Cloning and Sequence Assessment of VP3 Gene in Chicken Anemia Virus

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Abstracts **Purpose** Cloning the chicken anemia virus VP3 gene from the chicken´s bursa of Fabricius for the aim of isolating the tumor-specific-apoptosis protein. **Methods** To constructe and synthesize a pair of primers according to the Genbank concerning sequences, the DNA template was extracted from the chicken´s bursa of Fabricius at a poultry yard in Xi´an suburb. the cloning product was amplified specifically by PCR and inserted into the vector of pGEM-T-easy. transformed E coli DH5α was used to replicate this recombinated vector, then isolate the recombinated vector, enzymolysised and detected with restriction enzymes, the sequence was assessed by DNA sequencer, the practicing software of DNAsis was employed to confirm the consistence between our cloning and the one from genbank. **Results** Sequencing assay indicated that we succeeded to clone the VP3 gene (apoptin gene), comparing with the genbank, our sequence is completely in agreement with the report of ShanDong; s (GenBank AY 171617), and differential from the GenBank AF313470 in 6 sites. **Conclusion** Apoptin may make a foundation for the tumor therapy by inducing tumor specific apoptosis.

Key Words Chicken Anemia Virus; Apoptin; Clone; assessment of Sequence

Tumor, especially malignant tumor has been becoming one of the diseases threatening human health seriously. in the 90s of last century, many advances were made on inducing tumor cell apoptosis and cancer cell gene therapy. Scientists found that apoptosis is closely associated with many tumor physiological and pathogenic processes, such as: tumorigenisis, transformed cell eliminating, cell killed response, these properties make apoptin a potential agent for cancer therapy.

CAV, a circular virus, found by Yuasa in Japan in 1979 [1], can cause young chicken to death of anemia, the genome, long of 2.3kb [2], can encode three proteins of VP1, VP2, VP3 respectively, which of VP3 is functional protein that is the key pathogenic factor and can induce the cyto-apoptosis in infected young chicken, so it is named as apoptin. In recently, scientists have made great progress in the mechanism of apoptin inducing tumor apoptosis. Apoptin can induce apoptosis in tumor and transformed cells specifically [3], but not in normal cells, whereas can not be inhibited by p53 and over expression of Bcl-2. [4] so apoptin might be represent the highly expectation agent for tumoricidal specifically.

The objective of our study is to isolate and purified CAV DNA from the infected tissue in the yard chickens, emplify and extract the VP3 gene by the method of Polymerase Chains Reaction (PCR), then insert this segment into the PGEM-T plasmid vector. The sequences were analyzed by doubling -deoxygenated termination, we confirmed that we acquired the CAV VP3 gene clone comparing to the Genebank database, the successful cloning of the CAV VP3 gene made a foundation for further imploring the effect of apoptin tumor therapy.

MATERIALS AND METHODS

Experimental animals: 4 three months young female chickens weigh less than 1400 gram, purcased from a suburb poultry yard in Xi´an; the plasmid PGEM-T-easy, E coli DH5α were provided by Hua Guang Bio-Engieering Company.

Reagents: thermo-resistance Taq-DNA polymerase, Nae Ⅰ, Hind Ⅲ, DNA polymerase, T4-DNA ligase, proteinase-K, DNA marker were purchased from the Hua Mei Bio-Tech Company,

E. Coli DH5α from Hua Guang Bio-Engieering Company.

Apoptin gene clone

Isolation of CAV DNA: 4 yard young chickens, with anemia indications, were bleed to death through jugular vein, anesthesized with bentobarbital as usual. The chicken bursa of Fabriciuses were excised immediately, and snap-frozen in liquid nitrogen. 100mg the above freezed tissue was grinded into homogenate in 1ml common buffer (50mM Tris-HCl, 100mM NaCl 2mM EDTA pH 8.0). SDS and proteinase K were dropped into 500ul homogenate at the final concentration of 0.1% and 200ug/ml respectively. incubated and digested at 37℃ for 120 minutes, then equal volume phenol and chloroform were added to the digested solution for extracting the target gene. Recover the supernatant and after adding RNase, the solution was incubated at the temperature 37℃ for 60 minutes. extracte the solution with phenol and chloroform once again, twofold volume dehydrated alcohol add to the collective supernatant, the precipitation was washed with 70% alcohol, after dry completely, and dissolved in 100ul TE storing until use.

Primer designation: design the CAV sequence emplified primers. the forward primer (5'-CGG CCG GCG TGG GAT GAA CGA TCT CCA AGA AGA TAC-3'), and the reverse primer (5'-CAA GCT TCA GTC TTA TAC GCC TTC TTG CGG TTC-3')were synthesized according to the reported from Genebank which containing restriction enzyme sites of Nae I and Hind III in the 5′-terminal in the primers expressed in bold italic. The protective basio-CG and C were inserted into the forward and reverse primer, in additional, terminator was deleted in the reverse primer 3′-terminal for the expression of apoptin correlative fusion proteins.

PCR response

The apoptin gene was amplified by PCR, the procedures were as following: predenaturation first, at 94℃ 5min, then denaturation 94℃ 60s, annealing 60℃ 60s, polymerization 72℃ 90s, 30 cycles, elongation at 72℃ 5min.

DNA recover and analysis

The PCR product was ligate to the linearized vector PGEM-T, replicate small-scale the vector in transformed E coli DH5α. after lysis by alkali, apoptin DNA was recovered by agarose gel electrophoresis and confirmed by sequencing.

Screen and detect the positive recombination destinated segment

Transformed E coli DH5α was incubated in LB plates containing X gal and IPGT. The white clone was picked out, the PGEM-T-Apoptin vector was replicated emplified in E coli DH5α, recovered by agarose gal electrophoresis.

Sequence analysis

The recombinated cloning sequence was sent to Shang Hai JiKang Company for measuring. the cloning CAV VP3 gene fragments and its encoding protein were analysised with DNAsis 2.5.

RESULTS

PCR result analysis

The PCR product was confirmed by agarose gel electrophoresis, the amplified fragments were consistent with the target ones. the results were showes Fig.1.

Restriction analysis of recombinated plasmid PGEM–T/Apoptin

A larger fragment (2659bp) and a small fragment (393bp) were harvested by digested with restriction enzyme and agarose gel electrophoresis. the desired sequence was identified with the target fragment. the results were showed in Fig. 2.

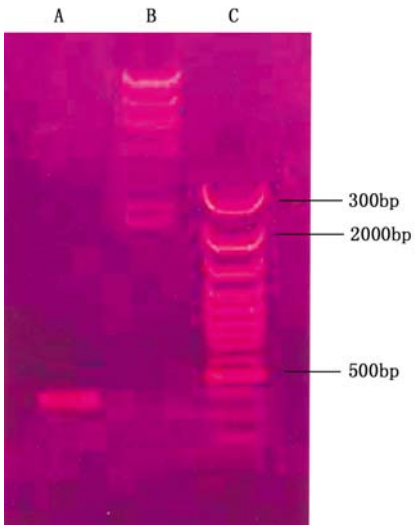

Fig.1 Result of apoptin PCR product
A. Amplified PCR product
B. DNA/Hind Marker
C. 100bp DNA marker

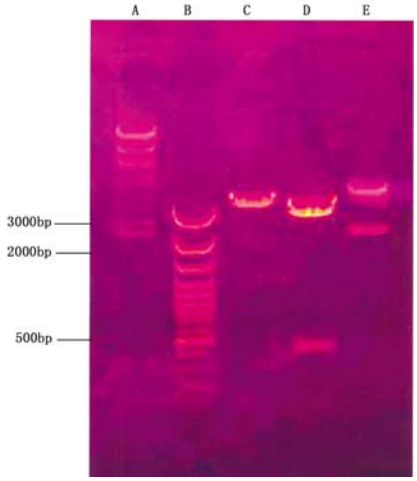

Fig.2 Restriction analysis of recombinated plasmid PGEM–T/Apoptin A. DNA/Hind Marker B. 100bp DNA Marker C. Plasmid PGEM–T–easy D. Replicated recombinated plasmid PGEM–T/Apoptin E. Recombinated plasmid PGEM–T/Apoptin

Analysis of apoptin protein primary structure

The CAV VP3 protein primary structure sequences deduced by cloning apoptin gene have 6 nucleus mutational sites compared with the reported from Shan Dong (AY 171617), whereas identified completely with the sequences from GenBank (AF 313470). see Fig. 3.

DISCUSSION

Apoptin, derived from CAV (chicken anemia virus), can induce apoptosis in tumor cell and transformed cell specifically, but not in normal cell, and VP3 can not be blocked by overexpression of Bcl-2. So apoptin might be served as a useful model for cell apoptosis research, moreover may be a high expectation candidate for new anti-tumor drug.

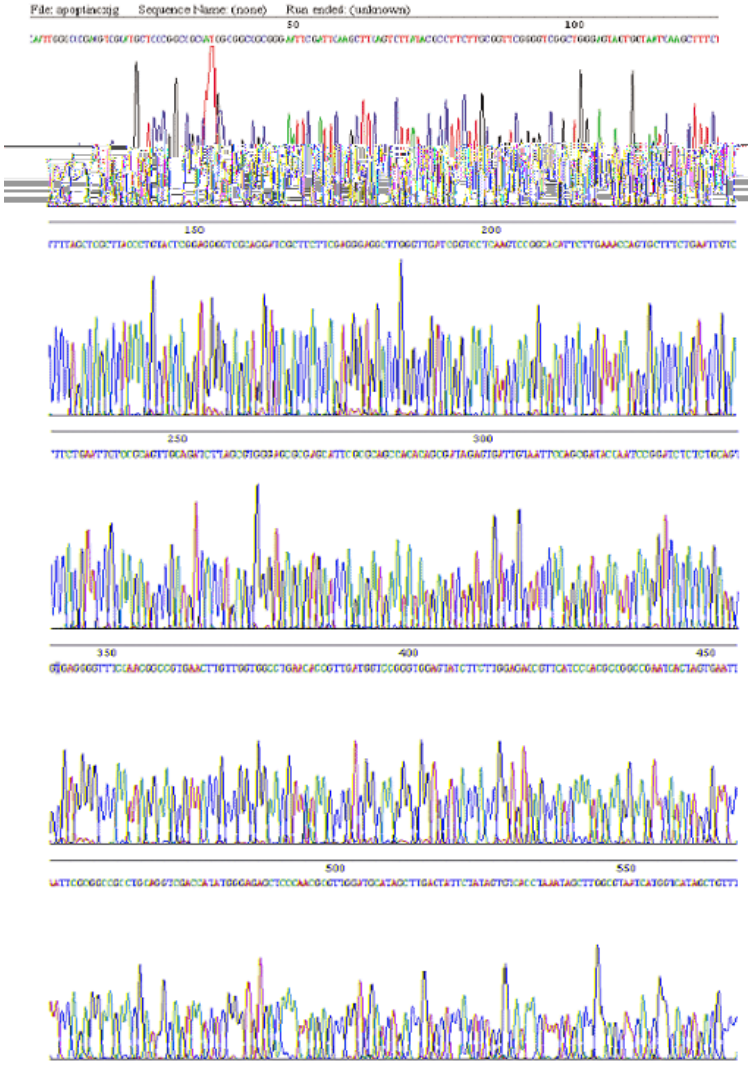

Fig.3 CAV VP3 gene sequence

Cell apoptosis, namely cell programmed death, is the mainly form of cell senescence or death procedures and differential from cell pathogenic death, no accompanying inflammatory reactions. Apoptin induced apoptosis is characterized with the spherical shaped, blebbing structure cellular membrane and the strongly condensing DNA in the nucleus. Somewhat later, the cell disintegrated into several fragments, becoming so-called apoptotic bodies. These bodies are taken up and digested by neighboring cells and macrophages, which prevent the release of cellular debris that cause inflammatory reactions in the body.

To identify whether our sample is the target apoptin gene, our cloning segment is presented to the GenBank, it is found that there is 98% homologous of gene between our sample and the 46 strains engaged in GenBank, moreover, about the proteins primary structure deduced by the apoptin gene opening reading frame (ORF), there is also 82% identical amino acids sequences with the 29 strains from GenBank, so it is confirmed that our cloning segment is apoptin gene indeed. CAV is highly conservative with only one serotype, a series of studies reported that it is almost half of Cys, the other of Arg at the 118th site, some experiments showed that in case of Arg in place of Cys, apoptin activity takes a great changes with lower reproductive efficiency, decreasing nucleus localization ability leading apoptin to localized at cytoplasma predominantly, then resulting in impairing apoptin inducing tumor cell apoptosis activity. Our cloning apoptin VP3 gene encoding protein reveal highly activity because of Arg at the 118th site in the sequence. Differential from many other functional protein, the mechanism of apoptin inducing cell apoptosis may be related or connected with other compenents at a form of ectopic and homologus multimerization. Leliveld[5] et al. Constructed a soluble recombinant fusion MBP-apoptin protein by connecting maltose binding proteion (MBP) with apoptin.Repeated experiments confirmed that apoptin exited in a stable, uniform and active globular mutimeric aggregates in vivo comprising 30 to 40 subunits. Zhang[6] et al. ever experienced in way of microinjecting MBP-apoptin into tumor and normal cells, MBP-apoptin was shown to be highly efficiency and specificity. However in normal cells, cell epitope was protected by unknown mechanism, MBP-apoptin forms heavily multimers, disappear and enzymolysises in cytoplasma eventually[7]. Many reports demonstrate that CAV derived apoptin can induce specifically apoptosis in tumor or transformed cell but not in untransformed cell, moreover, it can act function at a state of absence of tumor-inhibitor p53 and no repression of bcl-2 overexpression. A series of experiment reveal that apoptin appear no toxic and no transformed activity to normal cell through a long term expression in human fibroblast cell, and not hurt the cell life span.[8] These properties further enhance the safety for apoptin as tumoricidal agent, in the meantime, may represent a highly expectation for solving the contradiction between high efficiency and specificity. having been received widely attention, apoptin may be a promising new tumoricidal drug accompanying with huge social and economic efficiency.

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