Expression of Biologically Active Neurotrophin - 4 of Giant Panda in Escherichia coli

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Abstract: The neurotrophin - 4 (NT - 4) gene plays an important role in the protection of the damages resulted from epilepsy, which is an important cause of death in the pandas. The genomic DNA coding for the NT - 4 gene of the giant panda was cloned into expression vector pGEX - 4T - 3 under the control of a tac promoter, and expressed in *Escherichia coli*. After purification, the recombinant NT - 4 of the panda was found to be biologically active in the PC12 neurite outgrowth assay. We suggest that this technique may offer a feasible means of gene therapy for the giant pandas suffering from epilepsy.

Key words: Epilepsy; Neurotrophin - 4; Gene expression; Purification; Giant panda (*Ailuropoda melanoleuca*)

大熊猫神经营养素 - 4 基因在大肠杆菌中的表达

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摘要: 本文通过 PCR 技术, 直接从大熊猫基因组 DNA 上克隆得到其神经营养素 - 4 的成熟肽编码序列, 通过序列分析发现, 该基因在进化上具有较高的保守性。将神经营养素 - 4 成熟肽完整编码序列克隆至 pGEX - 4T - 3 表达载体, 并经 IPTG 诱导在大肠杆菌中进行原核生物表达, 获得了大熊猫重组蛋白神经营养素 - 4。重组表达蛋白经纯化后, 进行大鼠肾上腺嗜铬瘤细胞神经营养因子的活性鉴定, 发现其能够诱导神经细胞分化产生突触, 具有预期的生物学活性。对大熊猫神经营养素 - 4 的基因工程研究, 为大熊猫癫痫的基因治疗奠定了基础。

关键词: 癫痫; 神经营养素 - 4; 基因表达; 纯化; 大熊猫

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The giant panda (*Ailuropoda melanoleucaone*) is the most endangered species in the world with only about 1000 individuals in the wild[1,2]. Epilepsy is one of the most important reasons for abnormal death of the panda[3]. Epileptic seizures could exert severe and lasting influence on the neural system such as neuronal cell death, neuron degeneration and muscle spasms[4]. Previous study on 50 wild pandas indicated that 8 % of the pandas suffered from epilepsy with a death rate of 10 %[5]. The disease has threatened the survival of panda due to its serious damage to the nervous system, and there is still no effective method for treatment[6,7].

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The NT - 4 gene is known to protect the hippocampus and cortical neurons from the damages of excitation toxin and traumatic brain injury[8 - 11]. Therefore, it may also be helpful to the plerosis and protection of the damages resulted from epilepsy. In humans, the biologically active NT - 4 is a noncovalent homodimer, with the monomers consisting of 130 aa with three intrachain disulfide bridges formed by six Cysteine (Cys) residues. The monomer begins after an Arg - X - X - Arg cleavage sequence, and the DNA sequence encoding the monomer is a single exon, which makes it feasible to clone the NT - 4 gene from genomic DNA directly[12].

To study the function of NT - 4 on the panda nerve system, we cloned the coding sequence of NT - 4 monomer and expressed it in *E. coli* with partially soluble product. The expressed product was purified and its neurotrophic activity was confirmed by *in vitro* assay. This study is a preliminary attempt to develop gene therapy for optical epilepsy in pandas.

1 MATERIALS AND METHODS

1.1 Materials

Two samples of frozen liver tissue were donated from the Chengdu Research Base for Giant Panda Breeding (Chengdu, Sichuan Province, China). The pGEM - T vector and *E. coli* strain were obtained from Promega (Madison, WI, USA.). Expression vector and *E. coli* strain was from Pharmacia (Hong Kong, China). PC12 cells were obtained from Shanghai Institute of Cell Biology, the Chinese Academy of Sciences (Shanghai, China). Glutathione sepharose 4B microSpin column was from Pharmacia.

1.2 Extraction of the genome DNA

High quality genomic DNA was isolated from frozen kidney tissue with routine phenol - chloroform method[13].

1.3 Isolation of the panda NT - 4

A pair of PCR primers (N1, 5′- CCCCGGCCAACCGCAGCCGGC - 3′; N2, 5′- CTCCAGGAGAACTCCTATTC - 3′) was designed with the program Primer Premier 5.0 to amplify a 609 bp fragment spanning the NT - 4 monomer coding sequence[14]. PCR was performed in a total volume of 50 μl containing 25 ng template DNA, 30 pmol of each primers, 1.25 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 ×Taq polymerase buffer, and 1.5 unit Taq™ polymerase, with cycling parameters as following: 35 cycles of 94 ℃ for 1 min, 50 ℃ for 1 min, 72 ℃ for 1 min, and then 72 ℃ for 10 min. Aseptic manipulation and negative control were used to prevent PCR contamination. The target product of PCR was recovered from 1.0% agarose gel using PCR fragment recovery Kit (Takara), and ligated into pGEM - T Vector, then transformed into the *E. coli* JM109. Positive recombinants were identified with blue/white screening. Sequencing was carried out on the Megabase 1000 sequencer with a dye - labelled dd - NTP system (Amersham Pharmacia Biotech).

1.4 Sequence analysis

Sequence alignment was performed using the MegAlign routine by Clustal Method in DNAStar package (DNASTAR, Inc., Madison, WI), and statistical analysis was performed with the DNAClub package (Xiongfeng Cheng, Ithaca, NY).

1.5 Construction of expression vector

Another pair of PCR primers (E1, 5′- GCGGATCCATGGGGGTGAGCGAGACAGCACCA - 3′; E2, 5′- GCGAATTCGGCCCGACCAGTCCG - 3′) were designed based on the initial sequencing results (PCR product 412 bp), tagging restriction sites of *BamH* Ⅰ and *EcoR* Ⅰ at the 5′ ends respectively. PCR amplification was performed to obtain the whole NT - 4 gene including the restriction sites. After the PCR product and pGEX - 4T - 3 (Pharmacia Biotech) were digested with *BamH* Ⅰ and *EcoR* Ⅰ, pGEX - NT - 4 was achieved with ligation, verified by PCR and enzyme digestion. The obtained pGEX - NT - 4 was then transformed into host bacterial *E. coli* BL21 cells.

1.6 Expression and purification of panda NT - 4 mature protein

A single recombinant colony containing pGEX - NT - 4 was picked out and inoculated in 5 ml of 2 ×YT medium (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl, pH 7.0), at 37 ℃ overnight with vigorous agitation. The culture was then diluted 100 times with fresh pre - heated 2 ×YT medium, and incubated at 28 ℃ with

shaking until the absorbance (600 nm) reached 0.6. IPTG was added to the cultural solution to a final concentration of 0.6 mM and incubation continued for an additional 2 hours. Bacterial collection and lysis was undertaken according to the GST Gene Fusion System Technical Manual (3rd Edition). GST - NT - 4 fusion protein was purified from the supernatant of the bacterial lysate using Glutathione Sepharose 4B, and digested with thrombin to remove GST. Expressed product was visualized on 10 % SDS - PAGE gel stained with coomassie brilliant blue.

1.7 Biological activity assay

The NT - 4 solution was sterilized by filtration through a 0.22 μm filter. The biological activity was assayed by observing neurite outgrowth from PC12 cells after the purified NT - 4 of the giant panda (10 ng) was added. Cells living in RPMI1640 with 15.0 % FBS were incubated at 37 ℃ in a CO_2 atmosphere. Blank assay was done with no NT - 4 added to the cells[15].

2 RESULTS

The DNA fragment including the sequence encoding the NT - 4 monomer and bilateral sequence was amplified successfully, using primers N1 and N2. The sequences determined from the 2 samples agree with each other completely. The result of alignment showed that the similarity of the whole precursor sequence between the panda gene and human homologue was 89.5 %. There were 41 base substitutions including 28 transitions and 13 transversions. In the deduced amino acid sequence analysis, only 6 sense mutations were found in all of the 41 mutations, with most of the amino acid substitutions occurring between two residues with similar side chains, that is, conservative substitutions. The monomer started after an Arg - X - X - Arg cleavage sequence, and the six Cys residues appeared in the same sites as the human protein. In addition, a threonine codon (including 3 consecutive nucleotides) was found inserted in panda NT - 4 sequence, and the mutation in 61st base introduced a new Cys residue (Fig. 1).

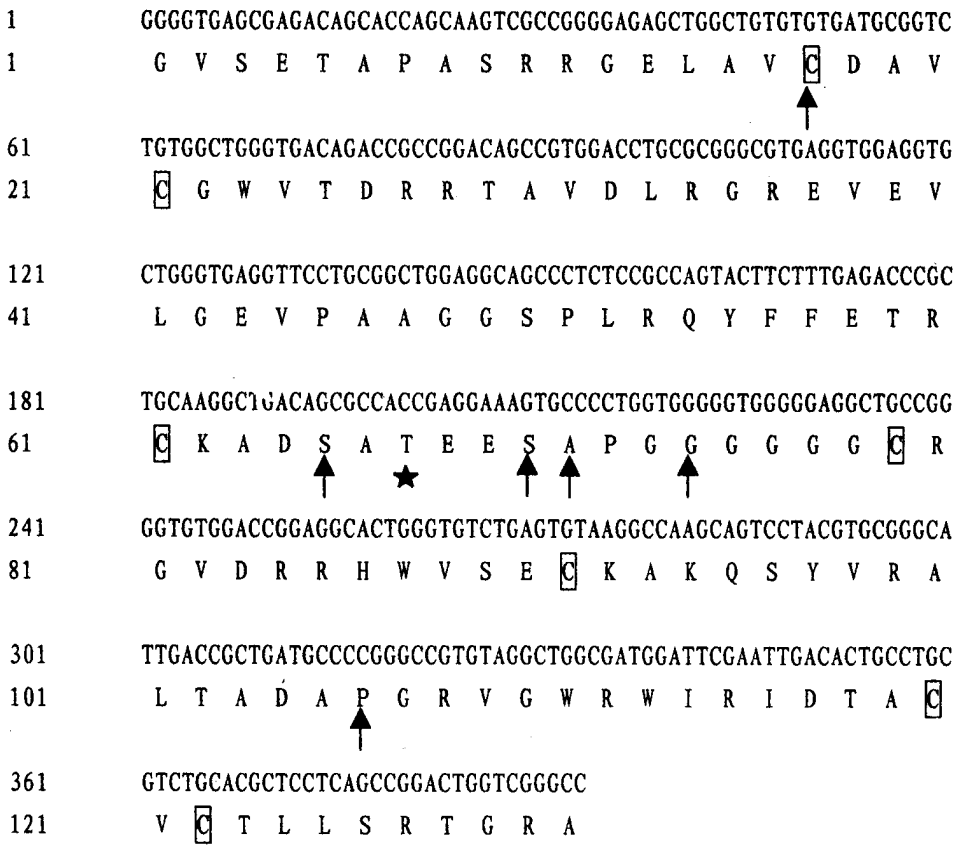

Fig. 1 Nucleotiode sequence and the deduced amino acid sequence of the NT - 4 monomer of the giant panda
The Cys residues are boxed; The star refers to the inserted Thr residue; The arrows refer to the sense mutations

Primer pair E1/ E2 introduced desired restriction sites of *BamH* I and *EcoR* I at the 5′ends of the NT - 4 gene successfully. After the PCR product and pGEX - 4T - 3 was digested with *BamH* I and *EcoR* I, the desired ligation forming pGEX - NT - 4 was achieved, and verified by PCR and enzyme digestion (Fig. 2). Induced by IPTG, the engineering bacteria (BL21/ pGEX - NT - 4) yielded the fusion protein GST - NT - 4 of 40 kD, agreeing perfectly with the expectation. Solubility analysis indicated the expressed fusion protein was partially soluble (Fig. 3). After digestion with thrombin and purification, we obtained panda NT - 4 at a concentration of 12 ug/ ml.

In the biological assay, PC12 cell division stopped following with augmentation of cell size. In addition, synapse was observed, which was most remarkable 72 hours after the recombinant protein was added. No synapse appeared in the blank control experiment (Fig. 4). The assays demonstrated that the purified NT - 4 protein exhibited normal activity, which is the first step in developing a comprehensive gene therapy for epilepsy in the giant panda.

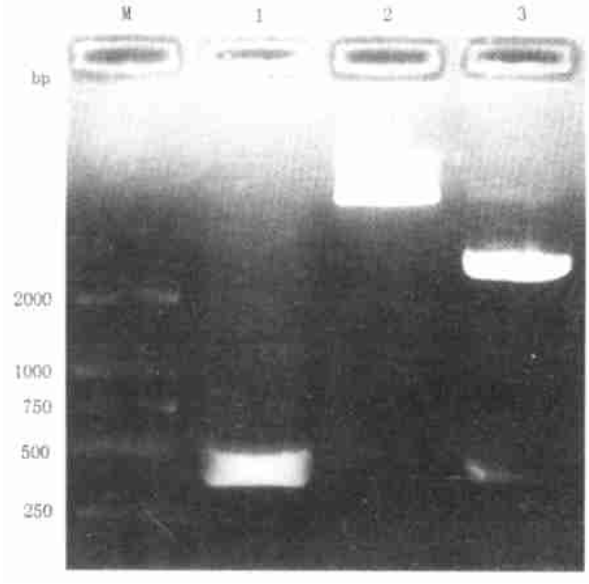

Fig. 2 PCR test and restriction map of pGEX - NT - 4
M. DNA molecular weight marker; 1. PCR product of NT - 4 (412bp); 2. pGEX - NT - 4; 3. pGEX - NT - 4 digested with *BamH I* and *EcoR I*

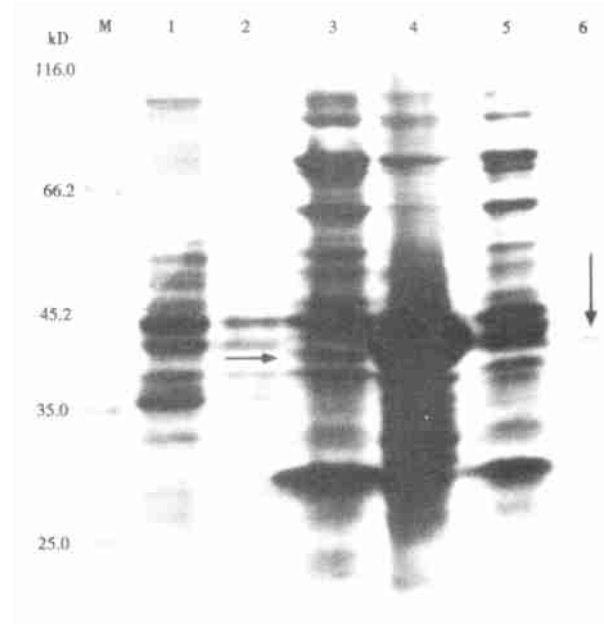

Fig. 3 SDS - PAGE analysis of the expression and purification of the giant panda NT - 4
M. Protein molecular weight marker; 1. Total cell lysate of *E. coli* BL21 containing pGEX - 4T - 3 with induction of IPTG; 2. Total cell lysate of *E. coli* BL21 containing pGEX - NT - 4 without induction of IPTG; 3. Supernatant of cell lysate of *E. coli* BL21 containing pGEX - NT - 4 with induction of IPTG; 4. Sedimentation of cell lysate of *E. coli* BL21 containing pGEX - NT - 4 with induction of IPTG; 5. Total cell lysate of *E. coli* BL21 containing pGEX - NT - 4 with induction of IPTG; 6. Purified NT - 4 of giant panda. The arrows refer to the expressed fusion protein GST - NT - 4

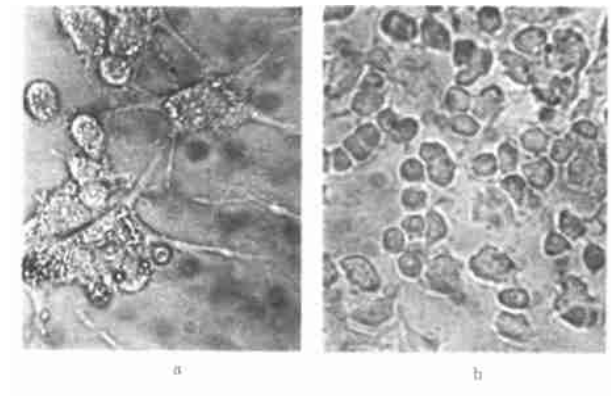

Fig. 4 Biological activity assay of the expressed NT - 4 of the giant panda
a: Synapse outgrowth from PC12 cells at three days after addition of NT - 4; b: No synapse outgrowth from PC12 cells in the blank control

3 DISCUSSION

NT - 4 belongs to the family of neurotrophic factors (NTs), which all play important roles in the differentiation, development, growth and regeneration of nerve system[16]. Different NTs work in different ways. The NT - 4

protein has two receptors, low - affinity p75 and high - affinity TrkB, although it mainly works through activating TrkB with autophosphorylation. The NT - 4 is found widely distributed in the central and peripheral nervous system[17,18]. During the development of nerve system, NT - 4 can be secreted not only by postsynaptic cells, but also by the neuron itself with autocrine[19]. In addition, the NT - 4 protein can also be obtained from the adjacent cells, such as astrocytes, oligodendrocytes and microglia, with paracrine. Previous studies indicated that the NT - 4 could protect both hippocampal and cortical neurons from damage by excitation toxin and traumatic brain injury[10,11]. However, it is cumbersome and time - consuming to extract NT - 4 from panda tissues due to limited quantities. Therefore, it is necessary to produce NT - 4 through genetic engineering methods.

Epilepsy is a group of related clinical syndromes, involving abnormal neuroelectrical activity. This heterogeneity poses many difficulties for therapy and prevention. Studies found that seizure could induce the expression change of the NTs and their receptors (especially BDNF, NT - 4 and their common receptor trkB) in the prosencephalon. This suggests that the NTs could play important roles in the control of seizure[20,21]. Thus, NT - 4 is potentially significant to cure the epilepsy if a proper form and method of application is developed.

In our study, the gene coding for the NT - 4 was cloned and expressed aiming at the epilepsy of the giant pandas. Sequence analysis showed that only six sense mutations occurred in the 41 base substitutions, compared with the mature panda NT - 4 protein of human. The expressed product in *E. coli* was partially soluble and the activity determined by PC12 cell line was definite. On this condition, extrinsic NT - 4 could be used directly, or the NT - 4 gene could even be implanted with gene therapy to accelerate nerve regeneration and induce the extension of damaged synapses. As a result, the damage resulted from the epilepsy could be recovered. The successful expression of recombinant NT - 4 with intact biological activity now makes gene therapy for the giant panda possible for the first time.

Panda is so sensitive to stimulation from environment. Panic, loudness and some other strong stimulation can all lead to the attack of epilepsy. This study of NT - 4 of the giant panda aims to make foundation for the gene therapy of the panda epilepsy. We have obtained recombinant NT - 4 protein of the giant panda with biological activity *in vitro*. Further studies will be carried out to test the protective and restorative effects of the NT - 4 on some model animals, like mouse that suffers from similar disease.

Present studies of the giant panda mainly focus on rearing, reproduction, and behavior, while very few efforts on functional genes have been reported[21]. Above all, this study may help enrich the conservation genetics studies related to the giant panda.

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