR, *In situ* hybridization, immunohistochemistry, Western blot and Northern blot analysis were repeated at least three times, one respective representative figure from at least three similar results was presented.

4. RESULTS

4.1. Cloning and sequence analysis of rat T6441 cDNA and protein

According to the sequence of a cDNA fragment from our SSH library of rat testis, we performed 5'- and 3'-RACE, which produced the cDNA fragments of 505bp and 398bp, respectively. The resulted full-length cDNA was 664 bp long, and the gene was named T6441 according to the clone number in our SSH cDNA library (bankit 710335, DQ080016). This cDNA contained a complete open-reading frame for a protein of 149 amino acids. The predicted molecular mass was about 16.7kDa. From the GenBank, the highly homogenous sequences were found and were aligned using DNAman 5.5 (Figure 1A). None of the homogenous sequences was annotated and no related publication was available in the literature. T6441 had a conserved domain which was composed of alpha helices (Figure 1B).

4.2. Temporal expression of T6441 in developing testis by Northern blot analysis

Northern blot analysis revealed that the T6441 had about 4 transcripts (Figure 2). The shortest transcript was about the same size as the cloned cDNA. Hybridization results from the rat testes at various days indicated that the expression level of T6441 gene was regulated in a stage-dependent manner during testicular development. The expression was low before postnatal stage on day 20, and dramatically increased between days 20 to 28, the defined period for the first wave of spermatogenesis in rat. The increased expression of the gene continued and maintained during the adult stage (Figure 2). The expression of the four mRNA variants had the similar profiles.

4.3. Multiple tissues expression pattern of T6441 and its *in situ* hybridization in spermatids

Total RNA from rat multiple tissues were extracted and reverse-transcribed to perform RT-PCR. From the result of RT-PCR, T6441 gene was expressed in various tissues. The strongest expression of T6441 was observed in testis.

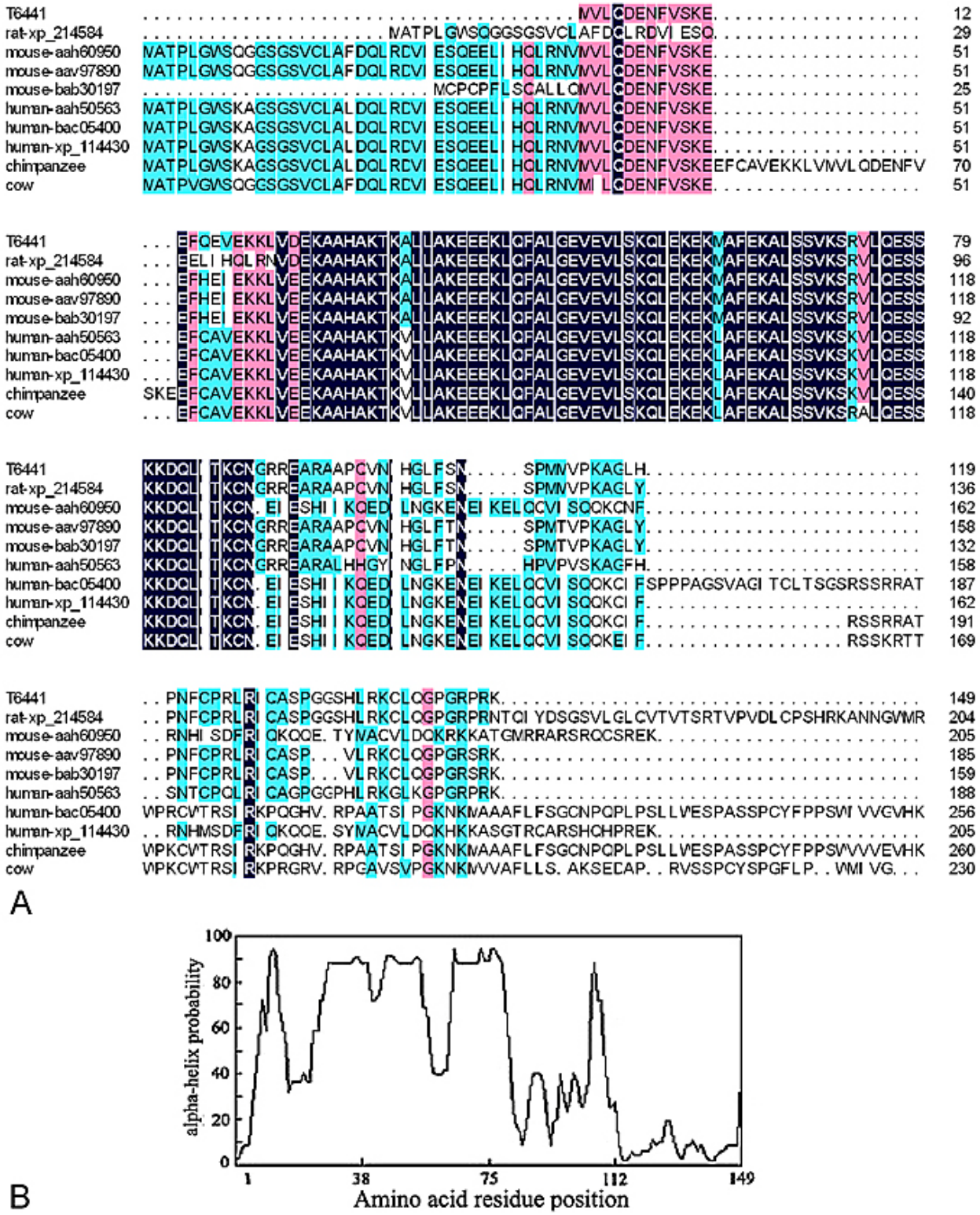

Figure 1. T6441 protein sequence analysis. (A) T6441 protein sequence alignment. T6441 was aligned with deposited sequences with accession number in GenBank as rat (xp_214584), mouse (aah60950, aav97890, bab30197), human (aah50563, bac05400, xp_114430), chimpanzee (xp_527025), cow (xp_582494). T6441 encoded 149 amino acids. (B) N-terminus 90aa was a conserved domain composed of alpha-helices.

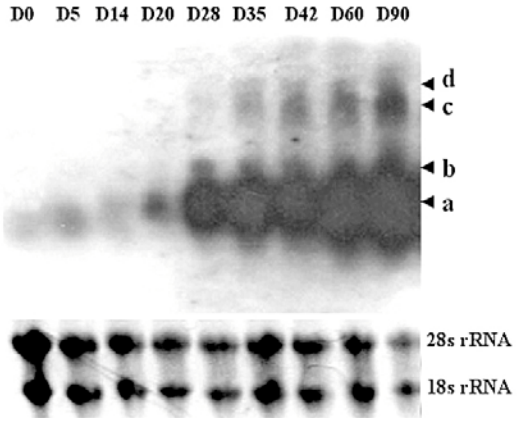

Figure 2. Northern blot analysis of total RNA from rat testis on day 0, 5, 14, 20, 28, 35, 42, 60, 90 after birth. T6441 had at least 2 more discernable transcripts: a (about 700bp), c (about 2000bp), and 2 less intelligible transcripts: b (about 800bp), d (about 2500bp), due to the small length variance and low transcription abundance. The abundance of 4 possible transcripts: a>b>c>d. 28s rRNA and 18s rRNA as the internal control.

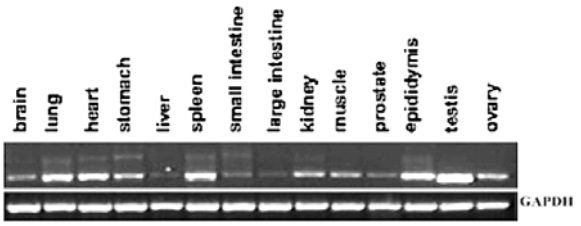

Figure 3. Multiple-tissue RT-PCR of T6441 with the GAPDH as internal control.

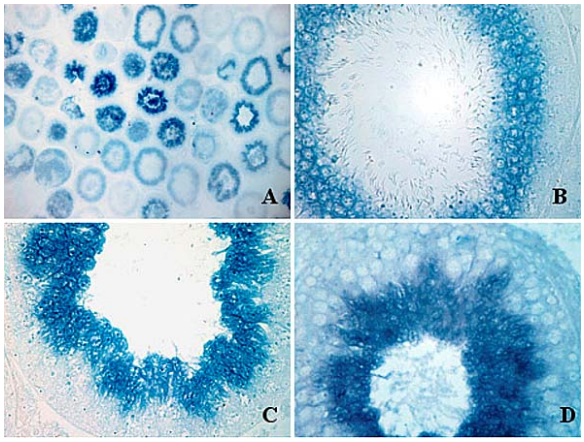

Figure 4. *In situ* localization of T6441 mRNA in adult rat testis. A, 4×amplification B; 40×, stage VI-VIII; C, 40×, stage IX-XI; D, 40×, stage XII-XIII.

Strong signals were also observed in lung, heart, spleen and epididymis, while relatively weak signals were detected in the other tissues (Figure 3).

To determine the cell types in which T6441 mRNA was expressed, paraffin-embedded sections of the adult rat testis were hybridized with a fragment of T6441antisense RNA. In situ hybridization results showed strong signals in the inner half-layer of the seminiferous epithelium, and the intensity of the hybridization signals markedly varied between the seminiferous tubules (Figure 4). The seminiferous tubules at stages VII-XIII showed strong signals exclusively in round spermatids and elongating spermatids, whereas at stage I-VI and at more differentiated spermatids (step 15-19) the T6441 mRNA showed faint or no signal at all. No obvious signal was detected in Sertoli cells, spermatogonia and spermatocytes. These data suggest that the transcription of T6441 was stage-specific, and mainly localized in the spermatids.

4.4. T6441 protein and its cellular localization in the cytoplasm and the residual bodies of late step spermatids

Antiserum was obtained from the immunized rabbit using the purified T6441 recombinant protein. Western blot analysis using the antiserum was performed with the proteins extracted from the adult rat tissues. In testis, four possible bands about 22kDa, 25kDa, 50kDa and 55kDa were respectively detected (Figure 5). In the other examined tissues, the reacted bands did not have the same size. The bands in the testis seemed to be testis-specific. That the smallest band was larger than the predicted molecular mass value (16.7kD) might be the results of side chain modifications in some amino acids.

Anti-T6441 serum was further utilized to examine the distribution of T6441 protein in the adult rat testis by immunohistochemistry analysis. At low magnification of the light microscope, T6441 immunostaining was detected at the luminal border of the seminiferous tubules, and the staining pattern varied among the tubules due to the stages of the spermatogenic cycle in the seminiferous epithelium (Figure 6). At the higher magnification, T6441 signal was observed as punctate staining in the cytoplasm of spermatids from step 10 to step 19 (Figure 6A, 6D). At step 19 spermatids (close to spermination), the immunostained small spheres appeared to be residual bodies in the caudal cytoplasm. In contrast, no obvious signal was detected in the other cell types of the seminiferous tubules.

In order to examine whether T6441 protein was present in mature epididymal spermatozoa, confocal laser scanning microscopy was performed on the spermatozoa immunostained for T6441. As shown in Figure 7, there was still rudimental immunolabeling of T6441 at the separated head cap segment (HCS) of spermatozoa from epididymis.

Construct of T6441 in frame with GFP was transiently transfected into Hela cells. The fluorescence of the fusion protein was located exclusively in the cytoplasm consistently with that of spermatids (Figure 8). The cells bearing T6441-GFP can be seen within 24 hours, but after 48 hours, the green signals disappeared completely, while plenty of those untransfected cells in the same well as well as those transfected with the empty vector of EGFP were still alive under high content of G418. Transient overexpression of T6441 had deadly effects on the cells.

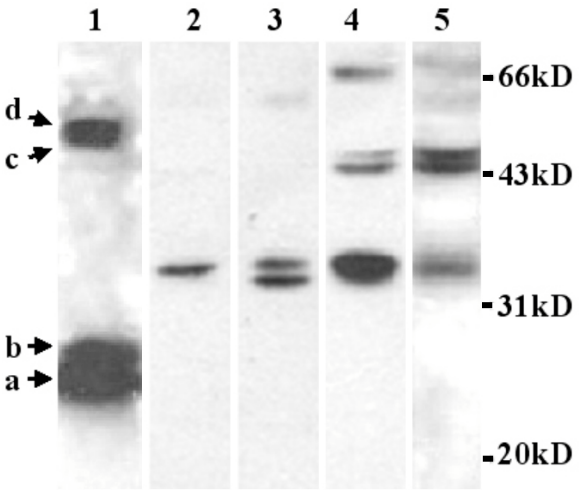

Figure 5. Western blot of T6441 protein in testis (1), liver (2), spleen (3), lung (4), ovary (5) from adult rat. T6441 has at least more than 2 discernable protein products: a (about 22kD), d (about 55kD) in adult rat testis. There are 2 less intelligible variants: b (about 25kD), c (about 50kD). The abundance of the 4 possible products: a>b>d>c.

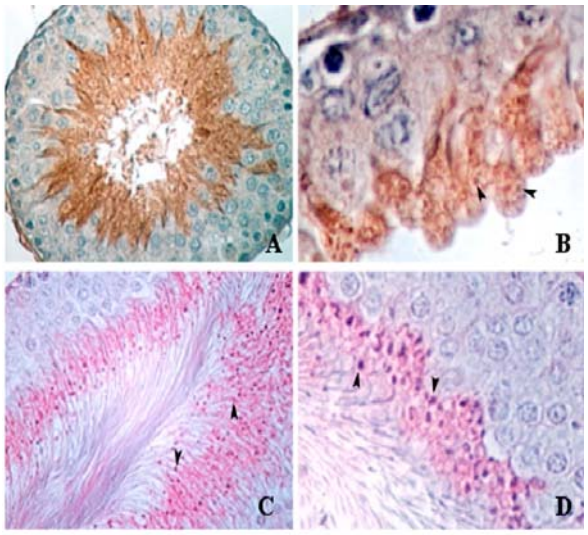

Figure 6. Immunohistochemical localization of T6441 protein within the rat testis. T6441 positive cells (brown stained with DAB or pink stained with AP red) are restricted to the cell layer close to the lumen. Many deeply stained granules could be distinguishable in the elongating and the elongated spermatid cytoplasm. (A) 40× positive cells are step 12 elongating spermatid (brown). (B) 100×oil object (partial field), step 11 elongating spermatid (brown). (C) 40×, step 17 spermatid (pink). (D) 40×, partial field, step 19 spermatid (pink).

5. DISCUSSION

T6441 may be an evolutionarily conserved gene among mammals, as homologues were found in mouse, rat, human, chimpanzee, cow in the 90 aa alpha helices domain (Figure 1). The protein does not bear significant similarity to any previously reported proteins, therefore, suggesting that this protein is novel. The gene has about 4 possible transcripts in the testis and the 4 possible transcripts have the similar temporal and spatial expression pattern along the testicular developmental stages, suggesting that the different variants may have similar, synergistic or cooperative function in metamorphism of spermatids.

Based on our data of the Western blotting analysis in the multiple tissues, we postulate that the T6441 might have plenty of variants among which some may be tissue-specific. The fact that T6441 antiserum was able to recognize four proteins and that T6441 cRNA probe detected 4 transcripts implies that these four proteins all contain the 90 aa alpha helices domain against which the antiserum and cRNA probe were prepared. We realize that this is still an open question to be answered in a more confirmative way in the future experiments by cloning of the various transcripts.

T6441 protein was specifically expressed in the spermatids from step 10 to step 19 and localized in their cytoplasm, it is suggested for the first time that T6441 gene might participate in the cytoplasm deformation and movement. At steps of 10-19, the central task of the spermatid is to deform its structure, such as to compact the nuclei, to shed most of the spermatid cytoplasm and to form acrosome and tail, and ultimately to acquire capability of motility and fertilization. In the cytoplasm of the spermatid at step 12, the T6441 gene might have attained its high level, and many punctulate structures in the spermatid at this step have been stained more positively. T6441 was also expressed in a pattern that partially and temporarily overlayed with that of the manchette, a cylindrical collection of microtubules extending from the nuclear region to the caudal region of spermatid (10-13). At this period, nuclear condensation is underway and transition protein may appear, and spermatid-specific histones are gradually disappearing (14-16). Moreover, Sertoli cell processes start invading spermatid cytoplasm and undergo considerable changes at this phase (17). Two types of Sertoli cell processes have been reported by Morales and Clermont (18). Both processes could be observed from the spermatid at the step 11 of spermiogenesis. One kind of the two processes is associated with a tight network of ER cisternae, the other is not associated with such a spermatid subsurface ER network (18). Breucker and colleagues have reported (19) that at late steps of spermatid maturation, the endoplasmic reticulum has gathered with other cell organelles to form aggregates. Golgi complex, mitochondria, annulate lamellae, a chromatoid body, flower-like structures, ribosomes, a few large vacuoles, myelin-like membrane profiles and sporadic lipid droplets have been detected to locate in the caudal cytoplasmic mass of the late spermatid. The cytoplasmic mass might be the candidates for the punctulate structures for the T6441 location. The cytoplasm of the elongated spermatid becomes progressively attenuated along axis of the sperm and thus forms a slender cytoplasmic lobe (future residual cytoplasm) extending

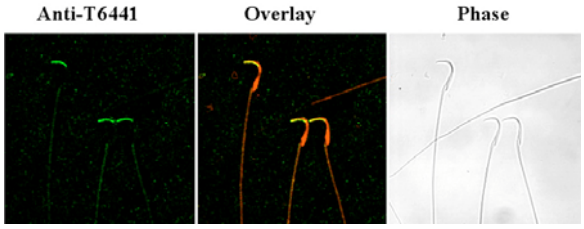

Figure 7. Indirect immunofluorescence of T6441 in sperm from caput epididymal. Residual protein of T6441 (green) was localized in the head cap of rat sperm. It was also localized in corpus and caudal epididymal sperm (data not shown). Condensed sperm nuclei were counterstained with PI (red).

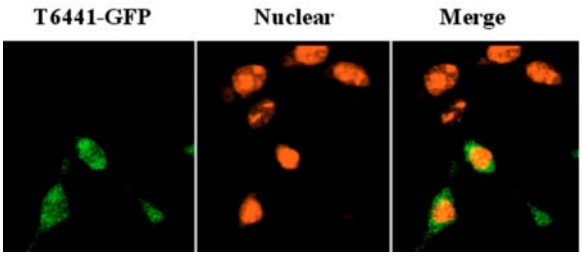

Figure 8. Overexpression of T6441 caused cell death in somatic cells. T644l-GFP was transfected into Hela cells with positive signal (green) in cytoplasm at 24 h. All positive cells underwent cell death within 48 h after transfection. The non-transfected cells in the same well or the empty cells transfected with EGFP vector were remained viable within the same duration. Nuclei were counterstained with PI.

from the neck of the spermatid and coursing toward the apical regions of the Sertoli cell (17,20-22). T6441 was apparently involved in the processes. However, at this step, we still do not know whether the T6441 participates in the residual body formation or the earlier events of spermatid deformation, which was pinched off with the residual cytoplasm just before spermiation.

From our data, some staining of T6441 protein was observed in the concave face of the sperm head, implying the possible role of this gene in the later events in sperm maturation or fertilization. Because of the fact that over-expression of the T6441 in Hela cells could result in death of the transfected cells, the function of T6441 protein might be specific to spermiogenesis. Moreover, apoptosis might be responsible for the formation of residual bodies like the formation of apoptotic bodies (23, 24). We can not rule out that T6441 might also play a role in such a mechanism, as observed that over-expression of the T6441 protein in Hela cells had deadly effects on the cells.

. The results reported in the present study have demonstrated that the T6441 is an abundently expressed novel gene at the late steps of spermatid development, and might play an important role in the movement and the removal of the spermatid cytoplasm during the late steps of spermiogenesis. However, we know only a little about the gene function, there are still many secrets that should be elucidated. To understand the detailed function of the T6441 gene, further investigation should be performed.

6. ACKNOLOWLEGEMENTS

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