Construction of Recombinant Vector Containing Fusion Gene NT4–Ant–Shepherdin

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ABSTRACT **Objective** To investigate Survivin as a anticancer therapeutic target by use of Shepherdin[79 - 87] ,a novel peptide carrying the Survivin sequence from Lys-79 through Leu-87,we constructed an recombinant vector containing fusion gene NT4-Ant-Shepherdin [79 - 87]. **Methods** The gene of Ant-Shepherdin [79 - 87] was obtained by PCR and T-vector method. After cloned and digested with restricted enzyme, Ant-Shepherdin [79 - 87] was inserted in PBV220/NT4 vector. The recombinant vector was transformed into the competent cell, E.coli DH5α. The fusion gene of NT4-Ant-Shepherdin [79 - 87] was identified by agarose gel electrophoresis. **Results** DNA sequencing results verified that the sequence of Ant-Shepherdin[79 - 87] was consistent with that we had designed. After transformed E.coli DH5α, a fragment of 321bp was confirmed. **Conclusion** The recombinant vector containing fusion gene NT4-Ant-Shepherdin [79 - 87] was successfully constructed in this experiment by molecular biology techniques, which provided the basis of further research of Survivin for cancer gene therapy.

Key words Survivin; Ant; NT4; Fusion gene; Vector

Survivin is one of important members of inhabitor of apoptosis protein (IAP). Ambrosini et al screened cDNA using EPR-1 in human genomic library and cloned a new gene being named apoptosis protein inhabitor 4 (API4) in 1997 [1]. The human Survivin gene spans 14.7 kb on the telomeric position of chromosome 17.It codes 16.5KD cytoblastema protein containing 142 amino acids . Survivin expression was found to be absent or low in most terminally differentiated tissues while strong survivin expression in most human solid tumor types and hematologic malignancies [2]. Survivin plays an important role in inhibiting apoptosis [3], controlling mitosis ,participating cell proliferation[4], regulating tumor angiogenesis and so on [5]. Survivin takes a cross action in a variety of network system as a oncogene. In recent years, considerable efforts have been made to validate Survivin as a new target in cancer therapy, including: anti-sense oligonucleotides, "dominant-negative" mutants, hammerhead ribozymes, small interfering RNAs (SiRNA), cancerVaccine/immunotherapy, small molecule antagonists and so on[6]. The recent studies have found that high expression of Survivin is related to the high expression of heat shock protein (Hsp90) in tumour cells. Hsp90 is a molecular chaperone . The Survivin - Hsp90 complex prevented proteasomal degradation of Survivin in cancer cells[7]. Survivin binding domain located at its Lys-79 to the Leu-87 region, thus, the researcher designed a cell-permeable peptide derived from the Survivin sequence Lys79 - Leu87, Shepherdin [79 - 87]. Shepherdin [79 - 87] competitive interfered Survivin combining with Hsp90 and promoted degradation of Survivin as a antagonist. Experiments have shown a good anti-tumor effect, with no obvious side effect[8]. On the basis of these findings, we designed a fusion gene, NT4-Ant-Shepherdin [79-87], which contains cell-penetrating peptide and signal peptide functions. We hope that it can realize the genetic therapy taking Shepherdin as anti-tumor target peptide by constructing this fusion gene.

MATERIALS AND METHODS

Materials

pGEM-T-Easy plasmid (50ng/μL)vector by Promega corporation ; restricted enzyme Nae I、BamH I、EcoR I,Taq DNA polymerase, T4 DNA ligase and so on by Huamei Bio-engineering Company; pBV220-NT4plasmid ,escherichia coli TOP10、E.coli DH5αs-

train provided by Xi'an Huaguang Bio-engineering Company.

Methods

Design of fusion gene Ant–Shepherdin[79 - 87]

Designed fusion gene based on human Survivin 79-87 amino acid sequence KHSSGCAFL and cell-penetrating peptides Ant(Antennapedia)analogues amino acid sequence GRQVKVWFQNRRMKWKK and preferred codons of mammalian.

Design and Synthesis of primer

Designed positive and negative primer/template strand using asymmetric primer/template method. Added NaeI restriction site to 5-end of positive primer while added terminator and BamH I restriction site to 5-end of negative primer. Primer synthesis completed by Sanbo Yuanzhi bio-engineering Limited Company of Beijing and purified by PAGE . Designed primers by DNASIS software as follows :

Positive primer / template F:

5′-C GCCGGC GTGGG AAG AAG TGG AAG ATG CGC CGG AAT CAG TTC TGG GTA AAG GTA C;

Negative primer /template R:

5′- C GGATCC TCA AAG GAA AGC GCA ACC GGA CGA ATG CTT TCC GCG CTG TAC CTT TAC C

cDNA Clone and Sequence Analysis

Amplified target gene by PCR .Reaction conditions were as follows : 94℃denatured 60s, 37℃annealed 60s, 72℃extended 80s,30 cycles later , 72℃ extended 5min .Took 5μL PCR reaction products and identified by agarose gel electrophoresis (AGE). Ant- Shepherdin[79 - 87] was inserted in pGEM-T-Easy vector. The recombinant vector was transformed into the competent cell, TOP10.Small amount of plasma was extracted by alkaline lysis method. After enzyme digestion analysis by EcoRI , pGEM-T positive clones were selected. DNA sequencing was done by Shenggong bio-engineering Company of Shanghai .

Construction of pBV220/NT4–Ant–Shepherdin [79–87] vector

Recombinant plasmid pGEM-T/ Ant-Shepherdin [79 - 87] was extracted by alkaline lysis method ,digested by BamH I after Nae I digestion. Ant-Shepherdin[79 - 87] was separated by gel electrophoresis and eluting . Using T4 DNA ligase, Ant-Shepherdin [79 - 87] was inserted in pBV220-NT4 plasmid vector , which digested by Nae I and BamH I , carrying the signal peptide of neurotrophic factor 4 (NT4).The recombinant vector was transformed into the competent cell, E.coli DH5α,then inoculated with LB culture media containing ampicillin.Small amount of plasma was extracted by alkaline lysis after culture at 37℃, digested

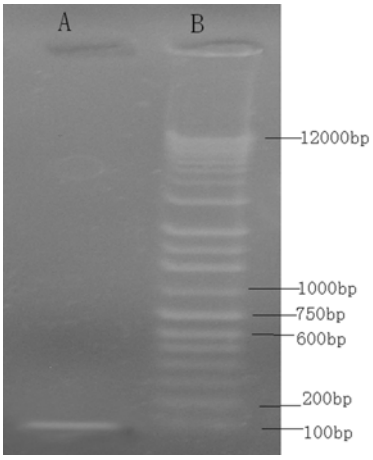

Fig.1 PCR product of Ant-Shepherdin[79 - 87]
A. Ant-Shepherdin[79 - 87]
B. HBI 1.0Kb plus DNA ladder

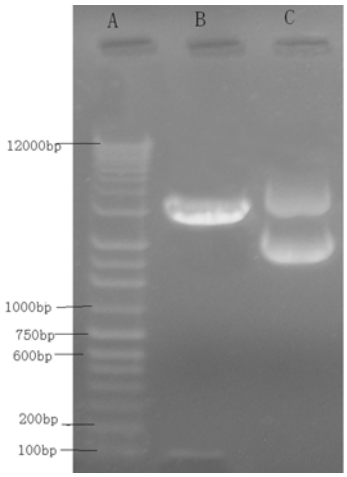

Fig.2 Restrictive enzyme analysis of pGEM-T/ Ant-Shepherdin [79 - 87] by EcoRI
A. HBI 1.0Kb plus DNA ladder B. vector digested by EcoR I
C. pGEM-T easy vector

by EcoR I and BamH I , identified by agarose gel electrophoresis (AGE). Target band for 321bp, the recombinant plasmid pBV220/NT4–Ant–Shepherdin [79–87] is selected.

RESULTS

PCR products

Compared PCR products to PCR molecular weight marker , a 100bp band of fusion gene Ant–Shepherdin [79 – 87] was obtained by AGE .The result corresponded with the theoretical value (as Fig . 1)

Enzyme Digestion of recombinant plasmid pGEM–T/ Ant–Shepherdin[79 – 87]

The recombinant plasmid pGEM –T/ Ant –Shepherdin [79 – 87] spans 3115bp.We can see target fragment of 100bp after EcoRI enzyme digestion and AGE . The result corresponded with the theoretical value .It showed that the positive recombined plasmid had been cut completely and Ant–Shepherdin [79 – 87] recombined in pGEM–T Easy successfully (Fig.2).

Nucleotide sequence analysis of Ant–Shepherdin[79 – 87] chimeric pepetide cDNA fragment

Selected the Ant –Shepherdin [79 – 87] chimeric pepetide cDNA fragment sequence from DNA sequencing ,compared to the fusion gene using DNASIS software .The cloned cDNA fragment from Nae I enzyme sites to stop codon was 100bp and had the same sequence.

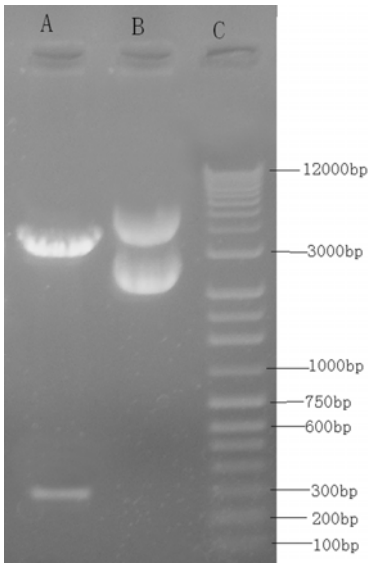

Fig.3 Restrictive enzyme analysis of pBV220/NT4 –Ant – Shepherdin[79 – 87] by EcoRI and BamH I

A. vector digested by EcoRI and BamH I

B. pBV220 vector

C. HBI 1.0Kb plus DNA ladder

Enzyme digestion of pBV220/NT4–Ant–Shepherdin [79–87]

The cloned recombined plasmid of 3906bp named pBV220/NT4–Ant–Shepherdin [79 – 87] . In theory, by the restriction enzyme EcoR I and BamH I , pBV220/NT4–Ant–Shepherdin [79 – 87] should have 3666bp and 321bp fragments. After 10g/L AGE , it showed the size of the recombinant plasmid and restriction fragments corresponded with the theoretical value and suggested that NT4 signal peptide and Ant–Shepherdin[79 – 87] had successfully combined(as Fig.3).

DISCUSSION

Survivin had become the new target in diagnosis and treatment of tumor at present by its specificity of expression and higher expression rate as well as its important roles such as controlling cell proliferation, anti – apoptosis and promoting angiogenesis . In a short period,many targeting Survivin strategies had been confirmed quickly ,some of them even entered into the clinical study .Survivin antagonist provided a new idea and resulted in the specific deactivation of molecular channel relating to tumor survival .It also used to all kinds of tumor.

Shepherdin [79 – 87] had good anti–tumor effect and no significant toxic and side effect as a protein drug interfering Survivin combining with Hsp90. Protein transduction domain (PTDs), that is cell–penetrating peptides (CPPs) is a new and effective way that transport the exogenous macromolecules or charged compounds into the living cells. The common PTD is HIV TAT(47–57), Antennapedia(43–58), HSV VP22, poly arginine and PTD –5.They are all positively charged short peptides and are rich in arginine and lysine. The mechanism of protein transduction may be related to some endocytosis channel [9].It can increase the Shepherdin [79 – 87] effect by adding Ant (43–58) protein .

However, there still exist some problems such as short biological half−life, easy to degradation, low bioavailability, difficult to achieve effective dose of concentration in vivo tumor and less expensive which limit its application .

Studies also showed that expressing proteins and peptides by gene engineer can clone foreign gene in a proper expression system and transcript, translate, process and purify in suitable host cell or transfect to mammalian cells. It can realize the secretory expression of foreign protein, prevent the host cell to degradate expression product, restore the natural conformation of product, and secret the target protein to extracellular to kill the nearby untransfected tumor cells for enlarging the treatment effect. The lack of study on secretory expression of short bioactive peptides mainly resulted from that signal peptide and target short peptide must keep the same sequence between the connected part and the amino acid sequence that peptidase recognized . In the laboratory, we have successfully applied the signal peptide sequence and leading sequence of neurotrophic factor 4 (NT4) to realize secretion and expression of short bioactive peptide in the eukaryotic cells[10]. The recombinant vector containing fusion gene NT4−Ant−Shepherdin [79 – 87] was successfully constructed in this experiment by molecular biology techniques, which provided the basis of further research of Survivin for cancer gene therapy.

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