Mutations of t-complex testis expressed gene 5 transcripts in the testis of sterile t-haplotype mutant mouse

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Abstract

Aim: To determine the possible roles of the t-complex testis expressed gene 5 (Tc tex 5) on sperm functions, the full-length sequence of mRNA was studied and compared in the testis between the normal wild-type and the sterile t-haplotype mutant mice. Methods: We applied rapid amplification of cDNA ends, Northern blot and reverse transcription polymerase chain reaction to analyze the full length of Tc tex 5 mRNAs isolated from testes of the wild-type and the t-haplotype mice. Reverse transcription polymerase chain reaction was used to semi-quantitatively compare expression of Tc tex 5 transcripts in the 16 tissues and 9.5 day stage embryos in the wild-type mice. E-translation was applied to estimate the amino acid sequences. Results: One long and one short transcript of Tc tex 5 mRNA were discovered in mouse testis of wild-type (Tc tex 5long-+ and Tc tex 5short-+) and t-haplotype (Tc tex 5long-t and Tc tex 5short-t) mice, respectively. Being enhanced only in the testis, Tc tex 5long-t had 17 point mutations and one 15-bp-deletion in the exon 1 region, comparing with the Tc tex 5long-+, whereas the Tc tex 5short-t was similar to the Tc tex 5short-+. The short isoforms of Tc tex 5 mRNAs in the two models encoded exactly the same peptides, but the long isoforms did not. The estimated peptide encoded by Tc tex 5long-t had significant mutations on putative sites of phosphorylation and PP1 binding. Conclusion: We established that mutations that occur in the Tc tex 5 long transcript of the t-haplotype mice are important for normal sperm function, whereas the short transcript of Tc tex 5 might have a conserved function among different tissues. (Asian J Androl 2008 Mar; 10: 219–226)

Keywords: t-complex testis expressed gene 5; transcripts; testis; mice

1 Introduction

Protein phosphorylation and dephosphorylation take part in spermatogenesis, especially for the G2/M transition evidenced by the fact that pachytene spermatocytes entering metaphase within 4–6 h under stimulation in vitro with the protein phosphatase 1 inhibitor okadaic acid [1]. In contrast to protein kinases that add a phosphate group to the hydroxyl oxygen of serine, threonine or tyrosine residues, protein phosphatases (PP) remove the phosphate group. There are mainly four groups of PP according to their catalytic subunits: PP1, PP2A, PP2B and
PP2C. Until now, PP1 (including subtypes PP1α, PP1β, PP1γ1 and PP1γ2), PP2A and PP2B have been found to be expressed in the testis [2]. The regulators of PP, such as inhibitor 2 (Inh2), inhibitor 3 (Inh3), sds22, 14-3-3 and hsp90, are also associated with spermatozoa both in the testis and in the epididymis [3–6].

Tctex5 is defined as t-complex testis expressed 5 [7]. The human homologue of Tctex5 was discovered in 1996, and has been confirmed to be a protein phosphatase 1 regulator 11 (PP1g1) and has been confirmed to be a protein phosphatase 1 γ [8, 9]. Inh3 in humans is co-localized to the nucleoli and centrosomes with PP1γ1 and PP1α, respectively [10]. In a sterile male mice model (t-haplotype mutant mice), Tctex5 and its function on sperm have drawn our attention as a candidate for the “Curlicue” and “Stop” (sperm oolemma penetration of t-haplotype mice) phenotype [11]. T-haplotypes are mutations in the proximal one-third of mouse chromosome 17. The true homozygous embryos will die at very early developmental stages in the uterus, whereas the secondary “t homozygotes”, which are homozygous for two non-complimentary t haplotypes (like t w32 and t w3 in the present study) originated from different genetic background (B6 and 129 mice, respectively), can survive. However, the male “t-homozygotes” (t w32/t w3) are absolutely sterile because of the expression of mutation-bound factors that perturb numerous and distinct sperm functions, such as motility, zona-pellucida binding and oolemma penetration [12, 13]. “Curlicue” and “Stop” are phenotypes shown by individual spermatozoa isolated from t-homozygotes male mice epididymis in vitro. The t sperm show extra curvatures (“Curlicue”) when exposed to increased levels of Ca2+ in the medium, and they can not penetrate the oolemma (“Stop”) when exposed to the zona-free oocytes. The genetic factors leading to these two phenotypes have been mapped to “Ccua” (include “Ccua” and “Ccub”) and “Stop” (include “Stop1p” and “Stop1d”) regions in chromosome 17 [14]. Being located to both the “Ccub” and “Stop1d” locus, Tctex5 might be involved in the regulation pathways for sperm function, including motility control, sperm flagella structure development and sperm-oolemma penetration. In our previous studies, Tctex5 has shown a specific expression pattern in the testis undergoing spermatogenesis and a similar distribution of PP1γ2 in the spermatozoa [4]. In addition to the binding of Tctex5 to PP1γ2 in vitro [15], our previous research indicates the important role of Tctex5 in normal sperm function through PP1γ2 or other isoforms of PP1. To reveal the role(s) of Tctex5 during spermatogenesis, in the present study, we have investigated the Tctex5 expression pattern in the testis and compared the sequences of each transcript between the wild-type and the t-haplotype mice.

2 Materials and methods

2.1 Production and genotyping of mice

Wild-type male mice were produced by crossing 129-+/+ and C57BL/6-+/+ mice. All t w32/t w3 mice (hereafter we called t-haplotype mice) were obtained by breeding of t w32/+ and t w32/+ mice and were congenic to the +/- mice. The proximal portion of mouse chromosome 17 was genotyped by restriction fragment length polymorphism analysis of genomic DNA extracted from tail-tip biopsies. Briefly, digested genomic DNAs were separated by electrophoresis in agarose gels and blotted to nylon membrane. DNAs were bound to the membrane by ultraviolet light and hybridized with radio-labeled probes that were informative for the chromosome 17 genotype. The presence of complete t haplotypes was detected using the marker DNA clones Tu48, Tu89. Pim1A was used to distinguish between t w32 and t w3 haplotypes.

2.2 mRNA preparation and rapid amplification of cDNA ends

mRNA from the testes of the wild-type and the t-haplotype (t w32/t w3) mice was isolated using the Oligotex Direct mRNA Mini Kit (Qiagen, Valencia, CA, USA). Full-length 5’ and 3’ ends of Tctex5 were obtained using the GeneRacer kit (Invitrogen, Carlsbad, CA, USA) based on RNA ligase-mediated and oligo-capping rapid amplification of cDNA ends (RACE). Referring to genomic Tctex5 sequences in the GeneBank (No. AK005379), Tctex5 specific 5’ and 3’ primers and nested primers were designed (Table 1). Sequencing of full length cDNA was carried out by Macrogen (Seoul, Korea). Experiments were carried out twice.

2.3 Reverse transcription polymerase chain reaction (RT-PCR), semi-quantitative analysis of Tctex5 transcripts in tissues and northern blot

After the long and short transcripts of Tctex5 mRNA were found by RACE in the wild-type mice and the short transcript in the t-haplotype mice, we designed two primer sets that discriminate the long and short Tctex5 transcript: the Tctex5 complete and Tctex5 lower.
respectively (Table 1 and Figure 1A). RT-PCR was performed using mRNA isolated from the wild-type and t-haplotype testis as template to confirm the existence of the transcripts. A new long transcript in the t-haplotype mice testis was discovered. Expression of each transcript of Tctex5 mRNA was screened using the mouse rapid scan gene expression panels (Origene MSCB-101, Rockville, MD, USA) in three dilutions (100 ×, 10 × and 1 ×) with the lowest dilution concentration (1 ×) with approximately 1 pg cDNA. Using the panels as templates, we carried out RT-PCR using the following cycle conditions: 95ºC for 3 min; 95ºC for 30 s, 55ºC for 30 s and 72ºC for 90 s for 35 cycles; 72ºC for 10 min, and 4ºC thereafter. Beta-actin was amplified as an internal control. Experiments were carried out twice. After washing, the blot was exposed to Kodak BioMax MS film (Kodak, Rochester, NY, USA).

2.4 Computer-assisted sequence analysis
Sequence analysis and Tctex5 intron/exon analysis used in the present study were obtained from http://www.ensemble.org. E-translation of known mRNA and protein structure prediction were carried out using the following websites: http://ca.expasy.org/tools/dna.html and http://www.predictprotein.org, respectively.

3 Results
3.1 Isoforms transcribed in the testis
There were two isoforms of Tctex5 mRNA transcribed in the testis. Significant mutations occurred in the long transcript of Tctex5 between the t-haplotype and the wild-type mice. The long isoform mRNA was enhanced in the testis whereas the short isoform was more ubiquitous.

Using RACE and RT-PCR, we identified a long and a short isoform of Tctex5 (Tctex5long-+ and Tctex5short-+ in wild-type, Tctex5long-t and Tctex5short-t in t-haplotype mutant) transcribed in the testis. The cDNA sequences of the Tctex5long, Tctex5short (Tctex5shortt) had exactly the same

### Table 1. Primers used in this study. RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; Tctex5, t-complex testis expressed gene 5. F, forward; R, reverse.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td><strong>Primers used for RACE</strong></td>
<td></td>
</tr>
<tr>
<td>A(F): GeneRacer 5’ primer</td>
<td>5’-CGACTGGAGCACGAGGACACTGA-3’</td>
</tr>
<tr>
<td>B(F): GeneRacer 5’ nested Primer</td>
<td>5’-GGACACTGACATGGACTGAAGGATGA-3’</td>
</tr>
<tr>
<td>C(F): GeneRacer 3’ primer</td>
<td>5’-GCTGTCACAGTACGCTGACACG-3’</td>
</tr>
<tr>
<td>D(F): GeneRacer 3’ nested primer</td>
<td>5’-CGCTACGTAACCGCATGACAGTG-3’</td>
</tr>
<tr>
<td>A(R): Tctex5 5’ race reverse primer</td>
<td>5’-TAATACGACCATTTGACGAG-3’</td>
</tr>
<tr>
<td>B(R): Tctex5 5’ race nested reverse primer</td>
<td>5’-CATATGCTCATGTTCACATGTC-3’</td>
</tr>
<tr>
<td>C(R): Tctex5 3’ race forward primer</td>
<td>5’-ACCAGAGTGATGAGGATGAGA-3’</td>
</tr>
<tr>
<td>D(R): Tctex5 3’ race nested forward primer</td>
<td></td>
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<tr>
<td><strong>Primers used for RT-PCR</strong></td>
<td></td>
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<tr>
<td>E(F): Tctex5 complete forward primer</td>
<td>5’-CTCGAGGTCTCATCCGTuttTTCCT-3’</td>
</tr>
<tr>
<td>E(R): Tctex5 complete reverse primer</td>
<td>5’-CTCGAGATGAATGGTAGAGGGAT-3’</td>
</tr>
<tr>
<td>F(F): Tctex5 lower forward primer</td>
<td>5’-AGGTGAGTGTCAGCTGACG-3’</td>
</tr>
<tr>
<td>F(R): Tctex5 lower reverse primer</td>
<td>5’-GGGCTTAGAGGATCAGGAG-3’</td>
</tr>
<tr>
<td>Beta actin forward primer</td>
<td>5’-AGCCATGTACGTAGCCATCC-3’</td>
</tr>
<tr>
<td>Beta actin reverse primer</td>
<td>5’-CTCTCAGCTGTGGTGGTAA-3’</td>
</tr>
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t-complex testis expressed gene 5 transcription in mice

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(to be continued)
Figure 1. Transcripts of t-complex testis expressed gene 5 (Tctex5) expressed in the testis of the wild-type and the t-haplotype mice. (A): A long and a short transcript of mRNA were discovered in each type of mice using GeneRacer and reverse transcription polymerase chain reaction. Sequence alignments of the four cDNA isoforms (the long-+, the long-t, the short-+ and the short-t) are shown. In the alleles, absent or unknown sequences are represented by dashed lines. ATG were boxed and the ending code TAA is underlined. Mutations occurring in the long t isoform are highlighted yellow. Sequences in accord with primers described in Table 1 are highlighted in the long-+ isoform only with their names labeled accordingly. Exons are separated by vertical lines and indicated by arrows. (B): Northern analysis of Tctex5 transcribed in the testis of the wild-type and the t-haplotype mice, respectively. The transcripts show three sizes, which were indicated as long, medium and short transcript, respectively. (C): Estimation of the expected promoters of Tctex5 in the mouse chromosome 17. The sizes of each exon and intron are not in the appropriate ratio, as shown in the diagram. Estimated promoters 1 and 2 are indicated. The wild-type alleles of Tctex5 are identical and are represented by green bars. The t-haplotype alleles are different and are denoted as 1 and 2. Mutations are represented by star symbols. (D): Estimated mutations of Tctex5 peptide in the wild-type and the t-haplotype mice. The estimated peptide was E-translated from the three Tctex5 mRNA transcripts. Mutations are highlighted and compared with the wild-type long peptide. Synonymous and non-synonymous mutations are distinguished by lighter (green) and darker (black) highlighting. Estimated influenced secondary modification sites are marked above and the PP1 binding site is underlined.
Figure 2. Transcription of t-complex testis expressed gene 5 (Tctex5) long and short transcripts in different tissues in wild-type mice. Reverse transcription polymerase chain reaction was performed using primer sets (Tctex5 complete and lower primer sets, respectively) specific for the long and short transcripts of Tctex5. In the 18 tissues analyzed, expression of the long transcript of Tctex5 was enhanced only in the testis, whereas expression of the short transcript of Tctex5 was enhanced in heart, kidney, liver, stomach, small intestine, muscle, lung and testis.

As Tctex5long-t was discovered by RT-PCR, its full length was not known. The full length transcript (Tctex5long-t) of Tctex5 contained three exons. In the Tctex5long-t mRNA, exon 1 spanned from 169 bp upstream of the first initiating ATG to 91 bp downstream of ATG; exon 2 spanned 92–310 bp downstream of the first initiating ATG; exon 3 spanned 311–481 downstream of the first initiating ATG (Figure 1A). Seventeen point mutations and one 15-bp-deletion in exon 1 (from 44 to 58 bp downstream of the first initiating ATG) occurred in the Tctex5long-t mRNA. The most intensive point mutation (hereafter called the highly mutational region) occurred in an area 7–23 bp downstream of the first initiating ATG, which was just 11 bp upstream of the deletion region. Tctex5short-t and Tctex5short-+ shared a second initiating codon ATG in exon 2 (103 bp downstream of the first initiating ATG in Tctex5long-+ and Tctex5long-t) without the amino acid reading frame shift comparing with their long isoform counterparts. They were similar in sequences except in the 5' UTR region.

Northern blot analysis of the Tctex5 transcripts transcribed in the testis of the wild-type and the t-haplotype mice showed that the sizes of Tctex5long-+, Tctex5short-+ and Tctex5short-t were approximately 1.2 Kb, 0.6 Kb, 1.0 Kb and 0.6 Kb, respectively. The short isoforms were much more abundant than the long isoforms in both models (Figure 1B). Rapid-scan gene expression panel showed that, in wild-type mice, only the long transcript of Tctex5 was enhanced in the testis. In contrast, the short transcript of Tctex5 was not just enhanced in the testis, but also in other tissues, such as heart, kidney, liver, stomach, small intestine, muscle and lung (Figure 2).
3.2 Estimated mutations of Tctex5 peptide

The short Tctex5 mRNAs encoded for a shorter truncated peptide compared with the long mRNAs with no reading shift. Estimated peptide sequences from the two short Tctex5 mRNA transcripts were exactly the same in the wild-type and the t-haplotype mice. In contrast, estimated peptide sequences from the two long Tctex5 mRNA transcripts were different. Two out of three sites for casein kinase II phosphorylation, one N-myristoylation site, one ASN-glycosylation site, one protein kinase C phosphorylation site, one Amidation site and the estimated PP1 binding site were mutated in the site, one ASN-glycosylation site, one protein kinase C for casein kinase II phosphorylation, one N-myristoylation mRNA transcripts were different. Two out of three sites estimated peptide sequences from the two long Tctex5 mRNA transcripts were mutated in the Tctex5 long-+ compared with its Tctex5 long-+ counterpart (Figure 1D).

4 Discussion

Among the two verified Tctex5 mRNA transcripts in GenBank, only 29 bps of the AK077455 (1170 bp in length) isolated from day 8 mouse embryo whole body shared 100% identity with the genomic sequences of Tctex5 in GenBank; whereas the BC027737 (652 bp in length) isolated from mouse mammary tumor was very similar to the long isoform mRNA sequence discovered in the present study (with only 21 bp discrepancies in the most 3' end). The long and short transcripts of Tctex5 in the wild-type mice discovered here might be either consequences of alternative splicing in the exon1/exon2, or products of different promoters. The long transcript covered all three exons, whereas the short transcript covered only partial exon 2 and complete exon 3 in the wild-type mice (the Tctex5 short covers partial exon 1 and complete exon 2 and 3 in the t-haplotype). In the t-haplotype mice, there were also two transcripts of Tctex5. There were several important mutations in the Tctex5 long+ compared with Tctex5 long-. Unlike its wild-type counterpart, Tctex5 short did not have mutations, but had a different 5' UTR. The two transcripts in both mouse models were not equal. Therefore, we assumed that: (i) the two transcripts in the wild-type and t-haplotype mice might not originate from alternative splicing of mRNAs but from different promoters and (ii) there were two different Tctex5 alleles (t32 and t33, respectively) in the t-haplotype mouse testis that might be under the control of different promoters (Figure 1C).

We have searched the transcription factor binding sites using bioinformatics tools available at http://www.cbrc.jp/research/db/TFSEARCH.html. There were putative trans-acting binding sites covering both the transcription start site of the long and the short transcripts in the wild-type mice. We estimated that promoter 1 and promoter 2 located just upstream of exon 1 and exon 2, respectively, produced the long and short isoform of Tctex5 in the wild-type mouse. Using northern blot analysis, the Tctex5 long+ mRNA was found to be significantly shorter than the Tctex5 long-+ mRNA. Although the RNA ladder on the northern blot shifted the difference in size (approximately 0.2 Kb) between the Tctex5 long+ and Tctex5 long-+ was even longer than the whole upstream sequences of the initiating ATG in the Tctex5 long+ (169 bp). Therefore, we assumed that the Tctex5 long-, which was obtained from RT-PCR, had both shorter 5' and 3' ends than those of Tctex5 long+. We anticipated that in the t-haplotype allele 1, promoter 2 might be inactivated by mutations; therefore, only promoter 1 worked and Tctex5 long-+ was produced. The Tctex5 long- might be either much less abundant than the Tctex5 long+, or be a splice variant of the longer transcript or hold a modified 5'-Cap structure because we could not detect it using RACE. Instead, the Tctex5 long+ was obtained by RT-PCR using mRNAs isolated from the t-haplotype testis. In the t-haplotype allele 2, promoter 1 might be inactivated by some unknown mutations; therefore, only promoter 2 worked and the Tctex5 short was produced (Figure 1C).

On average, there were 3.1 human putative alternative promoters (PAP) per gene and the richest tissue sources of the tissue-specific PAP were testis and brain [16]. Tctex5 was expressed on both the germ cells and somatic cells isolated from testicular seminiferous epithelial cells (Han et al., unpublished data). Functions of the two transcripts of Tctex5 in the testis might be different and we anticipated that the long transcript might be a relatively testis-specific isoform. Enhanced expression of the long isoform in primary breast tumor cell lines and metastatic colon tumor cell lines cultured in vitro [17] might indicate a relation of Tctex5 expression and cell division. The long transcript of Tctex5 might have an important role in sperm function. As a candidate gene for “Curlicue” and “Stop”, Tctex5 long+ showed a variety of mutations when compared with Tctex5 long+. The normal Tctex5 short could not compensate the function loss caused by the Tctex5 long-+. Therefore, we predicted that the secondary structure of the Tctex5 long peptide might be important for this function. The phosphorylation state of Tctex5 might be important and had an on/off function for the signal-
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...ing pathway(s). Although which pathways Tctex5 might be involved in during spermatogenesis in the testis it is so for unknown, PP1 or other PP pathways were expected to be candidates, implied by the importance of PP1-biding site mutation in the Tctex5long-t. Tctex5 was very much like a signaling factor itself connecting PP1 or other PP to the signaling pathway(s) important for pachytene-to-metaphase transition in spermatogenesis.

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References

10 Huang HS, Pozarowski P, Gao Y, Darzynkiewicz Z, Lee EY. Protein phosphatase-1 inhibitor-3 is co-localized to the nucleoli and centrosomes with PP1γ and PP1α, respectively. Arch Biochem Biophys 2005; 443: 33–44.