Conserved expression of ubiquitin carboxyl-terminal esterase L1 (UCHL1) in mammalian testes
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Received 12 May 2014; revised 19 August 2014

Spermatogonia, the adult germ cells that initiate spermatogenesis in mammalian testis, are capable of dividing both mitotically and meiotically. Isolation and preservation of spermatogonia helps in preserving genetic pool of endangered animals. In this context, identification of marker(s) that can distinguish spermatogonia from other cells in testis gains significance. Here, we examined the expression of ubiquitin carboxyl-terminal esterase L1 (UCHL1) gene and protein in the testes of several mammals, including highly endangered species. Semi-quantitative-reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed presence of UCHL1 amplicon of 442 bp in all the 18 mammals studied. Nucleotide sequence analysis of these amplicons and their predicted protein sequences revealed 88-99% and 95-100% homology with available human UCHL1 and UCHL1 sequences of other available species in the GenBank, respectively. Western blot analysis showed that UCHL1 protein size was unique in all wild mammals. Immunohistology results confirmed UCHL1 expression in the spermatogonia/gonocytes in testes of several mammals belonging to eight distinct families including highly endangered Felidae, Canidae and Cercopithecoidae. These findings suggest that UCHL1 expression is conserved in the mammalian testis, and could be used as a specific marker for gonocytes/spermatogonia for developing male germ-cell based conservation techniques.

Keywords: Conservation, Endangered animals, Germ cells, PGP9.5, Spermatogonia

Neonatal and prepubertal deaths in animals are of concern as they are lost before contributing their genetic information to their descendants and to the population as a whole. Routine ex-situ conservation techniques such as semen collection and artificial insemination are irrelevant for prepubertal and juvenile males because of the uncommenced or incomplete spermatogenesis. Moreover, spermatozoa being terminally differentiated haploid cells cannot self-replicate and thus, a finite resource.

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Note

Another approach to preserve the genetic potential of immature individuals involves harvesting and preservation of testis from neonatal and immature males (as well as adults). Using testis xenografting, the immature germ cells can later be made to differentiate into sperm by spermatogenesis in recipients. This is advantageous in species that suffer from high rates of neonatal/juvenile mortality. Male germ cells can also be used for preservation of the reproductive potential of individual males that receive known insults (such as chemotherapeutic agents) to their testis.

The male germ line stem cell technology developed during last decade has not only facilitated an increased understanding of the fundamental biology of male germ cell but also has found application in the genetic modification of rodents. It can also be used for targeted genetic modification of livestock in which, no germ line-competent embryonic stem cell line is known to date. Testis tissue xenograft is a potential practical application for conservation of endangered species. Testis xenografting maintains the complex architecture of the testis unlike in spermatogonial stem cells (SSCs) transplantation assay in which, SSCs are dissociated from their niche. Cryopreserved immature mice testis as well as neonatal pig testis tissue have been shown to produce viable offspring following testis tissue xenografting.

Effective application of either of these techniques depends up on spermatogonia-specific-marker(s) whose expression is conserved in endangered species and which can be utilized for the identification of spermatogonia during isolation, in vitro cultivation or in xenografts. This marker can facilitate monitoring of the survival, proliferation and development of these germ cells in vitro or in vivo. Use of spermatogonia-specific-markers for effective isolation of spermatogonia from the testis has been reported in rodents, domestic and wild animals.

The highly conserved ubiquitin-proteasome complex (UPC) is associated with the regulation of cell growth, differentiation, modulation of membrane receptors and cellular stress responses. UPC is comprised of enzymes involved in the protein ubiquitination/deubiquitination. The ubiquitin carboxyl-terminal esterase L1 (UCHL1), also termed...
protein gene product 9.5 (PGP 9.5), is a deubiquitinating enzyme, is a soluble 25 kDa protein with both ubiquitin hydrolase and dimerization-dependent ubiquitin ligase activities\textsuperscript{16-18}. The gracile axonal dystrophy (gad) mouse is an autosomal recessive spontaneous mutant with a deletion on chromosome 5 within the gene encoding Uch1\textsuperscript{18}. In vivo analysis of gad mice revealed that UCHL1 functions as a regulator of apoptosis in germinal cells and it is essential for the early apoptotic wave of germinal cells and for sperm quality control during spermatogenesis\textsuperscript{39}.

The ubiquitin pathway plays a critical role in the progression of spermatogenesis through the mitotic, meiotic and post-meiotic phases\textsuperscript{19-21}. UCHL1 is highly expressed during gonadal and germ cell differentiation by Sertoli cells, spermatogonia, spermatocytes, and spermatids\textsuperscript{22-26}. In mice, Uch1 is expressed in both spermatogonia and Sertoli cells\textsuperscript{26,27} while in domestic animals such as bovine\textsuperscript{28,11}, pig\textsuperscript{9,29}, sheep\textsuperscript{10} and water buffalo\textsuperscript{13}, it is expressed in gonocytes and spermatogonia. Interestingly, rat spermatogonia that have been reported to proliferate continuously do not possess UCHL1\textsuperscript{30,31}. Uch1 protein plays an important role in degradation of unnecessary proteins in the process of embryonic ectodermal differentiation in mouse\textsuperscript{32}. Over-expression of Uch1 in transgenic mice leads to male infertility, due to blockage of spermatogenesis\textsuperscript{33}. Uch1 protein has been implicated in anti-polysemry defense in both mouse\textsuperscript{34} and pig\textsuperscript{35}. Impaired function of UCHL1 correlates to failure in migration of cortical granules (CGs) to the oocyte cortex leading to insufficient anti-polysemry defense\textsuperscript{36}. Cytoplasmic localization of UCHL1 has an important role in suppression of proliferation and serves as a marker for the non-proliferating spermatogonia in primates\textsuperscript{37}.

In the present study, we analyzed the expression of UCHL1 transcript and protein in the testis of several mammalian species, to assess if its expression remains conserved.

**Material and Methods**

**Collection of testes**—Testes samples of dead wild mammals were collected from Nehru Zoological Park, Hyderabad, India; samples of cattle, buffaloes and goats from local Municipal Slaughterhouse; and the mice testes from CCMB animal house. In addition, testes were also collected from stray dogs that died in road accidents. Permission to use the testes for study was obtained from the respective authorities. A piece of testis tissue was submerged in RNA later\textsuperscript{®} (Ambion, Inc; www.ambion.com) immediately after collection and stored at −20 °C until isolation of RNA. For histochemical analysis, the sample tissues were immediately fixed in Bouin’s fixative following collection and processed, embedded, sectioned and stored until further analysis. Only tissue sections that were preserved optimally and, which showed fewer degenerative changes were chosen for immunohistochemical analysis. Similarly, testes samples from which, good quality total protein could be isolated were used for Western blot analysis. Since age of all animals could not be determined, their maturity status was identified based on the identification of most advanced germ cell in the testis section. The list of animals, age (if available)/maturity status and month at which the testis was collected is given in table 1.

**Isolation of RNA and semi-quantitative RT-PCR analysis**—Total RNA was isolated from the samples (testes tissues of 18 mammals) as mentioned in table 1. Testes tissue pieces stored in RNA later\textsuperscript{®} were removed and processed for RNA isolation using TRIZOL reagent (Invitrogen; www.invitrogen.com) according to the protocol provided by the manufacturer. The RNAs were diluted with DEPC-treated water and treated with 10 units of RNase free-DNase (Roche, www.roche.com) for 30 min at room temperature. To inhibit DNase activity, the samples were incubated at 70 °C for 15 min and then stored on ice. The RNAs were quantified using NanoDrop (Thermo Scientific; www.nanodrop.com). Random primers and RNase OUT (both from Invitrogen) were added to 1 μg RNA solution, incubated for 5 min at 65 °C. MMLV high performance reverse transcriptase (Epicentre biotechnologies, www.epibio.com) was added to the RNA solution for reverse transcription, and incubated for 10 min at 25 °C, for 60 min at 37 °C and for 5 min at 90 °C (RT+). At the same time, the reactions without the addition of reverse transcriptase enzyme were done to check genomic DNA contamination (RT–). PCR amplification was carried out on 1 μL of the cDNA per 19 μL of PCR reaction mixture containing 2 mM MgCl\textsubscript{2}, 0.25 mM dNTPs, 1 × PCR buffer, 5 pmol of each primers and 1 U of Taq DNA polymerase (AmpliTaq GoldTM, Applied Biosystems, www.appliedbiosystems.com). The quality and integrity of RNAs sample were checked using gene specific to β-ACTIN (ACTB)
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<th>Month of testis collection</th>
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<th>Nucleotide sequence similarity to Homo sapiens (%)</th>
<th>Nearest phylogenetic neighbour/GenBank Accession No./Amino acid sequence similarity (%)</th>
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NA, data not available; juvenile, no meiotic cells present; prepubertal, pachytene stage spermatocyte present; adult, elongated spermatid/spermatozoa present
5'-CGATCCACACAGAGTACTTGCG, 5'-CGAGCGTGGCTACAGTTCACC, 451 bp (GenBank accession no. NM_001101; annealing at 58 °C, 30 cycles). The sequence of the UCHL1 antisense primer was 5'-CCCGAGATGCTGAACAAAGT and the sense primer was 5'-CCAATGTCGGGTAGATGACA, 442 bp (GenBank accession no. NM_004181.4; annealing at 54 °C, 28 cycles). The PCR products were separated and visualized by 2% agarose gel electrophoresis containing 0.5 μg/mL ethidium bromide.

DNA sequence analysis of UCHL1 transcripts—ExoSAP-IT (GE healthcare; www.gelifesciences.com) was used for purifying the PCR products according to the manufacturer’s instructions and product were directly sequenced using Big dye Terminator and ABI 3700 DNA automated sequencer (Applied Biosystem; www.appliedbiosystems.com). To promote accuracy, both strands were sequenced. DNA sequences were edited initially by removing the primer sequences and subjected to BLAST sequence similarity search (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the nearest phylogenetic neighbor and aligned using Autoassembler software (Applied Biosystem). UCHL1 mRNA sequences of all 18 species were translated to respective amino acid sequences using EditSeq (DNA star; www.dnastar.com). The edited mRNA and protein sequences along with the other UCHL1 mRNA and protein sequences downloaded from the Genbank database were then aligned using CLUSTAL X. All 18 partial UCHL1 mRNA and predicted protein sequences were submitted to GenBank and the respective accession numbers are presented in Table 1.

Western-blot analysis—Total protein from testes of 13 wild mammals, and mice as control was extracted upon homogenization by sonication in a dissolving buffer (7 M urea, 2 M thiourea, 4% CHAPS, 18 mM Tris-HCl, 14 mM Tris-Base, 2 tablets EDTA protease inhibitor, 0.2% Triton-X, 50 mM DTT). Lysed samples (30 µg) were subjected to electrophoresis in 12% SDS-polyacrylamide gel. The gels were then transferred onto the polyvinylidene difluoride membrane (Millipore, www.millipore.com). The membranes were blocked with Starting BlockTM (TBS) Blocking Buffer (Thermo Scientific; www.piercenet.com) for 1 h at room temperature. The membranes were then incubated with rabbit polyclonal anti-UCHL1 antibody (DAKO, www.dako.com; 1:5000) at 4 °C overnight. The membranes were then washed with TBS-T and incubated with HRP-conjugated goat anti-rabbit IgG (Calbiochem, www.calbiochem.com; 1:10000) in TBS for 1 h at room temperature. After washing with TBS-T, immunoreactivity was revealed by chemoluminescence using a C-DiGit™ Blot Scanner (www.licor.com) against SuperSignal® West Femto Chemiluminescent Substrate (Thermo Scientific). To control protein loading on the gels, the membranes were further probed with mouse anti-GAPDH antibody (Thermo Scientifics; 1:10000).

Immunohistochemistry—Dilution of primary and secondary antibodies was done in PBS with 1% BSA (Sigma, www.sigmaaldrich.com). Sectioned (6 µm thick) testicular tissues were dewaxed, rehydrated and stained with anti-UCHL1 antibody as described. Briefly, sections were blocked with 15% fetal bovine serum (Gibco, www.invitrogen.com) in PBS for 30 min, incubated with anti-UCHL1 (1:300) antibody overnight at 4 °C, washed several times with PBS, incubated with 3% H2O2 for 10 min, washed three times with PBS, incubated with HRP-conjugated goat anti-rabbit IgG (1:300) for 30 min at 37 °C, rinsed three times with PBS, incubated for 3-5 min in DAB substrate kit (Vector Laboratories, www.vectorlabs.com) according to the manufacturer’s instructions, rinsed thoroughly in distilled water, dehydrated and mounted in Vectamount (Vector Laboratories) and observed under a Zeiss Axioplan 2 microscope (Carl Zeiss AG; www.zeiss.de). In negative controls, primary antibody was omitted and instead the section was incubated with 1% BSA in PBS.

All the animal experiments conducted for this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the CSIR-Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India.

Results and Discussion

In the present study, we analyzed the expression of *UCHL1* gene in the testes of selected 18 mammals. An amplicon of 442 bp representing partial *UCHL1* transcript was observed in all the tested species but with a variation in expression level; in spite of the primers used being designed in the coding region of human *UCHL1* gene (Fig. 1). The size of *UCHL1* amplicon was identical suggesting conserved transcript length across all 18 species. In our earlier study, we reported identical size of *VEGF*165 transcript in testes of several mammals. Identical length of *TLR3* gene has already been reported in water buffalo, nilgai and cattle. Presence of *UCHL1* transcript in the testis of all 18 species suggests...
UCHL1’s vital role in the testis. Importance of ubiquitin in modulating testicular germ cell death is evident by resistance to cryptorchid-induced apoptosis in testis of Uchl1 deficient gad mice, and mouse with K48R mutation in ubiquitin. Previous studies have shown that Uchl1 deficiency in mice decreases spermatogonial stem cell proliferation and lack of functional Uchl1 in the gad mutant mouse results in the absence of a physiological apoptotic wave in the testis that is important for male fertility. Alternatively, over-expression of Uchl1 arrests spermatogenesis in transgenic mice. Also, mechanisms controlling sperm-oocyte interactions during fertilization are ubiquitin-proteasome dependent and nuclear to cytoplasmic trans-localization of UCHL1 protein is reported to cause loss of proliferative activity of spermatogonia in the non-breeding seasons in the monkey testis. Thus, expression of UCHL1 gene in the testes of mammals can be concomitant to its role in testis development, germ cell proliferation, gametogenesis and regulation of sperm quality in mammals.

Amplified PCR products in all mammals were sequenced to confirm the identity of UCHL1 transcript. These sequences were subjected to BLAST analysis to identify the nearest phylogenetic neighbor. Further, these sequences were aligned with available UCHL1 mRNA sequences of human and eight other species present in GenBank database. Analysis showed highest similarity between UCHL1 mRNA sequences of Bovidae (cattle, water buffalo, black buck, nilgai and goat)/Cervidae (barasingha, sambar and spotted deer) and cattle (97-100%); Canidae (domestic and Indian wild dogs) and domestic dog (99-100%); Felidae (leopard, Asian lion, Indian tiger, jungle cat and Indian palm civet) and domestic cat (97-100%); Ursidae and giant panda (98%); Cercopithecoidae and human (99%). Partial UCHL1 mRNA from all the 18 species showed 88-99% sequence similarity with that of human UCHL1 mRNA sequence. Similarly, the male-enhanced antigen (Mea) gene, which plays an important role in mammalian spermatogenesis and/or testis development share at least 90% homology in the protein and mRNA coding sequences in human and mouse. The conserved sequence homology among the 18 mammals suggests important role of UCHL1 gene in mammalian evolution.

The partial UCHL1 nucleotide sequences of all 18 species were translated into amino acid sequences and were aligned with the existing UCHL1 amino acid sequences in GenBank, including with that of human (Table 1). The human UCHL1 protein is transcribed from a single gene that has 9 exons encoding 223 amino acids. The primers designed in the present study amplified 5 exonic sequences of UCHL1 gene encoding 147 amino acids in all 18 mammals. In the present study, results of amino acid sequences analysis were similar to that of mRNA sequence analysis. The highest similarity in UCHL1 amino acid sequences was seen between Bovidae/Cervidae and cattle (99%); Canidae and domestic dog (98-99%); the Ursidae and giant panda (97%); and the Cercopithecoidae and human (100%). The predicted partial UCHL1 amino acid sequences

![Fig. 1](https://example.com/fig1.png)

Fig. 1—Semi-quantitative RT-PCR analysis of testes tissue from 18 mammals. (A) Human UCHL1 gene-specific primers. A 442 bp amplified product corresponding to UCHL1 transcript could be seen in lanes 1-18: Bos taurus, Macaca silenus, Boselaphus tragocamelus, Rusa unicolor, Bubalis bubalis, Capra aegagrus hircus, Panthera tigris, Ursus arctos isbellinius, Paradoxurus hermaphroditus, Panthera pardus, Axis axis, Felis chaus, Canis lupus familiaris, Cuon alpinus, Cervus duvaucели, Antilope cervicapra, Panthera leo and Mus musculus testis, respectively. Note the variation in the expression as indicated by the intensity of amplified products. (B) β-ACTB was used for normalizing RNA samples. L, 100-bp DNA ladder; N, mouse RT-, where cDNA was omitted, serve as negative control.
from all 18 species showed 95-100% sequence homology to human UCHL1 amino acid sequence. Ubiquitin protein is reported to exhibit remarkable sequence conservation in which 72 out of 76 amino acids are invariantly present among fungi, plants, and animals. Similarly in the present study, UCHL1 amino acid sequences varied only in 19 positions in all 18 species. Anti-UCHL1 antibody identified 25 kDa protein in testis of 13 wild mammals identical to mice, by Western blot analysis (Fig. 2). The present study further substantiates our earlier report in which we reported a conserved expression of UCHL1 protein in a few wild and domestic bovids. Similar to UCHL1 transcript expression, UCHL1 protein level varied in testis of wild mammals. Nevertheless, these findings further corroborate that UCHL1 protein sequence and size is highly conserved in mammals.

Immunohistochemical examination of testis sections from 15 mammals further confirmed expression of UCHL1 protein in the testis of several mammals (Fig. 3). The expression of UCHL1 protein was detected in undifferentiated germ cells such as spermatogonia/gonocytes (arrows) in testis of: A, Boselaphus tragocamelus; B, Axis axis; C, Cervus duvaucelii; D, Cuon alpinus; E, Antilope cervicapra; F, Bos taurus; G, Macaca silenus; H, Panthera leo; I, Panthera tigris; J, Panthera pardus and K, Mus musculus testis, respectively. Note the variation is UCHL1 protein expression in different species. Membranes were probed with anti-GAPDH antibody to assess protein loading on the gels.
gonocytes/spermatogonia in all testes sections. This finding is in concordance with previous reports in cattle\(^1,2\), monkey\(^7,47\), mice\(^27\), pig\(^6,29\), sheep\(^10\), human\(^48\), water buffalo\(^13\) and wild bovids\(^13\) in which UCHL1 protein was found to be present in spermatogonia/gonocytes in the testis. The number of cells expressing UCHL1 protein and their staining intensities varied between the testis sections of various mammals. Results from earlier reports and present study conclude that expression of UCHL1 protein is present in spermatogonia/gonocytes of a wide variety of mammals.

The variation in expression level of UCHL1 transcript and protein and variation in staining intensity, and the number of cells expressing UCHL1 protein in mammalian testes in the present study could be attributed to species-specific differences, age/sexual maturity status of the animal or season during which the testes were collected.

In conclusion, UCHL1 expression is conserved in the testis of 18 mammals including many wild species. The sample size in the present study was small due to dearth of samples from wild animals. Therefore, this preliminary finding needs to be further carried out in this direction with required sample strength. Nevertheless, the findings from this study provides an insight into the role of UCHL1 in testis of a number of mammals and paves way for exploiting the male germ cell technology in conservation of endangered species.

Acknowledgements
This work was supported by grants from Council for Scientific and Industrial Research (CSIR) and Central Zoo Authority (CZA), Government of India, New Delhi. We thank Dr. M Naveen Kumar and Dr. P Srinivas, Nehru Zoological Park, Hyderabad, India for providing the testes samples post-mortem.

References


