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Isolation, identification and expression analysis of PD-1 gene in *Tupaia belangeri*

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Abstract: Programmed cell death 1 (PD-1) is an inhibitory receptor that belongs to the immunoglobulin (Ig) superfamily. Numerous studies have demonstrated that PD-1 expression can be induced in activated T lymphocytes, NKT cells, B lymphocytes and monocytes and play a critical role in the pathogenesis of chronic infections. *Tupaia belangeri* (tree shrew) represents an excellent model for some of the major human infectious diseases such as hepatitis B virus infection. To fully utilize tree shrew as a host immune response model to infectious diseases, tree shrew PD-1 was isolated. Full-length tree shrew PD-1 cDNA was cloned from spleen of tree shrew by rapid amplification of cDNA end-PCR (RACE-PCR). Sequence analysis revealed that the open reading frame (ORF) of tree shrew PD-1 cDNA encoded a predicted transmembrane protein of 242 amino acids with high homology with humans, primates and rodents. Expression of PD-1 in tree shrew was detected in spleen but not in other organs by RT-PCR. Stimulation of freshly isolated tree shrew peripheral blood monouclear cells(PBMCs) with PMA and ionomycin increased the expression of PD-1 mRNA. Our results may provide a foundation for future exploration of tree shrew immune function.

Key words: tree shrew; PD-1; RACE-PCR; expression; PBMCs

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树鼩 PD-1 基因的分离、鉴定及表达分析

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摘要:PD-1(Programmed cell death 1)是一种抑制性的受体,是免疫球蛋白超家族的成员. 大量研究证明

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PD-1 能被诱导而在活化的 T 淋巴细胞、B 淋巴细胞、NKT 细胞 和单核细胞上表达，从而在慢性感染的病因学中起着重要作用. 树鼩(*Tupaia belangeri*)做为一个理想的模型可被应用于许多人类感染性疾病如乙型病毒性肝炎. 为了充分利用树鼩对于感染性疾病的宿主免疫应答模型，我们分离出树鼩 PD-1 基因. 利用迅速扩增 cDNA 末端 PCR(RACE-PCR)技术，从树鼩脾组织中克隆了 PD-1 基因的全长 cDNA 序列. 序列分析显示树鼩 PD-1 cDNA 的开放阅读框编码一个由 242 个氨基酸组成的跨膜蛋白，并且和人类、灵长类和啮齿类中的同源基因有高度相似性. 组织分布分析表明在所检测的几种组织中 PD-1 基因只在脾中表达. 此外，淋巴细胞刺激实验显示，利用 PMA 和 ionomycin 刺激新鲜分离的树鼩外周血单核细胞(PBMCs)能够诱导 PD-1 mRNA 水平上的表达. 我们的结果为将来进一步探讨树鼩的免疫功能提供了良好的基础.

关键词：树鼩；PD-1；迅速扩增 cDNA 末端 PCR；表达；外周血单核细胞

0 Introduction

Exhaustion of virus-specific $CD8^+$ T lymphocytes has been considered as a principal reason for the inability of the host to eliminate persisting pathogen infections[1~4]. The exhaustion of virus-specific T cells was detected in persistent LCMV infection of mice[3,5] as well as in human chronic infections including HIV, HCV and HBV[6,7]. The inhibitory receptor programmed death 1 (PD-1) was inducibly expressed in $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells, B cells and activated monocytes[8~10] and functions as a negative regulator of activated T cells[10~13]. Recent studies have demonstrated that PD-1 plays an important role in inhibiting the function of virus-specific $CD8^+$ T cells in chronic viral infections in mice[4]. Evidence from chronic human viral infection also shows that PD-1 expression on HIV-specific as well as HCV-specific T cells is associated with virus-specific T-cell exhaustion, and blockade of the pathway augments virus-specific T cells function[14,15].

Hepatitis B virus infection is a major disease worldwide and approximately 350 million people are chronically infected. A high percentage of chronic HBV patients (25%) develop serious liver diseases including cirrhosis, liver failure, and hepatocellular carcinoma[16]. Due to a lack of suitable animal models for human chronic HBV infection, development of effective therapy to chronic HBV infection has been slow. Efficient infection by human HBV was documented in humans and chimpanzees[17]. The ethical constraints and high costs associated with using chimpanzees for HBV research severely restrict their use. Recent studies found that the Asian tree shrew, *Tupaia belangeri* can be a feasible animal model for infection of hepatitis virus. Two reports have described transient HBV infection of the *Tupaia belangeri* in vivo[18,19]. Moreover, successful infection of Tupaias with human herpes simplex virus and hepatitis C virus has been described[20,21]. To fully utilize tree shrew as a host immune response model to infectious diseases, we cloned the full-length cDNA of tree shrew PD-1 gene, compared the homology of putative protein sequence with other species and analyzed its expression in various organs as well as during the course of stimulation with PMA and ionomycin.

1 Materials and methods

1.1 Animals

Asian tree shrews (*Tupaia belangeri*) were obtained from Experimental Animal Centre, Kunming Institute of Zoology, The Chinese Academy of Sciences, Kunming, Yunan Province, China. Animal experiments were approved by local bioethics committee.

1.2 Cells and cell culture

Tree shrew PBMCs were isolated from a fresh blood sample by centrifugation using EZ-Sep™ Mouse 1 × lymphocyte separating medium (Dakewe Biotech co., Ltd., Shenzhen, China) according to manufacturer's instructions. Cells were maintained in RPMI1640 (Gibco)

supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 mg/L streptomycin. For activation, PBMCs were stimulated with 500 μg/L ionomycin plus 10 μg/L PMA (all from Sigma). Cells were collected at different time points after stimulation and used for further experiments.

1.3 3′RACE

Total RNA was extracted from tree shrew spleen by using an RNAsimple Total RNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to manufacturer's instructions. Single-strand cDNA was synthesized from 1.5 μg of RNA using 3′RACE System for Rapid Amplification of cDNA Ends (Invitrogen). Degenerate sense primer (5′- TGAGYCCCAGCAACCAGAC-3′) designed on the basis of conserved sequences of human and mouse PD-1 (GenBank accession number NM_005018, NM_008798 respectively) and antisense primer AUAP (5′-GGCCACGCGTCGACTAGTAC-3′) were used for PCR to amplify the 3′ end of the cDNA. Here, we performed Touch-down (TD) PCR[22] on a iCycler Thermal Cycler (BIO-RAD) using the following specific temperature cycling profile: 94 ℃ for 4 min; 15 cycles of 94 ℃ for 30 s, 68 ℃ to 54 ℃ for 30 s (annealing temperature decreased by 1 ℃ every cycle), 72 ℃ for 2 min; followed by 30 cycles of 94 ℃ for 30 s, 54 ℃ for 30 s, 72 ℃ for 2 min; and, finally, 72 ℃ for 10 min. The PCR products from three independent reactions were purified after agarose electrophoresis by using TIANgel Midi Purification Kit (Tiangen Biotech Co., Ltd., Beijing, China) and ligated into the pMD19-T vector (Takara) using the TA-cloning procedure, and sequenced in both directions with an ABI 3730 DNA Analyzer (Sunbiotech Co.,Ltd., Beijing, China).

1.4 5′RACE

After obtaining the 3′ end sequence of the PD-1 cDNA, we synthesized gene-specific primers (GSP) and performed 5′ RACE reactions using 5′ RACE System (Invitrogen, Life Technologies, USA). The first strand cDNA was synthesized from 1.5 μg of total RNA by a SuperScript™ Ⅱ Reverse Transcriptase with GSP1 (5′-GGCTCTGG- GGTCTTCTCTCG-3′). The 5′ end of the single strand cDNA was tailed with dCTP by terminal deoxynucleotidyl transferase (TdT). The tailed cDNA was then amplified with Abridged Anchor Primer (5′-GGGCACGCGTCGACTAG TACGGGIIGGGIIGGGIIG-3′) and GSP2 (5′-GGGTCTTCTCTCGCCACTGG-3′). Reaction conditions for PCR were: 94 ℃ for 4 min; 15 cycles of 94 ℃ for 30 s, 68 ℃ to 54 ℃ for 30 s (annealing temperature decreased by 1 ℃ every cycle), 72 ℃ for 1 min; followed by 30 cycles of 94 ℃ for 30 s, 54 ℃ for 30 s, 72 ℃ for 1 min; and, finally, 72 ℃ for 10 min. A nested 5′RACE-PCR was performed with nested Abridged Universal Amplification Primer (AUAP) and GSP3 (5′-TGCCGCTGTCATTGCGCCG-3′), 1 000-fold diluted 5′ RACE-PCR product as a template and the following conditions: 94 ℃ for 4 min; 30 cycles of 94 ℃ for 30 s, 57 ℃ for 30 s, 72 ℃ for 1min and a extension step of 72 ℃ for 10 min. The purified PCR products were ligated into pMD19-T vector and sequenced.

1.5 Semi-quantitative reverse transcription-polymerase chain reaction

Single-strand cDNA was synthesized from total RNA extracted from PBMCs 0, 24, 36, 48, and 72 h after stimulation. The cDNA obtained was then amplified by PCR with the sense primer 5′-CACGGAATTCATGCAGACCACGCAGACCTGG - 3′ and antisense primer 5′-GCCCAAGCTTTGGGGGCTCTGGGGTCTTCT-3′. The PCR amplification conditions were as follows: 94 ℃ for 4 min, 35 cycles of 94 ℃ for 30 s, 57 ℃ for 30 s, 72 ℃ for 1 min, and final extension at 72 ℃ for 10 min. Housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The annealing temperature for the GAPDH was 57 ℃ using the following primers, 5′-CCATCACCATCTTCCAGGAGCGAG-3′ and 5′-CAAAGGTGGAGGAGTGGGTGTCG -3′. The

PCR products were separated on 1% agarose gels using ethidium bromide for visualization. RNA isolation from organ samples (spleen, liver, kidney and lung) and subsequent PCR amplification were performed as described above. All reactions were repeated three times.

1.6 Bioinfomatics analysis

Similarity searches of the sequenced DNA fragments and deduced amino acid sequence were done by BLASTN and BLASTP using nr/nt database of NCBI. The alignment of PD-1 with its homologs was conducted in Clustal X. Phylogenetic tree was constructed using MEGA version 4.0[23].

2 Results

2.1 Isolation and sequence analysis of tree shrew PD-1 cDNA

Full-length PD-1 cDNA was cloned by a combination of RT-PCR, 3′ and 5′ RACE-PCR. Analysis of nucleic acid sequence indicated that tree shrew PD-1 cDNA (GenBank accesion number EU730948) contained an ORF consisting of 726 nucleotides and encoding 242 amino acid residues (Fig. 1). Coding sequence starts from ATG at position 61 and terminates at the stop codon TGA

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AGTTCTGCCAGAGCAGCAGAGGAGGGAGGAGCCGGCACGGGAGCGACAGACTCCACTGCC    60

ATGCAGACCACGCAGACCTGGCGACTGCTCTGGCCACCGGTCTGGGCCGCGCTACAGCTG   120
▲M  Q  T  T  Q  T  W  R  L  L  W  P  P  V  W  A  A  L  Q  L
GGCTGGAGGCCAGGACGGCTGCTAGACCTGCCGGGCGCACCCCGTGGCCTCCTCACCTTC   180
 G  W  R  P  G  R  L  L  D  L  P  G  A  P  R  G  L  L  T  F
TCCCCTGCCCAGCTCACGGTGCCTGAGGGGGAGAACGCCACCTTCGTCTGCAGCTTCTCC   240
 S  P  A  Q  L  T  V  P  E  G  E  N  A  T  F  V  C  S  F  S
AACACCACCGAGCGCTTCGTGCTGAACTGGTACCGCCTGAGCCCCAGCAACCAGACGGAC   300
 N  T  T  E  R  F  V  L  N  W  Y  R  L  S  P  S  N  Q  T  D
AAGCTGGCCGCCTTCCCCGAGGACCGCAGCCAGCCGGGCCACGATCGCCGCTTCCATGTC   360
 K  L  A  A  F  P  E  D  R  S  Q  P  G  H  D  R  R  F  H  V
ACGCTGCTGCCAGGCGGGCGCGACTTCCTCATGAGCATCGTGGCCGCCCGGCGCAATGAC   420
 T  L  L  P  G  G  R  D  F  L  M  S  I  V  A  A  R  R  N  D
AGCGGCACCTACCTCTGTGGTGCCATCTCCCTGCCCCCCAAGATCAACGAGAGCCCCCAC   480
 S  G  T  Y  L  C  G  A  I  S  L  P  P  K  I  N  E  S  P  H
GCAGAGCTCACGGTGACAGAGAGAGTCCTGGAGCCACCCACAGAGCACCCTAGCCCCTCG   540
 A  E  L  T  V  T  E  R  V  L  E  P  P  T  E  H  P  S  P  S
CCGGAGCCCCAGAGCCAGTCCCAAGGCCTGGTAGTGGGCGTCACGAGCACCCTGGTGGGC   600
 P  E  P  Q  S  Q  S  Q  G  L  V  V  G  V  T  S  T  L  V  G
ACCCTGCTGCTGCTGGCCTGGGTCCTGGCTGCCTCCTGCCTCAGGGCAGAGCGTGGTCCC   660
 T  L  L  L  L  A  W  V  L  A  A  S  C  L  R  A  E  R  G  P
CGAGGGCCCAGAGGAGCCAGCAGTCAGGACCCGCCCCCGAAGGAGGACCCCTCTGGCATG   720
 R  G  P  R  G  A  S  S  Q  D  P  P  P  K  E  D  P  S  G  M
CCTGCGGCCACTGTGGACTATGGAGAGCTGGACTTCCAGTGGCGAGAGAAGACCCCAGAG   780
 P  A  A  T  V  D  Y  G  E  L  D  F  Q  W  R  E  K  T  P  E
CCCCCATGAGGGTGTGGAGTTAGCCTTCGTCTCAATGCAGCGAGATGGCGTGCAGGCAGT   840
 P  P  ▲
GCCTATGGGGGCTCCACAGCGGGAGCCTGAGGGTCTCCCCACCACACAGACACCCGGCGA   900
GGAGCAAAGACTGACAGCAAGAAGCTTCACGCACGCATACGCAATTGAGACGTGTGTGCA   960
ACTGTGTATGCACATGATGTACATCTGTGTCTACGTGTGTGCACATCTGTGCACAGTATG  1020
TGTGCATGCGCACATGTGCATCCACATGTGTGTGCAGTGTGAGCATACATGTGTGTCCCT  1080
TAAACGATGAAATTTCAAACTGATGTTCAGTTCGAAATCATGCTCAAATTTGCTCATAAT  1140
GAGAAAAATAAAAATTAAAGATATGCCTAAAAAAAAAAAAAAAAA                 1185
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The nucleotides are numbered on the right. The start codon ATG and stop codon TGA are marked with ▲. The polyadenylation signal sequence is in italics. The putative IgV domain is underlined, and the boxed amino acids correspond to the transmembrane region. Potential N-glycosylation sites are shaded. The GeneBank accesion number of tree shrew PD-1 is EU730948

Fig. 1 Nucleotide and deduced amino acid sequences of tree shrew PD-1

at position 789 of the nucleotide (nt) sequence. The nt sequence from 1 144 to 1 185 was a typical polyadenylation signal sequence, indicating that the obtained sequence contained the full length of the 3′-UTR of the cDNA. We found four potential N-linked glycosylation sites using NetNGlyc 1.0 Server. Immunoglobulin (IG) domain and transmembrane domain were predicted with SMART program. The IgV region starts at nt 184 and ends at nt 498 while the transmembrane region starts at nt 565 and ends at nt 633. Application of SignalP 3.0 (http://www.cbs.dtu.dk/ services/ SignalP/) predicted that tsPD-1 has a 16-residue hydrophobic signal peptide and that the mature protein contains 226 amino acid residues.

2.2 Amino acid sequence analysis of tree shrew PD-1

The theoretical pI (isoelectric point) and Mw (molecular weight) of the mature tsPD-1 protein were computed by Compute pI/M (http://www.expasy.org/tools/pi_tool.html) with a calculated pI of 5.6 and an overall Mw of 25 kDa. The deduced amino acid sequence was compared for the highest scoring proteins by using the program BLASTP (http://www.ncbi.nlm.nih.gov/ BLAST/). The best match was found with *Homo sapiens* (NM_005018), *Equus caballus* (XP_001497914), *Macaca nemestrina* (ABR15757), *Bos taurus* (NP_001076975), *Canis familiaris* (XP_543338), *Rattus norvegicus* (NP_001100397) and *Mus musculus* (NP_032824). Amino acid sequence homology analysis reveals only moderate homology between the deduced amino acid sequence of tree shrew PD-1 and known homologous proteins (Fig.2(b)). Multiple sequence alignment of five homologous PD-1 amino acid sequences shows that all of the cysteine residues are conserved. In addition, three of the four potential N-linked glycosylation sites (consensus sequence NX(S/T)) are conserved (Fig.2(a)). The 68 amino acid residues between two cysteine residues (Cys57 and Cys120) in PD-1 bear resemblance to disulfide-linked immunoglobulin domain of the V-set sequences. Further analysis of the tree shrew PD-1 sequence predicts the presence of immunoreceptor tyrosine-based inhibitory motif ITIM (I/L/VXYXXL/V) that is also conserved. The conservation in structure and sequence between tree shrew and other vertebrate PD-1s suggests conserved functions and mechanism of action for this protein in vertebrates. In addition, a phylogenetic tree was constructed by aligning the amino acid sequences of tree shrew PD-1 with published PD-1 proteins of other species (Fig. 3). To generate a reliable phylogeny of PD-1, we have reconstructed the phylogenetic tree by neighbor-joining method with Poisson correction model[24]. The results show that the tree shrew PD-1 gene is closest to that in Bos Taurus and Canis Familiaris.

2.3 Organ distribution and expression analysis of Tree shrew PD-1 mRNA

To determine the organ distribution of PD-1 expression, total RNAs extracted from various tree shrew organs were analyzed by RT-PCR. The PD-1 mRNA was detected at a low level in the spleen but not in other organs such as liver, lung or kidney, indicating that PD-1 expression is lymphoid organ restricted. The amount and quality of the cDNA were verified by GAPDH expression in each organ (Fig. 4(a)). Next, we examined whether the expression of PD-1 mRNA could be augmented by activating the PBMCs in vitro. We extracted total RNAs from freshly isolated or PMA/ ionomycin stimulated PBMCs and determined PD-1 mRNA expression by semi-quantitative RT-PCR (Fig. 4(b)). The results show that PD-1 transcript was not detected in unstimulated PMBCs. Expression was found, although at a low level, in PBMCs 24 h post-stimulation, and then decreased to an undetectable level in PMBCs stimulated more than 24 h.

3 Discussion

PD-1 is an immunoreceptor that belongs to the immunoglobulin (Ig) superfamily and plays critical

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H.sapiens           1    MQIPQAP---WPVVWAVLQLGWRPGWFLDSPDRPWNPPTFFPALLVVTEG 47
M.nemestrina        1    MQIPQAP---WPVVWAVLQLGWRPGWFLESPDRPWNPPTFSPALLLVTEG 47
T.shrew             1    MQTTQTWRLLWPPVWAALQLGWRPGRLLDLPGAPRGLLTFSPAQLTVPEG 50
M.musculus          1    MWVRQVP---WSFTWAVLQLSWQSGWLLEVPNGPWRSLTFYPAWLTVSEG 47
R.norvegicus        1    MWVQQVP---WSFTWAVLQLSWQSGWLLEVLNKPWRPLTFSPTWLTVSEG 47
Clustal Consensus   1    *    *.     *.  .**.***.*:.* :*:   .  *     ** *:  * *.** 23

H.sapiens           48   DNATFTCSFSNTSESFVLNWYRMSPSNQTDKLAAFPEDRSQPGQDCRFRV 97
M.nemestrina        48   DNATFTCSFSNASESFVLNWYRMSPSNQTDKLAAFPEDRSQPGQDCRFRV 97
T.shrew             51   ENATFVCSFSNTTERFVLNWYRLSPSNQTDKLAAFPEDRSQPGHDRRFHV 100
M.musculus          48   ANATFTCSLSNWSEDLMLNWNRLSPSNQTEKQAAFCNGLSQPVQDARFQI 97
R.norvegicus        48   ANATFTCSFSNWSEDLKLNWYRLSPSNQTEKQAAFCNGYSQPVRDARFQI 97
Clustal Consensus         ****.**:** :*  :  ***  *:******:*  ***  :.  ***  :*  **::

H.sapiens           98   TQLPNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTER 147
M.nemestrina        98   TQLPNGRDFHMSVVRARRNDSGTYLCGAISLAPKAQIKESLRAELRVTER 147
T.shrew             101  TLLPGGRDFLMSIVAARRNDSGTYLCGAISLPPK--INESPHAELTVTER 148
M.musculus          98   IQLPNRHDFHMNILDTRRNDSGIYLCGAISLHPKAKIEESPGAELVVTER 147
R.norvegicus        98   VQLPNGHDFHMNILDARRNDSGIYLCGAISLPPKAQIKESPGAELVVTER 147
Clustal Consensus         **.  :**  *.::  :******  ********  **     *:**    ***  ****

H.sapiens           148  RAEVPTAHPSPSPRPAGQFQTLVVGVVGGLLGS--LVLLVWVLAVICSRA 195
M.nemestrina        148  RAEVPTAHPSPSPRPAGQFQALVVGVVGGLLGS--LVLLVWVLAVICSRA 195
T.shrew             149  VLEPPTEHPSPSPEPQSQSQGLVVGVTSTLVGT--LLLLAWVLAASCLRA 196
M.musculus          148  ILETSTRYPSPSPKPEGRFQGMVIGIMSALVGIPVLLLLAWALAVFCSTS 197
R.norvegicus        148  ILETPTRYPRPSPKPEGQFQGLVIVIMSVLVGIPVLLLLAWALAAFCSTG 197
Clustal Consensus          *  .*  :*  ***.*  .:  *  :*:  :  .  *:*     *:**.*.**.  *    .

H.sapiens           196  ARGTIGARRTG---QPLKEDPSAVPVFSVDYGELDFQWREKTPEPPVPCV 242
M.nemestrina        196  AQGTIEARRTG---QPLKEDPSAVPVFSVDYGELDFQWREKTPEPPAPCV 242
T.shrew             197  ERGPRGPRGASSQDPPPKEDPSGMPAATVDYGELDFQWREKTPEPP---- 242
M.musculus          198  MSEARGAGSKD---DTLKEEPSAAPVPSVAYEELDFQGREKTPELPTACV 244
R.norvegicus        198  MSEAREAGRKE---DPPKEAHAAAPVPSVAYEELDFQGREKTPE-PAPCV 243
Clustal Consensus              .   .             .  **    :.  *.  :* * **** ****** *

H.sapiens           243  PEQTEYATIVFPSGMGTSSPARRGSADGPRSAQPLRPEDGHCSWPL 288
M.nemestrina        243  PEQTEYATIVFPSGLGTSSPARRGSADGPRSPRPLRPEDGHCSWPL 288
T.shrew             242  ---------------------------------------------- 242
M.musculus          244  --HTEYATIVFTEGLGASAMGRRGSADGLQGPRPPRHEDGHCSWPL 288
R.norvegicus        243  --HTEYATIVFTEGLDASAIGRRGSADGPQGPRPPRHEDGHCSWPL 287
Clustal Consensus
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(a) Alignments of the predicted tree shrew PD-1 amino acid sequence with PD-1 from *H. sapiens* (NM_005018), *M.nemestrina* (pig-tailed macaque (ABR15757)), *M.musculus* (mouse (NP_032824)) and *R.norvegicus* (rat (NP_001100397))

Amino acids identity of PD-1(%)

	ts	hs	ec	mn	bt	cf	rn	mm
ts		69%	69%	68%	68%	65%	56%	55%

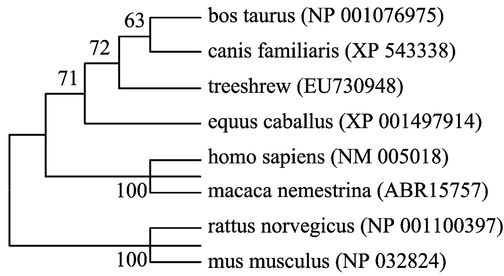
ts, *Tree shrew*; hs, *Homo sapiens*; ec, *Equus caballus*; mn, *Macaca nemestrina*; bt, *Bos taurus*; cf, *Canis familiaris*; rn, *Rattus norvegicus*; mm, *Mus musculus*

(b) Overall amino acid identity between tree shrew PD-1 and its homologs in other species, some of which are also included in (a)

Identical amino acids are marked by an asterisk and similar amino acids are marked with dots and semicolons. Dashes indicate gaps introduced for better alignment. The three conserved cysteine residues are marked by vertical box. The potential N-glycosylation sites are underlined. The immunoreceptor tyrosine-based inhibitory motif ITIM (I/L/VXYXXL/V) on the cytoplasmic domains of PD-1 is indicated by horizontal box

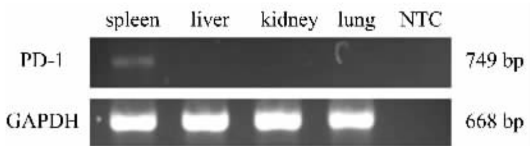
Fig. 2 Comparison of the amino acid sequences of PD-1 from tree shrew to other vertebrates

roles in immune regulations[11]. To fully utilize tree shrew as an animal model of human infectious diseases, we have cloned tree shrew PD-1 and analyzed its expression in tree shrew organs. The tree shrew PD-1 molecule had common characteristic tyrosine residues in its cytoplasmic region forming the immunoreceptor tyrosine-based inhibitory motif (ITIM), I/L/VXYXXL/V. This



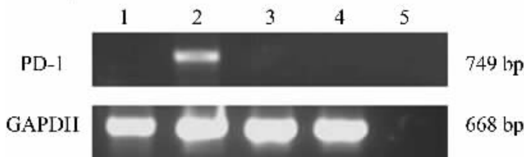
The tree was generated using the neighbour-joining Poisson-corrected distance matrix method(condensed tree, cut-off value is 50%). The numbers at relevant branches refer to bootstrap values of 1 000 replications

Fig. 3 Phylogenetic tree of evolutionary relationship between tree shrew PD-1 and its homologous proteins



5 μg total RNA prepared from spleen, liver, kidney or lung of tree shrew were subjected to PCR amplification of PD-1. NTC represents no template control. GAPDH was used as a control for cDNA template

(a) Tissue distribution of tree shrew PD-1 mRNA



Freshly isolated tree shrew PBMCs (1×10^6) were cultured for 72 h in the absence (lane 1) or presence (lanes 2~5) of stimulators (500 μg/L ionomycin and 10 μg/L PMA). Samples were collected at 24 h (lane 2), 48 h (lane 3) or 72 h (lane 4) after the stimulation. Lane 5 refers to negative control lacking template cDNA. The reaction products were analyzed by 1% agarose gel electrophoresis and visualized with ethidium bromide staining

(b) Expression of PD-1 mRNA in PBMCs with or without PMA and ionomycin

Fig. 4 RT-PCR analysis of tree shrew PD-1 mRNA expression in tree shrew PBMCs and organs

motif is conserved in all species in which the PD-1 sequence has been examined, further confirming a role for this motif in PD-1 signaling and function. Three cysteines in PD-1 were conserved among the mammalian counterpart molecules and two of which were found in the IgV-set region. The tsPD-1 protein had four potential N-linked glycosylation sites identified in the extracellular domains, three of which were found to be completely conserved in the human, primate and rodent counterpart molecules.

In summary, we have successfully cloned the tree shrew PD-1 cDNA and showed that the putative protein is a relatively conserved protein in vertebrates. Our studies also indicated that PD-1 mRNA was not detectable in resting lymphocytes. However, after stimulation of lymphocytes with mitogens such as PMA and inomycin, mRNA expression of tree shrew PD-1 in the activated lymphocytes could be readily detected by RT-PCR. The full length cDNA of tree shrew PD-1 reported herein will allow the synthesis of tree shrew PD-1 recombinant protein to raise monoclonal antibodies, which will facilitate future studies on immune responses in tree shrews.

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References

[1] Pantaleo G, Demarest J F, Schacker T, et al. The qualitative nature of the primary immune response to hiv infection is a prognosticator of disease progression independent of the initial level of plasma viremia [J]. Proc Natl Acad Sci USA, 1997,94:254-258.

[2] Moskophidis D, Lechner F, Pircher H, et al. Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells[J]. Nature, 1993,362:758-761.

[3] Gallimore A, Glithero A, Godkin A, et al. Induction and exhaustion of lymphocytic choriomeningitis virus-specific cytotoxic t lymphocytes visualized using soluble tetrameric major histocompatibility complex class I-peptide complexes [J]. J Exp Med, 1998,187:1 383-1 393.

[4] Barber D L, Wherry E J, Masopust D, et al. Restoring function in exhausted CD8 T cells during chronic viral infection [J]. Nature, 2006, 439: 682-687.

[5] Zajac A J, Blattman J N, Murali-Krishna K, et al. Viral immune evasion due to persistence of activated T cells without effector function [J]. J Exp Med, 1998, 188:2 205-2 213.

[6] Letvin N L, Walker B D. Immunopathogenesis and

immunotherapy in aids virus infections [J]. Nat Med, 2003,9:861-866.

[7] Rehermann B, Nascimbeni M. Immunology of hepatitis B virus and hepatitis C virus infection [J]. Nat Rev Immunol, 2005, 5:215-229.

[8] Greenwald R J, Freeman G J, Sharpe A H. The b7 family revisited [J]. Annu Rev Immunol, 2005, 23: 515-548.

[9] Okazaki T, Honjo T. The pd-1-pd-L pathway in immunological tolerance [J]. Trends Immunol, 2006, 27:195-201.

[10] Chen L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity [J]. Nat Rev Immunol, 2004, 4:336-347.

[11] Ishida Y, Agata Y, Shibahara K, et al. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death [J]. EMBO J,1992, 11:3 887-3 895.

[12] Nishimura H, Nose M, Hiai H, et al. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor [J]. Immunity, 1999, 11:141-151.

[13] Sharpe A H, Freeman G J. The B7-CD28 superfamily [J]. Nat Rev Immunol, 2002, 2:116-126.

[14] Day C L, Kaufmann D E, Kiepiela P, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression [J]. Nature, 2006, 443:350-354.

[15] Urbani S, Amadei B, Tola D, et al. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion [J]. J Virol, 2006, 80:11 398-11 403.

[16] Blumberg B S, London W T. Hepatitis B virus: pathogenesis and prevention of primary cancer of the liver [J]. Cancer, 1982, 50:2 657-2 665.

[17] Prince AM, Brotman B. Perspectives on hepatitis B studies with chimpanzees [J]. ILAR J, 2001, 42: 85-88.

[18] Walter E, Keist R, Niederost B, et al. Hepatitis b virus infection of tupaia hepatocytes in vitro and in vivo [J]. Hepatology, 1996, 24:1-5.

[19] Yan R Q, Su J J, Huang D R, et al. Human hepatitis B virus and hepatocellular carcinoma I. Experimental infection of tree shrews with hepatitis B virus [J]. J Cancer Res Clin Oncol,1996, 122:283-288.

[20] Darai G, Schwaier A, Komitowski D, et al. Experimental infection of Tupaia belangeri (tree shrews) with herpes simplex virus types 1 and 2 [J]. J Infect Dis, 1978, 137:221-226.

[21] Xie Z C, Riezu-Boj J I, Lasarte J J, et al. Transmission of hepatitis C virus infection to tree shrews [J]. Virology, 1998, 244:513-520.

[22] Don R H, Cox P T, Wainwright B, et al. "Touchdown" PCR to circumvent spurious priming during gene amplification [J]. Nucleic Acids Res, 1991, 19:4 008.

[23] Tamura K, Dudley J, Nei M, et al. MEGA4: molecular evolutionary genetics analysis (mega) software version 4.0 [J]. Mol Biol Evol, 2007, 24: 1 596-1 599.

[24] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees [J]. Mol Biol Evol, 1987, 4:406-425.