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Testis Selective Expression of NOR1 Gene in Rat and Down Regulation of NOR1 Protein in Human Testicular Germ Cell Tumors*

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Abstract Rat oxidored nitro domain containing protein 1 (rNOR1) was identified and characterized. Isolated rNOR1 cDNA consisted of 1 418 base pairs that encoded a 379-amino acid protein, and the amino acid sequence was 89% and 93% identical to that of human NOR1 (hNOR1) and mouse NOR1 (mNOR1), respectively. The rat NOR1 protein contained a putative conserved domain belong to OSCP1 superfamily. The message for rNOR1 is highly detected in rat testis. Furthermore, human NOR1 homologue is enriched in testis. By immunostaining human NOR1 protein was detected in human testicular tumors of different subtypes on tissue microarray. NOR1 was strongly immunostained in non-cancerous testicular tissues and embryonal carcinomas, while seminomatous tumour cells and differentiated nonseminomatous derivatives (teratoma, yolk sac tumor) stained less intensely. These data suggested that rNOR1 might serve as a testis-selective gene, and that altered NOR1 expression in testicular cancer may help us to elucidate the functions of NOR1 protein in germ line cells carcinogenesis.

Key words oxidored nitro domain containing protein 1, carcinogenesis, testicular cancer
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Testicular cancer is the most common malignancy among adolescent and young adult males. Testicular cancer occurs in a variety of histological patterns grouped together and described as testicular germ cell tumors (TGCTs) [1]. The incidence of TGCTs has doubled in the past 40 years [2-3]. Because of the availability of highly effective combination modalities of surgery, radiotherapy, and cisplatin-containing chemotherapy, disease-specific survival in TGCTs is > 90%. However, cure rates of patients with a higher stage at presentation and/or recurrent tumor, in particular those refractory to high-dose chemotherapy with stem-cell rescue, are low (5-year survival rate < 30%) [4]. TGCTs represent the model of a curable malignancy. Understanding the molecular biology of TGCTs could help improve treatment of other cancers[2].

More recently, the oxidored nitro domain containing protein 1 (NOR1) gene was identified as a potential testis/brain protein, based on its restricted expression in the testis and neurons [5-6]. Although human and mouse homologues have been isolated and

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well characterized, the rat homologue of NOR1 has not been reported. Here, we describe the isolation and expression profile of the rat homologue of NOR1. Our results indicate that rNOR1 is highly expressed in rat testis. Additionally, NOR1 protein was down regulated in human TGCTs. NOR1 protein expression was strongly associated with the histological type of TGCTs. These data warrant further study of the functions of NOR1 in testicular carcinogenesis.

1 Materials and methods

1.1 Analysis of rat NOR1 cDNA and genomic structure

The rat NOR1 mRNA sequence was identified from public database. The exon-intron organization of the rNOR1 gene was determined by the sequence alignment between the rNOR1 cDNA and the reported genomic sequences of rat.

1.2 The putative conserve domain analysis of NOR1 proteins in multiple species

NCBI Conserved Domain software was used to analyze the putative conserved domain in rat NOR1 protein (http://www.ncbi.nlm.nih.gov/cdd) [7]. Sequence alignments and matrix distance tree analysis were performed using Clustal W software *via* the European Bioinformatics Institute website (http://www2.ebi.ac.uk/clustalw) [8]. Using default parameter settings, and manual adjustments when necessary, we analyzed the following NOR1 homologue sequences (with their accession codes) from the GenBank database: *Rattus norvegicus* NOR1 (NP_001025094.1), Hsa (*Homo sapiens*) NOR1 (NP_659484.4), *Nomascus leucogenys* NOR1 (XP_003273330.1), *Drosophila sechellia* NOR1 (XP_002033483.1), *Mus musculus* NOR1 (NP_766289.2), *Gallus gallus* NOR1 (XP_001233001.1), *Danio rerio* NOR1 (XP_002667777.1), *Xenopus tropicalis* NOR1 (NP_001007858.1), *Ornithorhynchus anatinus* NOR1 (XP_001510354.2), *Caenorhabditis remanei* NOR1(XP_003111848.1).

1.3 Animals

Five male rat were obtained from the Laboratory Animals Science Department of The Central South University (Changsha, China). These animals were used according to the Guide for the Care and Use of Laboratory Animals as published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The protocol of this study was approved by the Committee of Animal Use and Welfare of Central South University. Tissue samples from these rat were extracted immediately after sacrificed by CO_2 asphyxiation and used for RNA and protein extractions or immunostaining.

1.4 Human tissues

Human adult testis tissue was obtained from donation of a victim (35 years old, disease free) in traffic accident at Xiangya Hospital [9]. Human fetal heart, thymus and muscle tissues were obtained from a 28- to 30-week gestation human fetus which was collected from termination of pregnancy material, with appropriate written consent and approval from Health Authority Joint Ethics Committee of the Central South University and relevant national guidelines. Tissues were collected into cold phosphate buffered saline (PBS containing 150 mmol/L sodium chloride, 150 mmol/L sodium phosphate, pH 7.2). Each type of tissues was obtained from three different sites.

1.5 RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from rat and human tissues using the Trizol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions and subjected to DNase (Roche, Mannheim, Germany) treatment to eliminate contaminating DNA. Different human tissues cDNA were prepared as previously reported [10]. To compare the expression levels of rat NOR1 in various tissues, the total extracted RNA from each tissue was subjected to oligo (dT)18-primed RT (Promega, Madison, WI, USA) and the product was used in PCR with the gene-specific primers (forward) 5′ CAAGCACCATGTCGGTGCGGA 3′ and (reverse) 5′ GCTGGCCTGGTTCAGTCGCA 3′. Rat GAPDH mRNA was also amplified, for use as an endogenous control, with the gene-specific primers (forward) 5′ GCCTCGTCTCATAGACAAGATGGTG 3′ and (reverse) 5′ AGCGGAAGGGGCGGAGATGA 3′. To compare the expression levels of human NOR1 in various tissues, RT-PCR was performed with the gene-specific primers (forward) 5′ TCAAGGGATTC-ATCCGAGAC 3′ and (reverse) 5′ CTGGCCAAGA-AATTCAGCTC 3′. Human β-actin mRNA was also amplified, for use as an endogenous control, with the

gene-specific primers (forward) 5′ AGCGAGCATCC-CCCAAAGTT 3′ and (reverse) 5′ GGGCACGAAG-GCTCATCATT′3′. The PCR reaction mix included Premix Taq™ DNA Polymerase (TaKaRa, Tokyo, Japan) and 0.4 μl of the RT products in a total volume of 20 μl. The cycling conditions were: 94℃ for 5 min, followed by 28 cycles of 94℃ for 15 s, 55℃ for 25 s, and 72℃ for 25 s. The PCR products (10 μl aliquots) were separated on a 2% agarose gel and the quantified by scanning using ImageJ software (NIH, Bethesda, MD). The NOR1 data are presented as percentage of GAPDH.

1.6 Protein extraction and Western blotting

Tissues were stored in liquid nitrogen before use. For protein extraction, the tissues were lysed with a lysis buffer [50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche, Mannheim, Germany)] [11]. The protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as the standard. The polyclonal anti-NOR1 antibody was obtained from Sigma-Aldrich Chemicals (St Louis, MO, USA) and polyclonal anti-GAPDH antibody was from Proteintech Group (Chicago, IL, USA).Western blotting was performed as described previously[12].

1.7 Tissue microarray and immunohistochemistry

Human testis tumor tissue microarray was purchased from Auragene Bioscience Corporation (Changsha, China), containing 46 cases of seminoma, 8 yolk sac tumor, 16 embryonal carcinoma, 5 teratoma, 3 tuberculosis, 6 atrophy, 15 adjacent normal tissue and 5 normal tissue, duplicate cores per case. Immunohistochemical staining of NOR1 expression, using the same specific antibodies as antibodies for Western blotting, was performed as described previously[5]. Briefly, the tissue microarray section were deparaffinized in xylene and rehydrated through graded alcohols (100%, 90%, 70%, and 50% alcohol for 5 min each). Endogenous peroxidase activity of the tissues was blocked with 3% hydrogen peroxide in phosphate buffered saline (PBS; 150 mmol/L sodium chloride, 150 mmol/L sodium phosphate, pH 7.2) for 10 min. For antigen retrieval, the sections were incubated in the sodium citrate buffer (0.01 mol/L, pH 6.0) for 20 min in a household microwave oven (600 W). The section were washed with PBS and incubated with a normal goat serum (Maixin, Fuzhou, China) in PBS for 30 min and then with the anti-NOR1 antibody (Sigma-Aldrich) at a dilution of 1∶200 at 4℃ overnight. The next day, polymerized HRP anti-rabbit IgG (Maixin) was added to the sections after washing with PBS. Color reaction was developed in the sections using diaminobenzidine chromogen solution (Maixin), and the section were counterstained with hematoxylin. Immunohistochemical staining of these sections was scored microscopically (Olympus; Tokyo, Japan). A staining index (values, 0 ～ 9) obtained as the intensity of the positive staining (scores: negative=0, weak=1, moderate=2, or strong=3) and the proportion of positive cells of interest (scores: < 10%=1, 10%～50%=2, > 50%=3) were calculated.

1.8 Statistical analysis

Comparisons of different groups were statistically tested with two-sided Fisher's exact tests.

2 Results

2.1 Characterization on cDNA and genomic sequence of NOR1 homologue in rat

In this study, we searched the NOR1 genes from the NCBI database (http://www.ncbi.nlm.nih.gov/) using the keyword "oxidored nitro domain protein 1". We identified and characterized the NOR1 family proteins in different species. The sequence analysis indicated that cDNA sequence (1 418 bp) of rat NOR1 gene contained the open reading frame (ORF) of 1 140 bp (the region from 50 to 1 189 nucleotides), flanked by 5′- and 3′-untranslated regions of 49 bp and 229 bp, respectively. The predicted NOR1 protein has 379 amino acids with a calculated molecular mass of 43 312.64 u (Figure 1). The amino acid sequence was 93% and 89% identical to that of mNOR1 and hNOR1, respectively. The exon-intron organization of the rNOR1 gene was determined by the sequence alignment between the rNOR1 cDNA and the genomic sequence of rat chromosome 5q36. The genomic sequence of rNOR1 gene is divided into 10 exons and 9 introns (Figure 2). Phylogenetic analysis with the maximum likelihood method revealed that the rat NOR1 grouped with the mouse homologue, while the platypus (*Ornithorhynchus anatinus*) NOR1 represents as a distinct group (Figure 3a).

```
1    ccgaaaccctggggctcttcattcacctgccctgcgggcctcaagcacc
50   atgtcggtgcggactctgccgctgctcttcttgaacttaggtggggagatgctttac
1    M  S  V  R  T  L  P  L  L  F  L  N  L  G  G  E  M  L  Y
107  gtcctagaccagcgactgagggcccagaacatcccgggagacaaggcccgcaaagtt
20   V  L  D  Q  R  L  R  A  Q  N  I  P  G  D  K  A  R  K  V
164  ctgaacgacatcatttcaaccatgttcaacaggaagttcacagaggagctgttcaaa
39   L  N  D  I  I  S  T  M  F  N  R  K  F  T  E  E  L  F  K
221  ccccaggagctgtactccaagaaggccttgaggactgtgtacgaccgcctagctcat
58   P  Q  E  L  Y  S  K  K  A  L  R  T  V  Y  D  R  L  A  H
278  gcctccatcatgcgactgaaccaggccagcatggataagctctatgacctgatgacc
77   A  S  I  M  R  L  N  Q  A  S  M  D  K  L  Y  D  L  M  T
335  atggctttcaaatatcaagtgctgctgtgcccacgccccaaggacgtgctgctggtc
96   M  A  F  K  Y  Q  V  L  L  C  P  R  P  K  D  V  L  L  V
392  actttcaaccatttagactccatcaagggtttcatccaagactcaccaaccatcatc
115  T  F  N  H  L  D  S  I  K  G  F  I  Q  D  S  P  T  I  I
449  catcaggtggacgagaccttccggcagctgacagaaatctacgggagtctctctgcc
134  H  Q  V  D  E  T  F  R  Q  L  T  E  I  Y  G  S  L  S  A
506  ggggagttccagctgatccggcagacactgcttatcttcttccaagacctgcatatc
153  G  E  F  Q  L  I  R  Q  T  L  L  I  F  F  Q  D  L  H  I
563  cgagtgtccacatttctaaaggacaaagttcagaattctaatggtcgctttgtgttg
172  R  V  S  T  F  L  K  D  K  V  Q  N  S  N  G  R  F  V  L
620  ccagtgtcggggcctgttccctggggaatagaagttcctggagtcattagagtgttc
191  P  V  S  G  P  V  P  W  G  I  E  V  P  G  V  I  R  V  F
677  aatgacaaaggtgacgaggtgaagaggatggaattccggcacggtggggactatgtt
210  N  D  K  G  D  E  V  K  R  M  E  F  R  H  G  G  D  Y  V
734  gctgcccacaaggaaggttcttttgagctttatggagaccgagtcttgaaactggga
229  A  A  H  K  E  G  S  F  E  L  Y  G  D  R  V  L  K  L  G
791  actaacatgtacagtgcgagtcgtcctgtggaaacccatatgtctgcaacatcaaag
248  T  N  M  Y  S  A  S  R  P  V  E  T  H  M  S  A  T  S  K
848  aactcagcctccagggcacaggaaaacattgctccaaaccctcttgccaaagaagaa
267  N  S  A  S  R  A  Q  E  N  I  A  P  N  P  L  A  K  E  E
905  ctgaatttcttggccaggctaataggagggatggagatcaagaagccaagtggccct
286  L  N  F  L  A  R  L  I  G  G  M  E  I  K  K  P  S  G  P
962  gaaccaggattccgactgaatctgtttaccaccgatgaggaagaggaacatgcagcg
305  E  P  G  F  R  L  N  L  F  T  T  D  E  E  E  E  H  A  A
1019 ctgtctcggcccgaggagttatcctatgaagtcatcagcatacaggccacacaggac
324  L  S  R  P  E  E  L  S  Y  E  V  I  S  I  Q  A  T  Q  D
1076 cagcaacggagtgaggagttggcccggatcatggaggagtttgagatgacagagcag
343  Q  Q  R  S  E  E  L  A  R  I  M  E  E  F  E  M  T  E  Q
1133 ccggagcggaacacaagcaagggagatgacttgctggccatgatggacaggttatag
362  P  E  R  N  T  S  K  G  D  D  L  L  A  M  M  D  R  L  *
1190 acaagcagatgggcacaccctaggcctccctcaagagactgactgccttatgaccac
1304 tgggagagacaaatggcctgcaccttctagtccattttctaagtcagaacaaactgt
1247 gaagatctctcagagagcatccatttctgaagccacacacgtttgtgtattttcagc
1361 aaaatatattcttgctcttaacccaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

Fig. 1　Nucleotide and deduced amino acid sequence of the cDNA sequence NOR1

The nucleotide sequence was presented over the deduced amino acid sequence, the start of the protein being at position 50. The translation stop codon was noted by *. The putative conserved domain of NOR1 protein was underlined.

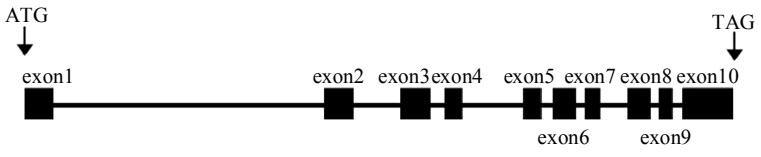
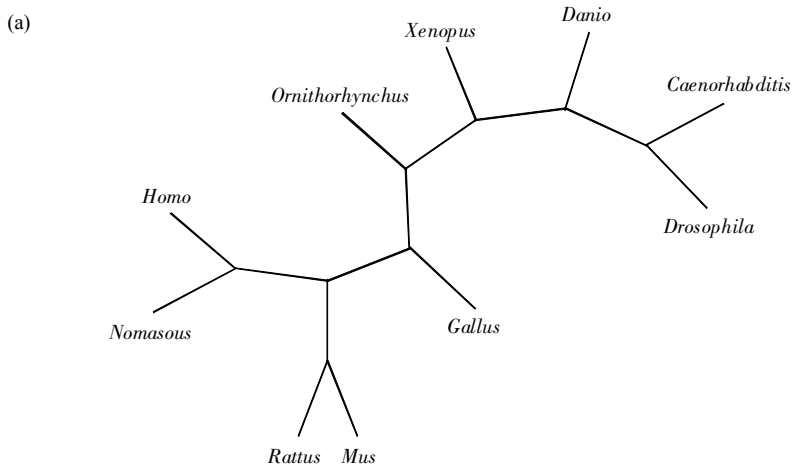


Fig. 2　The exon-intron organization of the rat NOR1 gene

The gene coding for rNOR1 contains 10 exons and 9 introns. Exons were indicated as black boxes and introns are shown as solid lines.



(b)

```
Xenopus       MLYILDQRLRAQNI---PPDKAKK----------VMNDIITTMFNKKFMEELFKPQ---- 43
Ornithorhynchus MLYILDQRLRAQNI---PADKARK----------VMNDIITTMFNKKFMEELFKPQ---- 43
Gallus        MLYILDQRLRAQSI---PGEKARK----------VMNDIITTMFNKKFMEELFKPQ---- 43
Mus           MLYVLDQRLRAQNI---PGDKARK----------VLNDIISTMFNRKFMDELFKPQ---- 43
Rattus        MLYVLDQRLRAQNI---PGDKARK----------VLNDIISTMFNRKFTEELFKPQ---- 43
Nomasous      MLYILDQRLRAQNI---PGDKARKDEWTEVDRKRVLNDIISTMFNRKFMEELFKPQ---- 53
Homo          MLYILDQRLRAQNI---PGDKARK----------VLNDIISTMFNRKFMEELFKPQ---- 43
Danio         MIYILDQRLRAPDE---SDEKTQRGCWTEDDRKRVMNDIVSTMFSKVFLEELLRPQ---- 53
Caenorhabditis MMYVLDQRLRVQKERIEDREKSDK----------VVKEIMLGFLAKQTLDEVFKGH---- 46
Drosophila    MLYVIDQRLKAQQI---AQDKSVQ----------VIHDVTSVLLEPKFIDSLLNGSKHNS 47
consensus     *:*::****:. .      :*: :          *::::   ::     :.::.

Xenopus       -ELYSKKALRTVFDRLAHASIMRLNQASMDKLYDLMTMAFKYQVLLCPRPKDILLVTFNH 102
Ornithorhynchus -ELYSKKALRTIYDRLAHASIMRLNQASMDKLYDLMTMAFKYQVLLCPRPKDILLVTFNH 102
Gallus        -ELYSKKALRTVYDRLAHASIMRLNQASMDKLYDLMTMAFKYQVLLCPRPKDILLVTFNH 102
Mus           -ELYSKKALRTVYDRLAHASIMRLNQASMDKLYDLMTMAFKYQVLLCPRPKDVLLVTFNH 102
Rattus        -ELYSKKALRTVYDRLAHASIMRLNQASMDKLYDLMTMAFKYQVLLCPRPKDVLLVTFNH 102
Nomasous      -ELYSKKALRTVYERLAHASIMKLNQASMDKLYDLMTMAFKYQVLLCPRPKDVLLVTFNH 112
Homo          -ELYSKKALRTVYERLAHASIMKLNQASMDKLYDLMTMAFKYQVLLCPRPKDVLLVTFNH 102
Danio         -DLYSHRALRTVLTRIAHTSIMRLNPASMDKLYDLMTMAFKYQLVLCPRPQDLLLITFNH 112
Caenorhabditis -GIPTRAGLKMFFEKVAHCSIMRLNENSMDKLFDLMQMSYKFALMKMTMPEQIMTITVNH 105
Drosophila    AQLLTAEHCKFMLNDIATCSLMRLDEQSMSKLWNLMTMVYKWQLFVSRHQHHLLEITFRH 107
consensus         :    : .   :*  *:*:*:  **.**::** * :*: :.     ..:: :*..*

Xenopus       MDAIKDFIRDSPSILNQVDETFRQLIDMYNCLPSGEFQLIRQTLLIFFQDMHIRVSIFLK 162
Ornithorhynchus LDAVKGFIRDSPTILNQVDETFRQLIDMYGSLCAGEFQLIRQTLLIFFQDLHIRVSIFLK 162
Gallus        LDAIKDFICDAPGILNQVDETFRQLIEMYGSLSAGEFQLIRQTLLIFFQDMHIRVSIFLK 162
Mus           LDAIKGFVQDSPTVIHQVDETFRQLSEVYGKLSEGEFQLIRQTLLNFFQDLHIRVSIFLK 162
Rattus        LDSIKGFIQDSPTIIHQVDETFRQLTEIYGSLSAGEFQLIRQTLLIFFQDLHIRVSTFLK 162
Nomasous      LDTIKGFIRDSPTILQQVDETFRQLTEIYGGLSAGEFQLIRETLLIFFQDLHIRVSMFLK 172
Homo          LDTIKGFIRDSPTILQQVDETLRQLTEIYGGLSAGEFQLIRQTLLIFFQDLHIRVSMFLK 162
Danio         TDAIKELVKDNPSLVNQINEAQRLLIEVYTPLSDGELQLIRHTLLLLFQDMQIRVSIFLK 172
Caenorhabditis LRALLDLVPLDKDIGTAVEHAYTMAFTFYRPLGPMGWFMLRNSLLVFFQDTRVKVSTFIK 165
Drosophila    LDAINRLYP-DAKRHMLIDFTKNTLLDFWNASGEDAQLSIYQTNRAWLQCFNTKISLLIR 166
consensus       ::  :          :: :      .:          : .:    :*  . ::* :::

Xenopus       DKVQNSNGRFVL 174
Ornithorhynchus DKVQNSNGRFVL 174
Gallus        DKVQNSNGRFVL 174
Mus           DKVQNSNGRFVL 174
Rattus        DKVQNSNGRFVL 174
Nomasous      DKVQNNNGRFVL 184
Homo          DKVQNNNGRFVL 174
Danio         EKIQNPNGHFVL 184
Caenorhabditis DCKQLPNGRFVI 177
Drosophila    MGFQAMDGSFIR 178
consensus        *  :* *:
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Fig. 3 Phylogenetic analysis of NOR1 homologues and sequence alignment of the putative conserved domain of NOR1 homologues using CLUSTALW software

(a) Phylogenetic relationship of NOR1 homologues in multiple-species. (b) Alignment of the putative conserved domain of NOR1 in multiple-species. The sequences of members were obtained from GenBank/EMBL/DDBJ database.

2.2 Domain organization in NOR1 protein

Using NCBI Conserved Domain software, rNOR1 protein contained a putative conserved domain of 174 amino acids which belong to the OSCP1 superfamily. To gain the conserved domain of NOR1 in multiple species, we aligned their sequences using the ClustalW program and showed the amino acid sequences of the conserved domains of NOR1 genes (Figure 3b). The

putative conserved domain of rNOR1 shared higher identities to other OSCP1 superfamily, indicating that OSCP1 doamin of NOR1 were highly conserved.

2.3 Rat NOR1 mRNA and protein is highly expressed in testis

To elucidate the tissue distribution of the rNOR1 gene in rats, RT-PCR analysis was performed. As shown in Figure 4a, RT-PCR data revealed that rNOR1 mRNA was highly expressed in the testis, but was undetectable or expressed at very low levels in most rat tissues, including brain, liver, spleen, stomach, lung and kidney. Western blot data demonstrated the rNOR1 protein was abundantly expressed in the rat testes and weakly expressed in the rat brain (Figure 4b). However, rNOR1 protein was undetectable in other tissues, including heart, liver, spleen, lung, kidney, thymus and colon(Figure 4b).

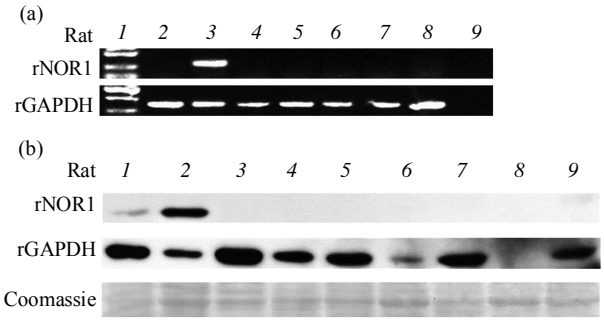

Fig. 4　Expression of rat NOR1 in various tissues

(a) RT-PCR analysis showed that testis has high expression levels of NOR1 mRNA. In contrast, the brain, liver, spleen, stomach, lung, and kidney do not express detectable levels of NOR1 mRNA. Rat GAPDH was detected as the control. *1*: DL-2000; *2*: Brain; *3*: Testis; *4*: Liver; *5*: Spleen; *6*: Stomach; *7*: Lung; *8*: Kidney; *9*: H_2O. (b) Western blot assay showed that NOR1 protein is highly expressed in the testis and the brain has weakly expressed. Rat GAPDH was detected as the control. Proteins were visualized by Coomassie blue staining and serve as loading control. *1*: Brain; *2*: Testis; *3*: Heart; *4*: Liver; *5*: Spleen; *6*: Lung; *7*: Kidney; *8*: Thymus; *9*: Colon.

2.4 Human NOR1 gene is highly expressed in testis

We analyzed the expression level of human NOR1 mRNA and protein. Expression pattern of human NOR1 mRNA was similar to the rat homologue expression detected in this study. As shown in Figure 5a, hNOR1 mRNA was highly expressed in the testis, and weakly expressed in heart, thymus and muscle. Western blot data demonstrated expression of the 43-kDa hNOR1 protein in the testis. However, hNOR1 protein was undetectable or expressed at very low levels in heart, thymus and muscle tissues(Figure 5b), similar to the RT-PCR data.

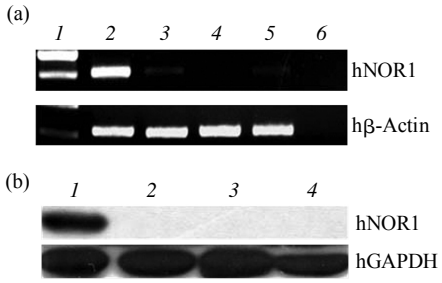

Fig. 5　Expression of human NOR1 in several tissues

(a) RT-PCR analysis showed that human adult testis has high expression levels of NOR1 mRNA. In contrast, the fetal heart, thymus and muscle do not express detectable levels of NOR1 mRNA. Human β-actin was detected as the control. *1*: DL-2000; *2*: Testis; *3*: Heart; *4*: Thymus; *5*: Muscle; *6*: H_2O. (b) Western blot assay showed that NOR1 protein is highly expressed in the adult testis. GAPDH was used as the loading control. *1*: Testis; *2*: Heart; *3*: Thymus; *4*: Muscle.

2.5 Down-regulation of NOR1 protein is strongly associated with histologic types of human testicular cancer

We used immunohistochemistry to investigate the expression of NOR1 in a human testis tumor tissue microarray containing 74 testicular tumors using the NOR1 antibody. The frequencies of positive tissues according to histological subtypes are illustrated in Table 1. These results further confirmed that the expression of NOR1 gene was high in the normal testis but it was markedly decreased in TGCTs. In 6 atrophy specimens, 15 adjacent normal tissue specimens and 4 normal tissue specimens, we observed high NOR1 expression as anticipated (Figure 6a). However, 3 testicular tuberculosis specimens were negative. In contrast, the percentages of positives among TGCTs was 40% (P=0.000, when compared to non-cancerous testicular tissues). Subsequently, we examined the expression of NOR1 in testicular tumours with different histologic types. The frequency of NOR1 immunoreactivity in seminomas(42%)(Figure 6b) was significantly different from that in embryonal carcinoma(81.3%, P=0.000)(Figure 6c). However, the frequency of NOR1 immunoreactivity in yolk sac tumor(Figure 6d) and teratoma was similar to that in seminomas, which signifies a significant loss of expression of NOR1 upon differentiation from embryonal carcinoma.

Table 1 NOR1 expression in normal and malignant testis subdivided by histological subtypes

	Positive(3~9) Number (%)	Negative(0) or weak(< 3) Number (%)	*P* value
NTGCT[a]	25(89.3)	3(10.7)	
Tuberculosis	0 (0.0)	3 (100.0)	
Atrophy	6 (100.0)	0 (0.0)	
Adjacent normal tissue	15 (100.0)	0 (0.0)	
Normal	4 (100.0)	0 (0.0)	
TGCT[b]	30 (40.0)	45(60.0)	$P^{a-b} < 0.01$
Seminoma[c]	12 (26.1)	34(73.9)	
NSE[d]	18 (62.1)	11 (37.9)	$P^{c-d} < 0.01$
Embryonal carcinoma[e]	13 (81.3)	3 (18.7)	$P^{c-e} < 0.01$
Yolk sac tumor	3 (37.5)	5 (62.5)	
Teratoma	2 (40.0)	3 (60.0)	
Total specimens	55	48	

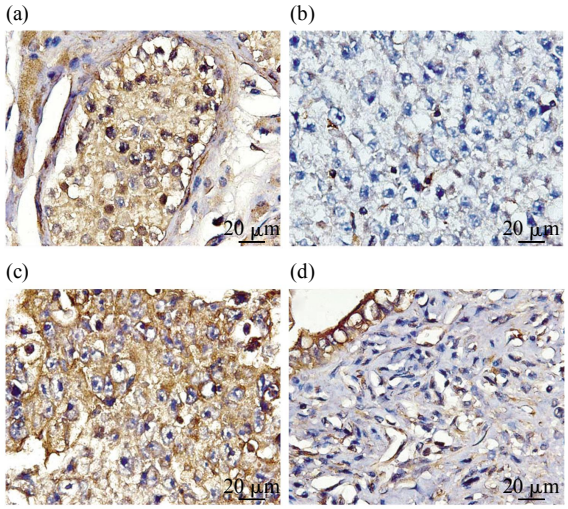

Fig. 6 Expression of human NOR1 protein in normal testis and testicular cancer

(a) Strong positive immunostaining in non cancerous testis specimens. (b) Negative immunostaining in seminoma specimens. (c) Strong positive immunostaining in embryonal carcinoma specimens. (d) Decreased immunostaining in yolk sac tumor specimens.

3 Discussion

The present study describes the characterization and expression profile of a rat homologue of oxidored nitro domain containing protein 1, rNOR1. Rat NOR1 shared high homology with human NOR1 and mouse NOR1. The data showed that rNOR1 and hNOR1 is highly expressed in testis. More important, we found that altered NOR1 expression in testicular cancer is strongly associated with the histologic types of testicular cancer, which may help to elucidate the functions of NOR1 protein in germ line cells carcinogenesis.

Many investigators have attempted to isolate and determine the functional characterization of tissue-specific genes, which are expressed only in a particular tissue [13]. Tissue-selective genes not only underlies tissue development and function, but also implicated in many complex human diseases [14], and identification of these genes may provide valuable information for developing novel biomarkers and drug targets. In this study, rat NOR1 mRNA and protein are predominantly expressed in the testis. Our previous works have reported that hNOR1 and mNOR1 mRNAs are predominantly expressed in the testis [5, 10]. All of these findings together with our published papers lead us to conclude that NOR1 is belong to a novel testis-selective protein family. Phylogenetic analysis with the maximum likelihood method revealed that the NOR1 superfamily is conserved from *Xenopus* to humans. Although the function of the NOR1 protein remains unknown, rNOR1 protein contained a putative conserved domain which belong to the OSCP1(organic solute carrier partner 1)superfamily. Kobayashi *et al*. have reported that hOSCP1 and mOscp1 mRNAs are predominantly expressed in the testis. The hOSCP1 and mOscp1 protein functions as a transporter of

several organic solutes into cells [6, 15–17]. This finding suggests that NOR1 might belong to solute carrier (SLC) superfamily and facilitate the transport of nutrients or drugs into germ cells across the blood-testis barrier. Phylogenetic analysis with the maximum likelihood method revealed that the rat NOR1 grouped with the mouse homologue, suggesting NOR1 is highly conserved in rodent animals.

The incidence of testicular germ cell tumours (TGCTs) has uniformly increased worldwide in successive generations born from around 1920 until very recently[3, 18–19]. However, molecular signatures for TGCTs do not yet exist. Methods that identify novel, tissue-specific and tumor-related genes therefore have potentially important implications for developing cancer diagnosis and immunotherapy. Similar to the rat NOR1 orthologs, human NOR1 mRNA and protein was highly enriched in testis. We then investigated the expression level of the human NOR1 protein in cancerous and normal testicular tissues with immunohistochemistry. The NOR1 protein expression in TGCTs was markedly reduced compared to non-cancerous testis tissues. We do not currently have any plausible biological explanation for loss of NOR1 expression in three testis tuberculosis samples. Human NOR1 gene is mapped to the human chromosome 1p34.3. A genome-wide DNA methylation profiling study revealed that chromosomes 1p34.3 was intensively hypermethylated in human testicular cancer[20]. The deletion of chromosome 1p is also one of the frequent genetic alterations found in testicular germ cell tumors [21–22]. NOR1 hypermethylation has been reported in nasopharyngeal carcinoma (NPC), acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and myelodysplastic syndromes (MDS) [23–26]. Given its location within a region frequently undergoing loss of heterozygosity in TGCTs[21–22], we surmise that promoter hypermethylation combined with allelic loss, as well as other unknown mechanisms, may contribute to the inactivation of NOR1 in TGCTs.

Our previous works have reported that hNOR1 is down-regulated in small samples of testicular cancers[5]. In contrast to the previous study, which did not address the question of whether reduced expression of hNOR1 has any influence on clinicopathological features, this study clearly shows that reduced hNOR1 expression is indeed significantly associated with the histological type of testicular cancers. TGCTs are classified into two main histological subgroups: seminomas(SEs) and non-seminomas (NSEs). The latter comprise several subtypes: embryonal carcinomas, yolk sac tumours, choriocarcinomas, and teratomas. Histologically, SE resembles primordial germ cells/gonocytes, embryonal carcinoma resembles the stem cell component, whereas teratoma and yolk sac tumor shows somatic or extra-embryonal differentiation respectively [2]. Here, we convincingly show that 81.3% of embryonal carcinoma specimens are positive for NOR1. In contrast, testicular seminoma, and the differentiated nonseminomatous derivatives (teratoma, yolk sac tumor) showed decreased of NOR1. This suggests that loss of protein expression is because of down-regulation of gene expression upon differentiation/ maturation.

In summary, the rat homologue of NOR1 has been identified and its expression profile was characterized. Rat NOR1 is highly expressed in testis. Altered human NOR1 protein expression is strongly associated with histologic types of human TGCTs. Our results suggest that NOR1 may contribute to testicular development and tumorigenesis, and the data show that rat could potentially be a pertinent model to study the role of NOR1 in testicular cancer.

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硝基还原酶结构域蛋白 1 在大鼠睾丸组织中特异性高表达在人类睾丸癌中表达下调 *

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摘要　通过克隆分离鉴定得到大鼠硝基还原酶结构域蛋白 1(rNOR1)，发现 rNOR1 cDNA 含有 1 418 个碱基，编码含 379 个氨基酸残基的 rNOR1 蛋白．rNOR1 与人类 NOR1 (hNOR1)和小鼠 NOR1 (mNOR1)的同源性分别为 89% 和 93%，这三种同源蛋白都含有 OSCP1 家族的保守结构域．rNOR1 基因在大鼠睾丸中选择性高表达，而且与之同源的人类 hNOR1 也选择性高表达于睾丸中．通过免疫组化检测人类不同睾丸癌中的 hNOR1 蛋白表达，发现 hNOR1 蛋白在非癌变睾丸组织和胚胎性癌组织中高表达，而在精原细胞癌和分化型非精原细胞癌(畸胎瘤，卵黄囊瘤)中低表达．这些数据表明，hNOR1 可能是一种睾丸选择性表达基因，睾丸癌 hNOR1 表达的改变或许可以帮助我们阐明 hNOR1 蛋白在生殖细胞系肿瘤发生中的功能．

关键词　硝基还原酶结构域蛋白 1(NOR1)，肿瘤发生，睾丸癌
学科分类号　Q7，R73　　**DOI**: 10.3724/SP.J.1206.2012.00460

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