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Science Letters:

Construction of a eukaryotic expression plasmid of Humanin*

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Abstract: Objective: To construct a eukaryotic expression plasmid pcDNA3.1(-)-Humanin. Methods: The recombinant plasmid pGEMEX-1-Humanin was digested with restriction endonucleases BamH I and Hind III and the Humanin gene fragments, about 100 bp length, were obtained. Then the Humanin gene fragments were inserted into eukaryotic expression vector pcDNA3.1(-) and the recombinant plasmids pcDNA3.1(-)-Humanin were identified by sequencing. Results: Recombinant plasmid DNA successfully produced a band which had the same size as that of the Humanin positive control. The sequence of recombinant plasmids accorded with the Humnain gene sequence. Conclusions: A eukaryotic expression plasmid of Humanin was successfully constructed.

Key words: Humanin, Alzheimer's disease, Eukaryotic expression
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INTRODUCTION

Alzheimer's disease (AD) is one of the most common causes of senile dementia and is a multi-gene hereditary disease. The typical symptomology of AD is characterized by gross and progressive impairments of cognitive function and often accompanied by behavioural disturbances such as aggression, depression, and wandering. AD is characterized by neuronal loss and neurofibrillary tangle formation in circumscribed regions of the neocortex and hippocampus, primarily affecting pyramidal neurons and their synapses. In recent years, some peptides, such as presenilin 1 (PS1), presenilin 2 (PS2) and amyloid precursor protein (APP), were found related to AD by molecular genetics. The mutation of the peptides may increase β-amyloid (Aβ) level and further extracellular deposits of Aβ, which forms the amyloid plaque and leads to the death of neurocytes. Hashimoto *et al*.(2001b; 2001c) reported that a short peptide, named Humanin, was isolated from the brain of AD patients. Humanin had significant protective action on the neurocytes death induced by APP, PS1, PS2 and Aβ. The neurocyte protective mechanism of Humanin was not yet known. In order to study the protective mechanism of Humanin on AD and develop new anti-AD drugs, we will clone the Humanin gene into the eukaryotic expression vector on the basis of plasmid pGEMEX-1-Humanin we constructed previously, thus establish a fundamental basis for study on the action of Humanin and on clinical gene therapy for AD.

MATERIALS AND METHODS

Materials

1. Bacterial strains and plasmids: The plasmid pGEMEX-1-Humanin constructed in our lab included

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the Humanin gene fragment and was identified by sequencing (Luo *et al.*, 2004). Eukaryotic expression vector pcDNA3.1(-) and *E. coli* strain DH5α were provided by the Institute of Infectious Diseases, Zhejiang University.

2. Enzymes and kits: Plasmid DNA purification kit and gel extraction kit were purchased from Qiagen Incorporation and restriction endonuclease was from Fermentas Incorporation. T4DNA ligase was purchased from Roche Company, and Taq DNA polymerase and 100 bp DNA Ladder were both from Promega Company.

3. Primers:

Up-stream primer of Humanin:

5′>ATTGGATCCATGGCTCCACGAGGGTTCAG<3′

Down-stream primer of Humanin:

5′>TGGAAGCTTTTATGCCCGCCTCTTCACGG<3′

Primers were synthesized by Shanghai Shengyou Biological Engineering Corporation.

Methods

1. Amplification and enzymatic digestion of plasmid: pGEMEX-1-Humanin plasmids were amplified and purified and then were digested with restriction endonucleases BamH I and Hind III. The products were separated on agarose gel and the purified Humanin DNA fragments were obtained by gel extraction kits. The purity and concentration of Humanin DNA fragments were verified by gel electrophoresis. The pcDNA3.1(-) vectors were treated in the same way.

2. Clone of Humanin: Vector pcDNA3.1(-) and Humanin DNA fragments were ligated at molar ratio of approx. 3:1 by T4 DNA ligase at 20 °C for 30 min. Competent *E. coli* strain DH5α was transformed with 5 μl ligation reaction and plate aliquots of the transformation reaction on LB agar containing 50 μg/ml ampicillin and the cultures were incubated at 37 °C for 16 h. Appropriate clones were selected and the inoculate cultures were incubated overnight at 37 °C to obtain a saturated culture. Recombinant plasmid DNA was extracted and purified and PCR was performed to analyze Humanin up and down stream primers according to the following cycle program: 95 °C 3 min, 1 cycle; 94 °C 1 min, 60 °C 90 s, 30 cycles; 72 °C 7 min, 1 cycle. PCR amplification products were fractionated by electrophoresis through agarose gels and were analyzed on a UV table after ethidium bromide staining.

3. Identification of recombinant plasmids: The recombinant plasmids were sequenced by Amersham MegaBace^{TM} 1000 DNA Analysis System.

RESULTS

PCR screening of recombinant plasmids

Recombinant plasmid DNA was amplified by PCR analysis using Humanin primers and the product was identified by running it on the agarose gel. The result is shown in Fig.1.

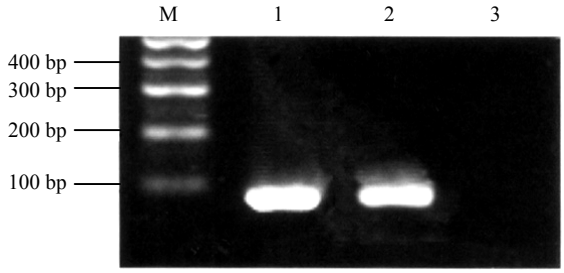

Fig.1 PCR screening of recombinant plasmids. M: 100 bp Marker; 1: Humanin PCR product; 2: Positive control; 3: Negative control

Identification of recombinant plasmids by sequencing

Two recombinant plasmids were identified by sequencing with the results according with what was expected. The sequence listed below:

ATGGCTCCACGAGGGTTCAGCTGTCTCTTAC-TTTTAACCAGTGAAATTGACCTGCCCGTGAA-GAGGCGGGCATAA

DISCUSSION

Humanin is a short linear peptide that distributes primarily in the heart, kidneys, liver and skeletal muscle. The content of Humanin in human brain and stomach is relatively low, but it has vital physiological action. Humanin was cloned from a cDNA library extracted from surviving neurons of the occipital lobe of Alzheimer (AD's) brain. Some researchers considered that the protective genes are expressed by the

occipital lobe of Alzheimer brain, although the occipital lobe is usually not involved in AD patients (Niikura *et al.*, 2002).

The following characteristics make Humanin the key player in the neurocyte protection of AD patients: (1) Cell toxicity induced by Aβ is related to the disorder of cell membrane stability and cell calcium channel. Humanin may combine with different receptors in the cell membrane and partially inhibit the toxic ultrastructure induced by Aβ and stabilize the cell membrane. Humanin may also interact with Aβ1-40 to reduce the neuronal calcium reflux by Aβ (Zou *et al.*, 2003); (2) Humanin and S14G-HN (HG) may prevent death of neuronal cells by Aβ and family Alzheimer's disease (FAD) mutants of APP, PS1, and PS2 with the effective concentration being as low as 10 nmol/L; (3) Humanin cDNA encodes a chemically synthesizable short polypeptide that acts on cells from the outside (Hashimoto *et al.*, 2001a; 2001c); (4) The activation of Humanin is regulated by the D-Ser and the Humanin with D-Ser at position 14 exerts more potent neuroprotection (Terashita *et al.*, 2003); (5) Humanin or HNG has no effect on genes caused death linked to Huntington's disease, spinocerebellar ataxia or familial amyotrophic lateral sclerosis (Hashimoto *et al.*, 2001c).

Humanin is gradually being recognized by researchers all over the world for its specific action on the genes related to AD and its neurocytes protection. It is hoped that Humanin and its derivatives can be used to develop new drugs for sporadic AD and FAD in the future. As Humanin is a 24aa short linear peptide, it will be very costly to chemically synthesize it. Eukaryotic expression plasmid contains promoter, enhancer and polyadenylation sequences and may be expressed transiently in the eukaryotic cell. It is a useful, simple and effective approach to study the functions of new genes. In this work, we successfully cloned Humanin gene fragments into the eukaryotic expression plasmid pcDNA3.1(-) to provide the basis for the development of new anti-AD drugs and for genetic treatment for AD.

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