

Overexpression, purification, and pharmacologic evaluation of anticancer activity of ribosomal protein L24 from the giant panda (*Ailuropoda melanoleuca*)

Y.L. Hou, X. Ding, W. Hou, B. Song, T. Wang, F. Wang, J. Li, J. Zhong, T. Xu, B.X. Ma, H.Q. Zhu, J.H. Li and J.C. Zhong

Key Laboratory of Southwest China Wildlife Resources Conservation (Ministry of Education), College of Life Sciences, China West Normal University, Nanchong, Sichuan Province, China

Corresponding author: W. Hou
E-mail: hwr@cwnu.edu.cn

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ABSTRACT. The ribosomal protein L24 (RPL24) belongs to the L24E family of ribosomal proteins and is located in the cytoplasm. The purpose of this study was to investigate the structure and anti-cancer function of RPL24 of the giant panda (*Ailuropoda melanoleuca*). The complementary DNA of *RPL24* was cloned successfully using reverse transcription-polymerase chain reaction technology. We constructed a recombinant expression vector containing *RPL24* complementary DNA and overexpressed it in *Escherichia coli* using pET28a plasmids. The expression product obtained was purified using Ni-chelating affinity chromatography. The results indicated that the length of the fragment cloned is 509 bp, and it contains an open-reading frame of 474 bp encoding 157 amino acids. Primary structure analysis revealed that the molecular weight of the putative RPL24 protein is 17.78 kDa with a theoretical

isoelectric point of 11.86. The *RPL24* gene is readily expressed in *E. coli*, and the RPL24 fused with the N-terminal histidine-tagged protein to give rise to the accumulation of an expected 23.51-kDa polypeptide. The inhibitory rate in mice treated with 0.1 µg/mL RPL24, the highest of 3 doses administered, can reach 67.662%, which may be comparable to the response to mannatide. The histology of organs with tumors showed that the tissues in the RPL24 group displayed a looser arrangement compared with that in the control group. Furthermore, no obvious damage was apparent in other organs, such as heart, lung, and kidney. The data showed that the recombinant RPL24 had time and dose dependency on the cell growth inhibition rate. Human laryngeal carcinoma Hep-2 cells treated with 0.3125-10 µg/mL RPL24 for 24 h displayed significant cell growth inhibition ($P < 0.05$; $N = 6$) in assays using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide compared with that in control (untreated) cells. By contrast, human hepatoma Hep G-2 cells displayed no significant change ($P > 0.05$; $N = 6$) from control (untreated) cells. RPL24 has time and dose dependency on Hep-2 cell growth inhibition. The data indicate that the effect at low concentrations is better than that at high concentrations, and the concentration of 0.625 µg/mL provides the best rate of growth inhibition. Further research is ongoing to determine the bioactive principles of recombinant RPL24 protein that are responsible for its anticancer activity.

Key words: Giant panda; Ribosomal protein L24; cDNA; Cloning; Overexpression; Purification; Anticancer activity

INTRODUCTION

Ribosomes are essential for protein synthesis. The composition and structure of ribosomes from several organisms have been determined, and ribosomal RNAs and ribosomal proteins (RPs) are the clear constituents of these important organelles. Many RPs also fill various roles, called extraribosomal functions, independent of protein biosynthesis. These functions include DNA replication, transcription, and repair, RNA splicing and modification, cell growth and proliferation, regulation of apoptosis and development, and cellular transformation. With the continuous advancement of science and technology, researchers are gradually learning the physiological functions of RPs that play important roles in human disease and its development (Wool, 1996; Wool et al., 1995). RPL24 protein belongs to the L24E family of RPs and is located in the cytoplasm. This gene has been referred to as RPL30 because the encoded protein shares amino acid identity with the RPL30 from *Saccharomyces cerevisiae*; however, its official name is RPL24. As is typical for genes encoding RPs, multiple processed pseudogenes of this gene are dispersed through the genome (Barkic et al., 2009).

The giant panda (*Ailuropoda melanoleuca*) is one of the oldest and rarest species on earth, and is currently found only in China, where it is known as the national treasure of China. It is the most endangered species in the world. In recent years, scientists have achieved tremendous success with the macrograph of the giant panda. Recently, functional gene study

has become a hot trends in giant panda research (Hou et al., 2008, 2009; Su et al., 2012; Sun et al., 2012). Therefore, more attention is being paid to RPL24 in giant panda because of its many biological functions, especially those related to anticancer activity. Cancer is one of the leading causes of mortality worldwide. In cancer, cells divide and grow uncontrollably, forming malignant tumors, and invade neighboring parts of the body. A cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream (Tolar and Neglia, 2003; Croce, 2008; Jemal et al., 2011).

According to related information about the designed primer for the RPL24 gene (*RPL24*) of some mammals, including *Homo sapiens*, *Bos taurus*, *Felis catus*, *Mus musculus*, and *Rattus norvegicus*, we used a reverse transcriptase-polymerase chain reaction (PCR) technique to amplify the complementary DNA (cDNA) of the *RPL24* gene from the total RNA of the skeletal muscle of the giant panda. We then analyzed the sequence characteristics of the protein encoded by the cDNA and compared it with those of humans and other animals. We constructed a recombinant expression vector containing *RPL24* cDNA and overexpressed it in *Escherichia coli* using pET28a plasmids. Under the optimized expression conditions, we obtained the recombinant RPL24 protein from giant panda, which we then purified using Ni-chelating affinity chromatography. The purpose of this study was to evaluate the anti-cancer activity of RPL24 from giant panda *in vivo* and *in vitro*.

MATERIAL AND METHODS

Skeletal muscle was collected from a dead giant panda at the Wolong Conservation Center of the Giant Panda (Sichuan, China). The collected tissue was frozen in liquid nitrogen and then used for RNA isolation. Hep-2 cells from a human laryngocarcinoma line and human hepatoma Hep G-2 cells were purchased from the Department of Biochemistry and Immunology, North Sichuan Medical College (NSMC; Nanchong, China). Total tissue/cell RNA extraction kits were purchased from Sigma Company (Shanghai, China). Reverse transcription kits were from Promega Company (Beijing, China). Gel extraction mini kits were purchased from OMEGA Corporation (Kanpur, India). PMD-18 T vector systems and the restriction enzymes *EcoRI* and *HindIII* were obtained from TaKaRa Bio Group (Dalian, China). DNA polymerases were purchased from Sangon Co. (Shanghai, China). Host bacteria *E. coli* DH5 α were stored in the Key Laboratory of Southwest China Wildlife Resources Conservation (Nanchong, China). CW0009 Ni-agarose His-tag protein purification kits were purchased from Beijing Ealysino Biological Technology Co. (Beijing, China). Bradford Protein Assay Kits were purchased from Majorbio Biotech Co. (Shanghai, China). Penicillin/streptomycin (10,000/10,000 $\mu\text{g}/\text{mL}$) and Dulbecco's minimal essential medium reagent were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum was obtained from Sijiqing Co. (Huangzhou, China).

DNA and RNA isolation

A total of 500 mg muscle tissue was ground in liquid nitrogen to a fine powder and then suspended completely in 15 mL lysis buffer containing 10 mM Tris-HCl, pH 8.0, 100 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% sodium dodecyl sulfate (SDS). After treatment with proteinase K (100 mg/mL, final concentration) at 55°C for 3 h, the mixture was cooled to room temperature and mixed with an equal volume of saturated phenol, pH 8, before

being centrifuged at 5000 g at 4°C for 20 min. The supernatant was pooled and mixed with an equal volume of 1:1 (v/v) phenol-chloroform and then centrifuged as above, and the supernatant was collected, from which the DNA was precipitated using ethanol. The DNA obtained was then dissolved in Tris-EDTA buffer and kept at -20°C. Total RNAs were isolated from approximately 400 mg muscle tissue using Total Tissue/Cell RNA Extraction Kits (Waton Inc., Shanghai, China) according to manufacturer instructions. The extracted total RNAs were dissolved in diethyl pyrocarbonate water and kept at -70°C.

Primer design and RT-PCR

PCR primers were designed with Premier 5.0 based on the messenger RNA sequence of RPL24 from *Homo sapiens* (NM_000986), *Bos taurus* (NM_174455), *Felis catus* (NM_001128841), *Mus musculus* (NM_022515), and *Rattus norvegicus* (NM_024218). The specific primers of the cDNA sequences were as follows: RPL24-F: C [G/C] TGGAGCTGTC GCCATGAAG; RPL24-R: TAAAAATCTGATCCACCAA.

Total RNAs were synthesized into the first-stranded cDNAs by using a reverse transcription kit (Promega, Madison, USA) with Oligo dT as the primers according to manufacturer instructions. Twenty microliters of the first-strand cDNA synthesis reaction system was included in 1 mg total RNAs, 5 mM MgCl₂, 1 mM deoxyribonucleotide triphosphate, 0.5 mg Oligo dT15, 10 U/mL RNase inhibitor, and 15 U avian myeloblastosis virus reverse transcriptase, and incubated at 42°C for 60 min. The synthesized first-strand cDNA was used as a template. The total reaction volume for DNA amplification was 25 µL. Reaction mixtures contained 1.5 mM MgCl₂, 200 µM each deoxyribonucleotide triphosphate (Omega, China), 0.3 µM each primer, 5.0 U Taq plus DNA polymerase (Sangon Co., Shanghai, China). DNA amplification was performed using a thermocycler (Model PTC-200, MJ Research, Watertown, MA, USA) with a program of 4 min at 94°C, followed by 30 cycles of 1 min at 94°C, 0.5 min at 48°C, and 1.5 min at 72°C, and a final extension for 10 min at 72°C. After amplification, PCR products were separated via electrophoresis on 1.5% agarose gel with 1X Tris-acetate-EDTA buffer, stained with ethidium bromide, and visualized under ultraviolet light. The expected fragments of PCR products were harvested and purified from the gel using a DNA harvesting kit (Omega) and stored at -20°C.

Cloning and identification of the cDNA sequence

The harvested PCR products were ligated into a pMD19-T vector at 4°C for 12 h. The recombinant molecules were transformed into *E. coli* complete cells (DH5α) and spread on a Luria-Bertani plate containing 50 µg/mL ampicillin, 200 mg/mL isopropyl-beta-D-thiogalactopyranoside, and 20 mg/mL X-gal. Plasmid DNA was isolated and digested with *Pst*I and *Sca*II to verify the insert size. Plasmid DNA was sequenced by Huada Zhongsheng Scientific Corporation (Beijing, China).

Cloning the genomic sequence of RPL24

The PCR primers were the same as those described above for RPL24-F and RPL24-R. The genomic sequence of the RPL24 gene was amplified using touchdown-PCR with the

following conditions: 94°C for 30 s, 62°C for 45 s, and 72°C for 4 min in the first cycle, with the annealing temperature decreasing 0.5°C per cycle; after 20 cycles, conditions changed to 94°C for 30 s, 52°C for 45 s, and 72°C for 4 min for another 20 cycles. The fragment amplified was also purified, ligated into the clone vector, and transformed into *E. coli* competent cells. Finally, the recombinant fragment was sequenced by Invitrogen (Beijing, China).

Construction of the expression vector and overexpression of recombinant RPL24

A PCR fragment corresponding to the RPL24 polypeptide was amplified from the *RPL24* cDNA clone with the forward primer 5'-CAGAATTCATGAAGGTCGAGC-3' (*EcoRI*) and reverse primer 5'-CGAAGCTTAGCGTTTTCCAC-3' (*HindIII*), respectively. PCR was performed at 94°C for 3 min followed by 35 cycles of 30 s at 94°C, 45 s at 53°C, and 1 min at 72°C, and a final extension of 10 min at 72°C. The amplified PCR product was cut and ligated into the corresponding site of the pET28a vector (Stratagene, Shanghai, China). The resulting construct was transformed into *E. coli* BL21 (DE3) strain (Novagen, Shanghai, China) and used for the induction by adding isopropyl-b-D-thiogalactopyranoside at an optical density (OD) at 600 nm of 0.6 and culturing for an additional 4 h at 37°C using the empty vector-transformed BL21 (DE3) as a control. The recombinant protein samples were induced after 0, 1, 1.5, 2, 2.5, 3, and 4 h and then separated with SDS polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue R 250.

Purification of recombinant protein RPL24

Acquired genetic engineering recombinant protein has a tag comprised of 6 histidines (His-tag), making Ni-chelating affinity chromatography available as follows. The ultrasonic products were centrifuged to collect sediments and then suspended with soluble binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazole, pH 7.9) until the inclusion body was clean. The sediments were centrifuged and suspended with inclusion body binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, and 8 M urea, pH 7.9) on ice until the inclusion body was thoroughly dissolved. The supernatant was centrifuged and transferred to the chromatography column with Ni. The column was allowed to stand for 2 min after the supernatant was completely transferred so that the 6 His-tags and nickel in the padding could combine fully. An inclusion body-binding buffer in a volume 15 times that of the column was used to flush the column and wash the uncombined protein. The excurrent liquid was collected and inclusion body elution buffer in a volume 5 times that of the column was used to wash the combined protein. According to the amount of the column volume, the outflow liquid of one column volume was collected each time until all of the fluid were collected. The outflow liquid of one column volume was collected each time until all of the fluid were collected. SDS-PAGE was then used to detect the effects of purification. The concentration of the recombinant protein was determined with Bradford Protein Assay Kits (Majorbio). Dialysis was used to purify the elution protein. After 48 h of desalination, the purified protein with 10% glycerol was stored at -20°C.

Animals

S180 tumor cells were maintained in the peritoneal cavities of Kunming strain male

mice obtained from the Institute of Biochemistry and Molecular Immunology of NSMC. Male Kunming strain mice weighing 25.0 ± 1.0 g were purchased from NSMC and housed 6 per plastic cage with wood chip bedding in an animal room with a 12-h light and 12-h dark cycle at room temperature ($25^\circ \pm 2^\circ\text{C}$) and allowed free access to a standard laboratory diet (purchased from the Institute of Biochemistry and Molecular Immunology of NSMC). The animal experiments were conducted according to the guidelines for animal experimentation of NSMC.

Assay of anti-tumor activity *in vivo*

S180 tumor cells (3×10^6) were implanted subcutaneously into the right hind groin of Kunming strain male mice. Mice were randomly divided into 5 groups ($N = 6$). One day after inoculation, RPL24 was dissolved in distilled water and administered intraperitoneally to the mice at doses of 0.025, 0.05, and 0.1 $\mu\text{g}/\text{mL}$. Positive and negative controls were set for comparison. The positive control was given 0.2 mL 2 mg/mL mannate, and the negative control was given physiological saline instead of the test solution. The animals were killed after 2 weeks. The body weights were measured. Tumors, spleens, and livers were excised, and the tumor inhibitory ratio was calculated with the following formula: Inhibition ratio (%) = $[(A-B)/A] \times 100$, where A and B were the average tumor weights of the negative control and treated groups, respectively.

Histopathology and morphological observations

After treating the mice with RPL24 as described above, a portion of the tissues were cut into small pieces, fixed in Heidenhain's Susa fluid (4.5 g HgCl_2 , 0.5 g NaCl, 80.0 mL distilled water, 20.0 mL formalin, 4.0 mL acetic acid, 2.0 mL trichloroacetic acid) and then stained with hematoxylin and eosin, examined, and photographed under an OLYMPAS microscope (Janpan).

Cell culture

The human laryngocarcinoma line Hep-2 cells and human hepatoma Hep G-2 cells were grown in RPMI (Roswell Park Memorial Institute)-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid, pH 7.4. Cells were kept at 37°C in a humidified 5% CO_2 incubator.

Pharmacologic evaluation for anticancer activity on cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method

Cells were seeded into 96-well microculture plates at appropriate densities to maintain the cells in an exponential phase of growth throughout the duration of the experiment. Cancer cells were exposed to the recombinant RPL24 protein at doses of 10, 5, 2.5, 1.25, 0.625, 0.5, 0.3125, and 0 $\mu\text{g}/\text{mL}$ for 24 h, and each concentration was evaluated in 6 wells. At the end of the exposure, 20 μL MTT was added to each well, and the plates were incubated for 2–4 h at

37°C. Then, 150 μ L dimethyl sulfoxide was added to each well, and the plates were surged for 5 min. The OD was read with a plate reader (BIO-RAD Co., NY, USA) at wavelengths of 490 nm. Media alone as well as control wells in which RPL24 was absent were included in all experiments. The degree of inhibition of cell proliferation was calculated using the following formula: Growth inhibition (%) = (OD control - OD treated)/OD control \times 100. The 96-well plates were examined under an inverted microscope and photographed at the different concentrations to record cell morphology.

Data analysis

The sequence data were analyzed with GenScan software (<http://genes.mit.edu/GENSCAN.html>). Homology research of the giant panda *RPL24* compared with the gene sequences of other species was performed using Basic Local Alignment Search Tool 2.1 (<http://www.ncbi.nlm.nih.gov/blast/>). The open reading frame (ORF) of the DNA sequence was searched using ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The values of molecular weight and isoelectric point (pI) were computed using the Compute pI/molecular weight tool (http://www.expasy.org/tools/pi_tool.html). The protein structure of the cloned RPL24 sequence was analyzed using PredictProtein software (<http://www.predictprotein.org/>). Multiple sequence alignment was performed with the software DNASTar Lasergene and DNAMAN 6.0. Prediction of tertiary structure of recombinant protein RPL31 was simulated with the SWISS-MODEL software (<http://swissmodel.expasy.org/>).

RESULTS AND DISCUSSION

Analysis of the cDNA of RPL24 from giant panda

A ~500-bp cDNA fragment was amplified from giant panda. The length of the cloned cDNA was 509 bp (Figure 1). Given the high identity, we concluded that the cDNA isolated was that encoding the giant panda RPL24 protein. The *RPL24* sequence was submitted to GenBank (accession No. HQ318071). The 509-bp sequence of the giant panda *RPL24* sequence contains a 15-bp 5'-untranslated sequence and a 20-bp 3'-untranslated region. An ORF of 474 bp encoding 157 amino acids was found in the cDNA sequence (Figure 2). Alignment analysis of *RPL24* of giant panda and those of *H. sapiens*, *B. taurus*, *F. catus*, *M. musculus*, *R. norvegicus*, and *Danio rerio* indicated that both the nucleotide sequence and the deduced amino acid sequence are highly conserved. No deletion or insertion of nucleotides or amino acid residues occurs. As determined through Basic Local Alignment Search Tool analysis, the nucleotide sequence RPL24 cloned from giant panda shared high homology with those of *H. sapiens*, *B. taurus*, *F. catus*, *M. musculus*, *R. norvegicus*, and *D. rerio* of 94.30, 94.94, 97.68, 91.35, 91.14, and 74.68%, respectively; the homologies for amino acid sequences among the species are all 100%, except that for *D. rerio*, which was 89.17% (Table 1). This striking pattern of evolutionary conservation is considered reasonable, as RP genes are a group of highly conserved housekeeping genes. The phylogenetic tree was generated from 7 aligned sequences (Figure 3). In the tree, *A. melanoleuca* and *F. catus* were located in the same clade, and they showed a very close genetic relationship with other related species.

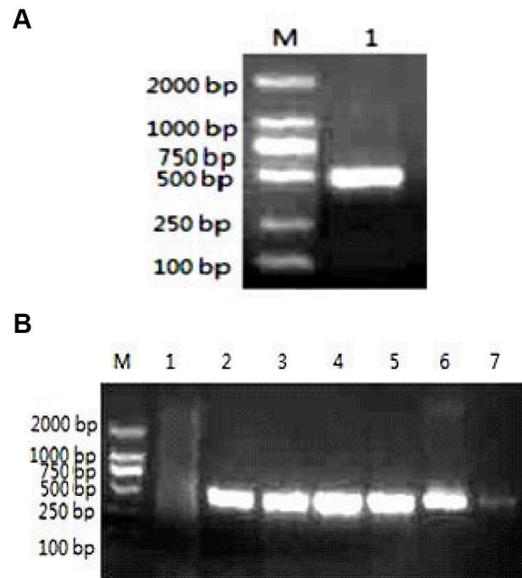


Figure 1. Electrophoresis analysis of RT-PCR amplified products (A) and identification of the recombinant by PCR (B). **A.** Lane M = marker; lane 1 = RT-PCR amplified products. **B.** Lane M = marker; lane 1 = blank group; lanes 2-7 = recombinant by PCR.

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1  cgtggagctgtcgcc  atg aag gtc gag ctg tgc agt ttc agt ggg tac aag att tac ccc
1  M K U E L C S F S G Y K I Y P
61  gga cat ggc agg cgc tac gcc agg act gac ggg aag gtt ttc cag ttt ctt aac gca
16  G H G R R Y A R T D G K U F Q F L N A
118 aaa tgc gaa tcc gca ttc ctt tcc aag agg aat cct cgg cag ata aac tgg act gtc
35  K C E S A F L S K R N P R Q I N W T U
175 ctc tac agg aga aag cac aaa aag gga cag tcg gaa gaa att caa aag aaa aga acc
54  L Y R R R K H K K G Q S E E I Q K K R T
232 cgc cgt gca gtc aaa ttc cag agg gcc atc act ggt gca tct ctt gct gat ata atg
73  R R A U K F Q R A I T G A S L A D I M
289 gcc aag agg aac cag aaa cct gaa gtt agg aag gct caa cga gaa cag gcc atc agg
92  A K R N Q K P E U R K A Q R E Q A I R
346 gct gcc aag gaa gca aaa aag gct aag caa gca tct aaa aag aca gca atg gct gct
111 A A K E A K K A K Q A S K K T A M A A
403 gct aag gct ccc acg aag gca gca cct aag caa aag att gtg aag cct gtg aag gtt
130 A K A P T K A A P K Q K I U K P U K U
460 tct gca ccc cga gtt ggt gga aaa cgc taa attggtgatcagattttta
149 S A P R U G G K R *
    
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Figure 2. Nucleotide sequence and putative amino acid sequence of *RPL24* cDNA from the *Ailuropoda melanoleuca*. *Termination codon.

Table 1. Comparison of nucleotide and protein sequence among 7 vertebrate species.						
	<i>Homo sapiens</i>	<i>Bos taurus</i>	<i>Felis catus</i>	<i>Mus musculus</i>	<i>Rattus norvegicus</i>	<i>Danio rerio</i>
Nucleotide sequence	94.30%	94.94%	97.68%	91.35%	91.14%	74.68%
Protein sequence	100%	100%	100%	100%	100%	89.17%

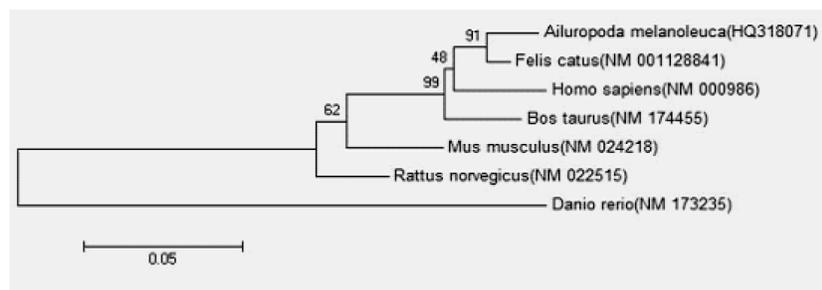


Figure 3. Phylogenetic tree calculated by the neighbor-joining (NJ) based on ORF sequence of *RPL24* gene from variety species.

Analysis of the genomic sequence of *RPL24* from the giant panda

A fragment of 4531 bp was amplified from genomic DNA of the giant panda using primers *RPL24-F* and *RPL24-R*. Comparison between the cDNA sequence and this DNA fragment indicated that the cDNA sequence is a full cDNA corresponding to 6 exons in the *RPL24* genomic sequence of giant panda (Table 2). The genomic sequence of the *RPL24* gene has been submitted to GenBank (accession No. HQ318072).

Table 2. Comparison of *RPL24* genomic sequence among six vertebrate species.

Species	Length (bp)	No. of exons	No. of introns	5'-UTR	3'-UTR	Join sites in the CDS	Accession No.
<i>A. melanoleuca</i> *	4531	6	5	0	20	1~5, 130~205, 752~862, 3086~3222, 2435~2498, 4431~4511	HQ318072
<i>A. melanoleuca</i>	4667	6	5	109	47	110~114, 239~314, 861~971, 3195~3331, 3744~3807, 4540~4620	NW_003217465
<i>H. sapiens</i>	5618	6	5	43	45	44~48, 163~238, 792~902, 3805~3941, 4220~4283, 5493~5573	AC_000135
<i>Bos taurus</i>	5503	6	5	38	39	39~43, 175~250, 765~875, 3514~2450, 4701~4764, 5384~5464	AC_000158
<i>M. musculus</i>	5163	6	5	159	46	160~164, 273~348, 786~896, 3224~3372, 3838~3901, 5037~5117	NC_000082
<i>R. norvegicus</i>	5209	5	4	0	39	1->76, 509~619, 3276~3412, 3877~3940, 5090~5170	AC_000079
<i>D. rerio</i>	5130	6	5	24	30	25~29, 155~230, 1589~1699, 2086~2222, 4825~4888, 5020~5100	NC_007112

*Sequent we cloned.

Prediction and analysis of protein functional sites in the RPL24 protein of giant panda

Primary structure analysis revealed that the molecular weight of the putative RPL24 protein of giant panda is 17.78 kDa with a theoretical *pI* of 11.86 (Table 3). Topology prediction showed 1 N-glycosylation site, 1 cyclic adenosine monophosphate- and cyclic guanosine monophosphate-dependent protein kinase phosphorylation site, 3 protein kinase C phosphorylation sites, 1 casein kinase II phosphorylation site, 1 N-myristoylation site, 1 amidation site, and 1 RPL24e signature in the RPL24 protein of giant panda (Figures 4 and 5). Further analysis detected a few polymorphic sites in the amino acid sequences of the 7 species compared.

In addition, most base transitions of the gene-coding sequence in these mammals were synonymous mutations that did not result in any changes in the corresponding DNA information or change the amino acid in the expression product. Consequently, the spatial structure of the corresponding protein is unaffected.

Table 3. Molecular weight and pI of *RPL24* of the *Ailuropoda melanoleuca* and six other vertebrate species.

	<i>A. melanoleuca</i>	<i>H. sapiens</i>	<i>B. taurus</i>	<i>F. catus</i>	<i>M. musculus</i>	<i>R. norvegicus</i>	<i>D. rerio</i>
Molecular weight (kDa)	17.78	17.78	17.78	17.78	17.78	17.78	17.87
Isoelectric point (pI)	11.86	11.86	11.86	11.86	11.86	11.86	11.85



Figure 4. Comparison of the *RPL24* protein sequences among the different Eukaryotic. ____ = Bipartite nuclear localization signal profile; _____ = Ribosomal protein L24e signature; □ = Amidation site; ■ = Protein kinase C phosphorylation site; • = N-glycosylation site; _____ = cAMP- and cGMP-dependent protein kinase phosphorylation site; — = N-myristoylation site; ~ = Casein kinase II phosphorylation site.

Overexpression and purification of the RPL24 gene in *E. coli*

The *RPL24* gene was overexpressed in *E. coli* using pET28a plasmids carrying strong promoter and terminator sequences derived from phage T7. For this purpose, *RPL24* was amplified individually using PCR and cloned in a pET28a plasmid, resulting in a gene fusion coding for a protein bearing a His-tag extension at the N terminus. Expression was tested with SDS-PAGE analysis of protein extracts from recombinant *E. coli* BL21 strains (Figure 6). The results indicated that the *RPL24* protein fusion with the N-terminally His-tagged form gave rise to the accumulation of an expected 24-kDa polypeptide that formed inclusion bodies. Apparently, the recombinant protein was expressed after 30 min of induction and after 3 h reached the highest level. The expression product could be used for purification and further study of its function. Under optimized expression conditions, we obtained substantial recombinant protein, which then was purified using Ni-nitrilotriacetic acid chelating affinity chromatography. SDS-PAGE

analysis clearly indicated ~24-kDa polypeptides in lanes 3-8 (Figure 7). Protein separation and purification are key steps in genetic engineering technology. Through affinity chromatography, we obtained purified protein. During this process, the protein solution pH was changed twice, enabling us to achieve high protein purity. In other words, the protein solution first goes through the column under acid conditions and then finally outflows from the column through a change in the pH of the effluent liquid. SDS-PAGE analysis clearly indicated ~24-kDa polypeptides in lanes 3-8. Size consistency of the purified protein and unpurified RPL24 protein suggested that the protein was only that encoded by *RPL24* of giant panda. The sequence of the acquired recombinant RPL24 protein from giant panda consists of 190 amino acid residues. The molecular weight of the recombinant RPL24 protein is 23.51 kDa.

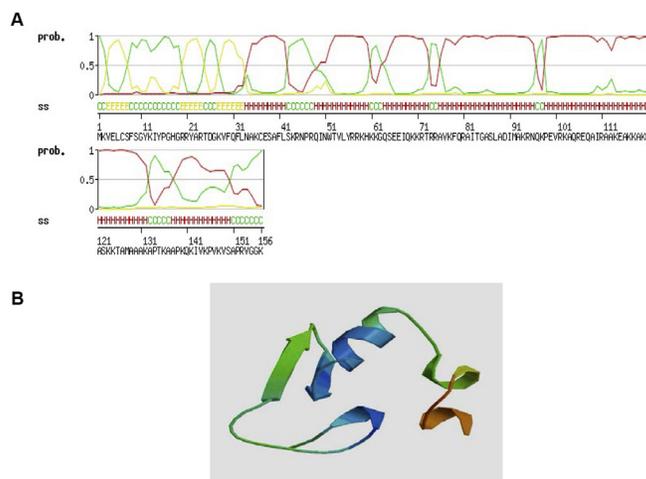


Figure 5. Secondary (A) and tertiary (B) structure of recombinant protein RPL24 from the giant panda. H = helix; E = extended-beta; C = coil.

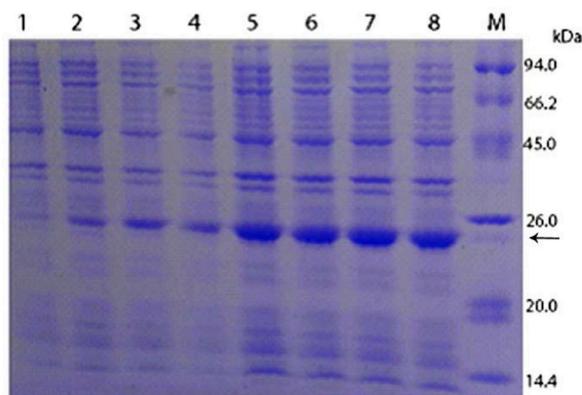


Figure 6. Protein extracted from recombinant *Escherichia coli* BL21 strains was analyzed by SDS-PAGE gel stained with Commassie blue R250. Numbers on right shows the molecular weight and the arrow indicates the recombinant protein bands induced by IPTG with 0, 1, 1.5, 2, 2.5, 3, 3.5, and 4 h (lane 2-8), respectively. Lane 1 = products of the *E. coli* strains with the empty vectors. Lane M = marker.

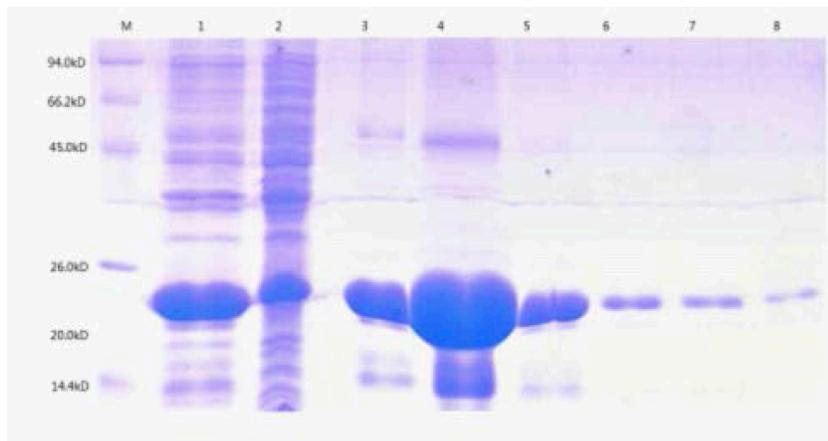


Figure 7. Analysis of purified recombinant RPL24 protein. *Lane 1* = production before the purified; *lane 2* = flow in collecting liquid; *lanes 3-8* = collecting liquid of purified recombinant RPL24 protein. *Lane M* = marker.

Anti-tumor activity of RPL24 *in vivo*

An increasing amount of recent research has indicated that the high-level expression of RPs is a prognostic factor in certain types of tumors, and several RPs have been observed at high levels in cancers (Barnard et al., 1992; Chiao et al., 1992; Denis et al., 1993; Ropolo et al., 2004; Clark et al., 2005). To detect the anti-tumor activity of RPL24 *in vivo*, we used mice-transplanted S180. The results are summarized in Table 1. The weight and histological preparations of the vital organs in each male mice in the control group were compared to that in the treated group to measure the effect of the drug. RPL24 inhibited the growth of the tumors ($P < 0.01$) in a dose-dependent manner (Table 4). The inhibitory rate in mice treated with 0.1 $\mu\text{g/mL}$ RPL24, the highest of 3 administered doses, was 67.662%. Furthermore, during the experiments, the appetite, activity, and coat luster of each animal in the RPL24 groups were better than those of mice treated with mannatide. Histology of the immune organs liver, spleen, and thymus showed that tissues were arranged more regularly and firmly, but the tumor tissue was arranged more loosely in the RPL24 group than that in the control group. Meanwhile, no obvious damage occurred to other organs such as heart, lung, and kidney (Figure 8). The results also showed little change in average liver weight in the test groups, indicating that RPL24 caused no serious liver damage (see Table 4). On day 14, the average tumor weight of negative control mice was 3.751 g, whereas the average tumor weight in RPL24 group mice that received a dose of 0.1 $\mu\text{g/mL}$ was 1.213 g. The weight was also significantly reduced in the RPL24 mice that received doses of 0.05 and 0.025 $\mu\text{g/mL}$ (2.181 and 1.693 g, respectively). Notably, the average spleen and thymus weights in the test groups were significantly greater at a dose of 0.05 $\mu\text{g/mL}$ than that in the negative control organs and even that in the mannatide mice, indicating that RPL24 may increase the weights of immune organs in moderate doses (see Table 4). These results suggested that activating immune responses in the host might be a mechanism of RPL24 anti-tumor activity, as many anti-tumor polysaccharides are known.

Table 4. Anti-tumor activities of RPL24 on S180 tumor (N = 8).

Group	Spleen index (mg/g)	Liver index (mg/g)	Thymus index (mg/g)	Average tumor weight (g)	Inhibitory rate (%)
N	5.694 ± 2.008	60.417 ± 9.495	1.549 ± 0.602	3.751 ± 0.373	-
L1	7.529 ± 2.314	55.568 ± 4.509	4.451 ± 0.311	2.181 ± 0.640	41.855 (**)
L2	7.647 ± 3.227	65.451 ± 8.702	7.870 ± 3.018	1.693 ± 0.530	54.865 (**)
L3	5.002 ± 0.889	59.877 ± 4.676	0.913 ± 0.411	1.213 ± 0.342	67.662 (**)
Man	7.139 ± 3.006	57.103 ± 5.323	2.662 ± 1.637	1.561 ± 0.736	58.384 (**)

Significant differences from negative and positive control group were evaluated using the Student *t*-test. *P < 0.05, **P < 0.01. N = negative control group; L1, L2, L3 = RPL24 groups of 0.025, 0.05, 0.1 µg/mL, respectively; Man = positive control group of mannate. Data are reported as means ± SD.

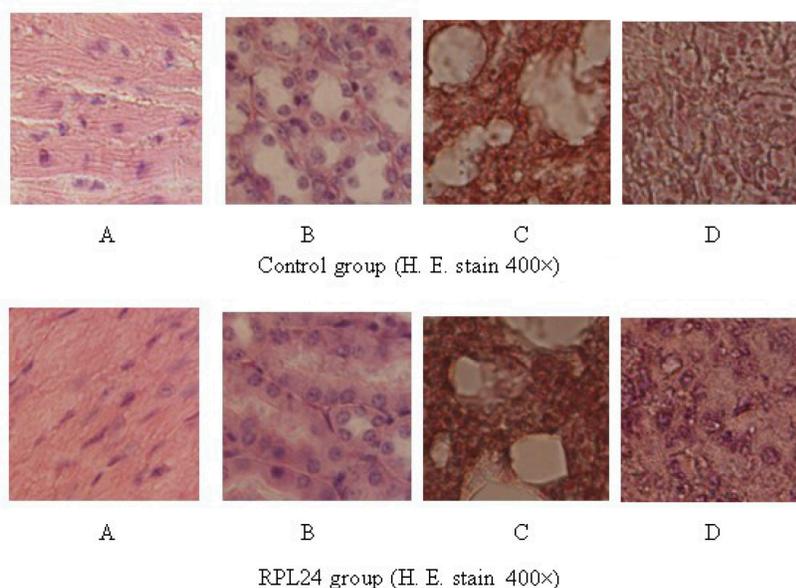


Figure 8. Histological preparations of the heart (A), kidney (B), lung (C), tumor (D) in LDG-A group (concentration of 80 mg/kg) compared with control group.

Anticancer activity of RPL24 *in vitro*

Laryngeal cancer is also called cancer of the larynx or laryngeal carcinoma. Most laryngeal cancers are squamous cell carcinomas, reflecting their origin from the squamous cells that form the majority of the laryngeal epithelium. Laryngeal cancer may spread by direct extension to adjacent structures, by metastasis to regional cervical lymph nodes, or more distantly through the bloodstream. Distant metastases to the lung are most common (Yu et al., 2009). A hardy cell line, Hep-2, resists temperature, nutritional, and environmental changes without losing viability. It has supported the growth of 10 of 14 arboviruses as well as the measles virus, and it has been used for experimental studies of tumor production in rats, hamsters, mice, embryonated eggs, and volunteer terminal cancer patients (Nutting et al., 2008). Hep G2 (hepatocellular carcinoma, human) cells are epithelial in morphology and secrete a variety of major plasma proteins - e.g., albumin, transferrin, and the acute phase

proteins fibrinogen, alpha 2-macroglobulin, alpha 1-antitrypsin, transferrin, and plasminogen (Chen and Chan, 2009). To assess whether RPL24 demonstrates anticancer activity in human laryngeal carcinoma Hep-2 cells and human hepatoma Hep G-2 cells, we cultured these cells in the presence and absence of various concentrations of RPL24 for 24 h. As shown in Figure 9, the human laryngeal carcinoma Hep-2 cells treated with 0.3125-10 $\mu\text{g}/\text{mL}$ RPL24 for 24 h displayed significant cell growth inhibition ($P < 0.05$, $N = 6$) compared with that in control (untreated) cells in MTT assays. For comparison, human hepatoma Hep G-2 cells displayed no significant change ($P > 0.05$; $N = 6$) compared with control (untreated) cells. RPL24 was time and dose dependent on Hep-2 cell growth inhibition. The data indicate that the effect at low concentrations was better than that at high concentrations, and the concentration of 0.625 $\mu\text{g}/\text{mL}$ had the best rate of growth inhibition (see Figure 9).

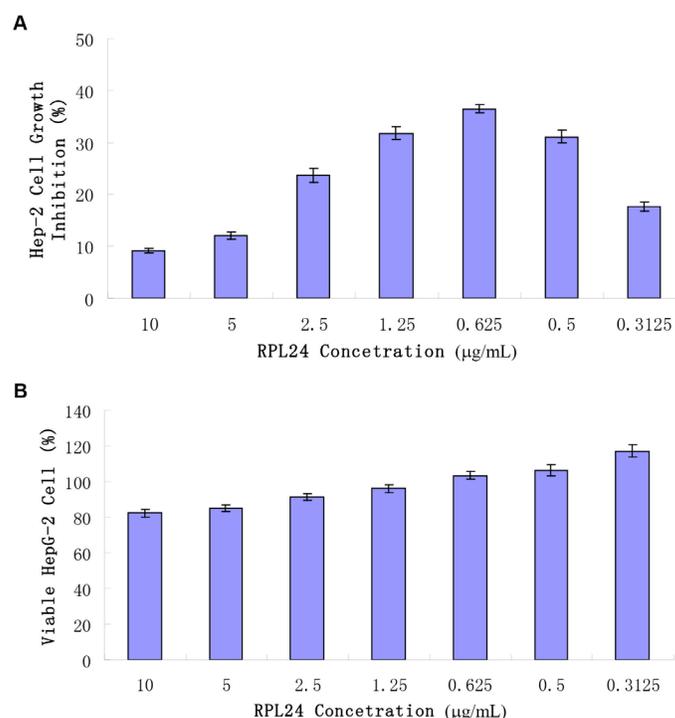


Figure 9. Cell growth inhibition of ribosomal protein L24 (RPL24) on human laryngeal carcinoma Hep-2 cells (A) and human hepatoma HepG-2 cells (B).

Effect of RPL24 on morphology of human laryngeal carcinoma Hep-2 cells and human hepatoma Hep G-2 cells

The 96-well plates were placed under an inverted microscope to record cell morphology changes at various concentrations of RPL24 to measure its effects. RPL24 exhibited the highest anticancer activity, as indicated by the cell morphology, which had rounded into a group and even cracked off in pieces in the Hep-2 group (see Figure 6), whereas Hep G-2 cells displayed no significant changes compared with those in the control group (Figure 10).

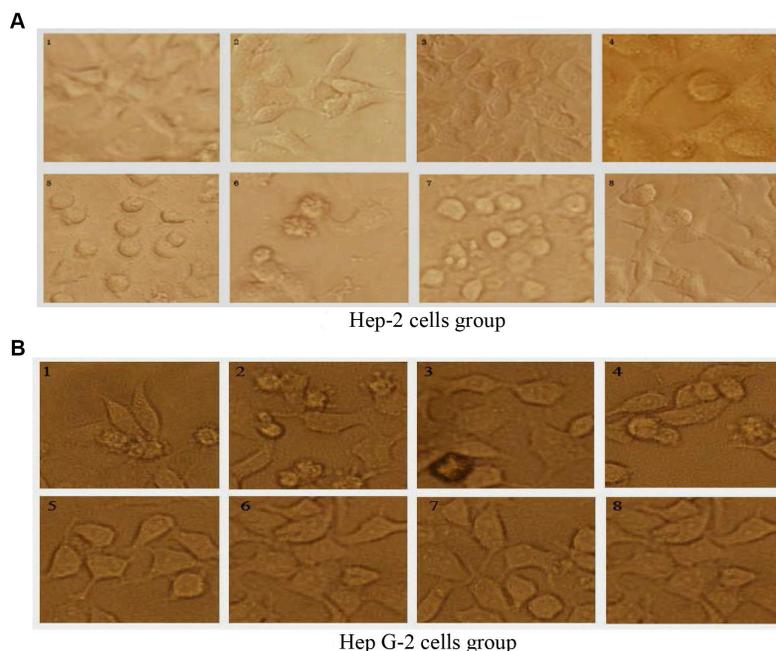


Figure 10. Effect of ribosomal protein L24 (RPL24) on morphology of human laryngeal carcinoma Hep-2 cells (A) and human hepatoma HepG-2 cells (B) for 0, 10, 5, 2.5, 1.25, 0.625, 0.5, 0.3125 µg/mL (pictures 1-8), respectively.

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