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Study on the mammalian cell lines of stable expression strain matrix protein of rabies virus CTN-1V

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ABSTRACT

Objective: To construct CHO cell line stably expressing rabies virus matrix protein. **Methods:** RT-PCR was used to amplify and isolate CTV-1V gene of rabies virus. After cloning into pCDNA5.0FRT vector, the recombinant plasmid pCDNA5.0FRT-M was constructed and then transfected into CHO cells with POG44 plasmid. The positive clones were screened by hygromycin B and the stable cell lines were identified by indirect immunofluorescence and Western blot. **Results:**After enzyme digestion and DNA sequencing, the recombinant expression plasmid pCDNA5.0FRT-M were transfected into CHO cells, get the visible positive cell clones, scraping positive clones were expanded in culture and defined as the second generation, After 10 generations, the results were still positive.**Conclusion** The CHO cell line stably expressing rabies virus matrix protein was successfully established, which lays a foundation for further study of the function of the matrix protein.

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Introduction

Rabies virus (RV) is a neurotropic virus that causes fatal disease in human and animals. Its genome is a single stranded, non-segmented negative strand RNA virus, encoding nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and transcription protein (L). M protein is located on the inside of the envelope membrane of a connexin, could be connected capsule and nucleocapsid. There exists an epitope between the M protein genes in 1-72 amino acid residues, associated with Immune response with RV. M protein plays an important role in maintaining the bullets morphology of rabies virus [1], and plays an important role in viral assembly and budding [2]. In

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addition, regulate the transcription and replication of the virus[3], And is associated with the induction of early apoptosis[4].

In order to study the function of rabies virus M protein. RT-PCR was used to obtain the full-length gene of rabies virus CTN-1V strain, cloned into pCDNA5.0FRT vector. pCDNA5.0FRT-M recombinant expression plasmid and POG44 plasmid were cloned into the CHO cell line and screened by hygromycin B to obtain the engineered cell lines that stably expressing M Protein of Rabies Virus CTN-1V Strain. The details are as follows.

Materials and methods

1. Reagents and Tools

Virus RNA extraction kit and reverse transcription kit were purchased from Invitrogen Corporation, USA.

Plasmid extraction kit was purchased from Qiagen Company, USA.

High-fidelity DNA polymerase, restriction endonuclease Nhe I and Not I and T4 DNA ligase were purchased from USA NEB company.

DNA fragment recovery kit, DNA molecules Marker DL2000, DL15000, were purchased from Dalian TaKaRa company.

IDEM medium purchased from the United States Gbico company, other conventional chemical reagents are of analytical grade.

2. Plasmids, strains, strains and cells

Plasmid pcDNA5.0FRT and CHO-FRT cell lines were purchased from Invitrogen Corporation, E. coli DH5α strain was Purchased from Sangon Biotechnology, rabies virus CTN-1V strain from the Chinese Academy of Food and Drug Administration.

3. Total RNA extraction and amplification of the full-length gene of M protein

Genomic RNA was extracted from Invitrogen's Trizol LS Reagent kit and dissolved in RNase-free water. Reverse transcription was performed using random primers. The primers were designed according to the sequence of the CTN-1V matrix protein gene of rabies virus [2]. NheI and HindIII were introduced into the template. The full-length gene of the rabies virus matrix protein was obtained by PCR amplification using cDNA as a template, and the primers were as follows:
5’-TCAGCTAGCATGAACTTTCTA-3’;
5’-AATGCGGCCGCCTATTCTAGGAG CA-3’.

4. Construction of recombinant plasmid pCDNA5.0FRT-M:

The recombinant plasmid pcDNA5.0 FRT-M was constructed and cloned into the pcDNA5.0FRT vector to construct the recombinant plasmid pcDNA5.0FRT-M. Plasmid extraction, digestion, fragment recovery, connection transformation reference [3].The recombinant plasmids were identified by restriction endonuclease, The recombinant plasmids were identified by restriction

endonuclease, The target gene was sequenced by Sangon Biotechnology. The recombinant plasmids for transfection were prepared according to the instructions of the Qiagen plasmid extraction kit.

5. hygromycin B lethal dose selection

The CHO-FRT cell line (10% fetal bovine serum IDEM) was subcultured and passaged to 24-well plate. Hygromycin B (final concentration of 0-1000 μ g / ml) was added to passaged at 37 ℃, 5% CO2. The cell growth status was recorded every day.

6. plasmid transfection and resistance of CHO cell clones screening

Resuscitation culture CHO-FRT cell line (containing 10% fetal bovine serum IDEM medium), spread to 24-well plate, to be covered with monolayer with Serum-free IDEM medium without antibiotics 3 times, Add 1ml of serum-free DMEM nutrient solution without antibiotics.Lipofectamin and pcDNA5.0FRT-M were mixed and incubated at room temperature for 30 min. The cells were shake-agitated at 37 ℃ and 5% CO2 for 48 h. Replace the hygromycin B-resistant medium (10% serum IDEM, 400 μg / ml hygromycin B) for 1: 100 high-dilution passages, changing the liquid every 3d until cell cloning occurred.

7. The expression of CHO cell line and the detection of matrix protein expression

The cell clones were scraped and passaged to 96-well plates. The cells were passaged with 800 μg / ml hygromycin B and 10% fetal bovine serum IDEM medium. The cells were passaged in 1 passages, And finally expanded to the cell bottle. The expression of rabies virus matrix protein was detected by indirect immunofluorescence and Western blot during cell passage, and the cell line with strong fluorescent expression was retained.

Results

1. Amplification of matrix protein gene and identification of pcDNA5.0FRT-G recombinant plasmid

The recombinant plasmid pcDNA5.0 FRT-M was constructed by RT-PCR. The recombinant plasmid pcDNA5.0FRT-M was constructed with the full length of the matrix protein gene of CTN-1V. The size of the gene was 609bp (Fig. 1, lane 1). After cleavage with NheI and HindIII, about 609bp of the target fragment and empty vector were cut out, as expected (Fig. 1, lane 2). The recombinant plasmids were sequenced by Shanghai Sangong Company. The results showed that the inserted frame was correct, indicating that the recombinant vector was successfully constructed. The sequence of the inserted fragment corresponds to the sequence of the original strain and the reading frame is correct.

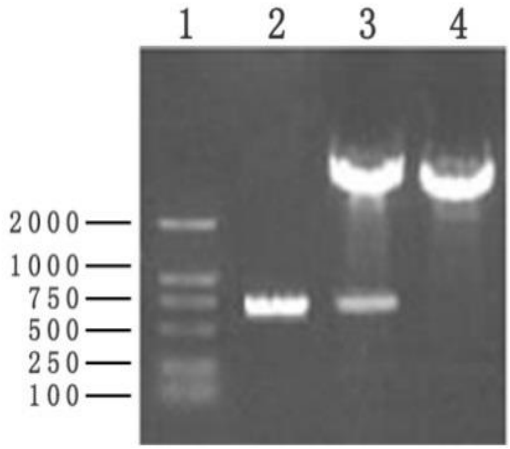


Fig 1 PCR and double digestion of recombinant plasmid pcDNA5.0FRT-M

1: DL2000 2: PCR product 3: double digestion result 4: empty plasmid double digestion results

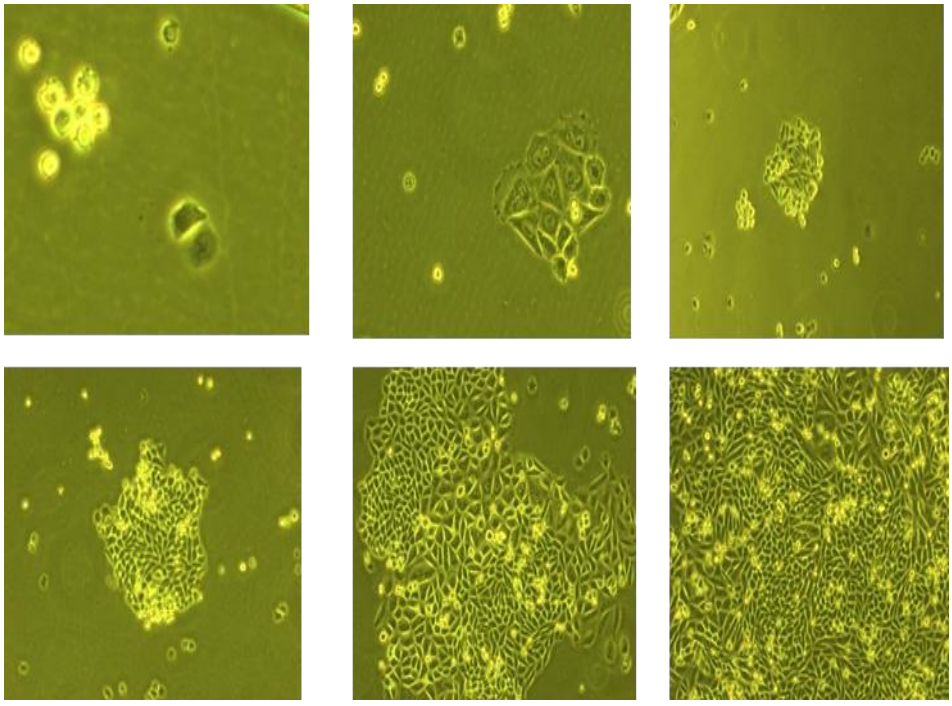
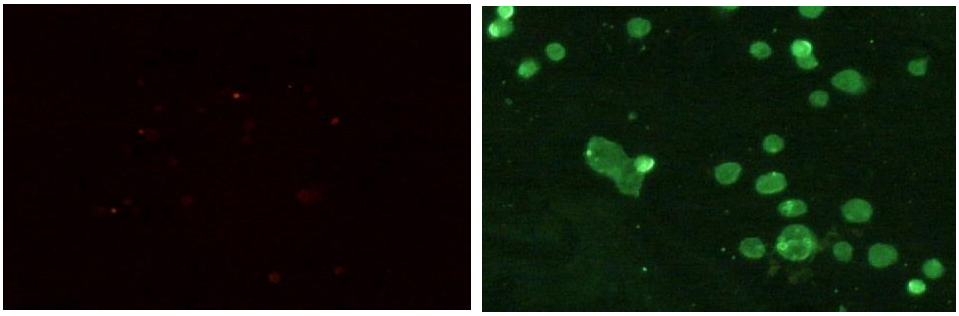


Fig.2 Screening of cell lines with stable expression of M protein

2. Stable expression of stromal protein CHO engineered cell lines

Screening of Stable Expression matrix Protein Cell Lines to ensure that positive cells as a single cell line, see Figure 2.The total DNA of M-CHO cells was amplified by M-F / R primers, and the specific bands of about 609bp were obtained. The total DNA of the recessive CHO cells was not amplified. The cell pre-cooling 80% acetone, the use of M protein rabbit polyclonal anti-indirect immune-fluorescence detection of cells, showing positive cell fluorescence signal, shown in Figure 3.



Negative positive

Figure 3. Detection of positive clonal M protein cell lines by indirect fluorescent assay

Discussion

Rabies is a serious global public health problem. Rabies is a naturally fatal disease of the acute lethal central nervous system caused by rabies virus (RV) in humans and all mammals. Rabies mortality rate is almost 100%.In recent years, China's rabies epidemic continued to rise year after year. The rabies virus genome is a single-stranded negative-strand RNA of about 12 kb in size. Matrix protein is located in the inside of the capsule membrane of a connexin, connecting capsule and nucleocapsid, can directly affect the glycoprotein in the virus envelope surface, plays an important role in the formation of virus morphology [5], and has an immunological response associated with RV antigenic determinant [6].

With the development of biological experimental techniques, the research on rabies virus is becoming more and more

clear. However, the structural proteins of rabies virus and its pathogenicity are relatively vague. More research is the relationship between G protein and RV pathogenicity. Many studies have shown that G protein is the key to determine the pathogenesis of RV protein [7-8]. G protein mutation of certain amino acid sites will change the pathogenicity of RV and some of the biological properties of the virus (such as intercellular diffusion, membrane Fusion, induction of apoptosis, etc.) [9].However, the effect of M protein on the pathogenicity of RV is poorly understood. In 2006, Shimizuk et al. [10] used reverse genetics to obtain Ni-CE (derived from a fixed strain of Nishigahara in chick embryo fibroblasts for 100 passages without pathogenicity in adult mice) M protein gene was replaced by Nishigahara strain (fixed strain, inoculated adult mice with death). The recombinant virus CE (NiM) was re-acquired virulence and inoculated into the adult mice. This result opens up a new chapter in the study of pathogenesis of RV.

Zheng Guanglai et al [11] expressed the M protein of rabies virus BD06 strain through baculovirus expression system, but the protein obtained by baculovirus expression system is simple and cannot meet the requirement of eukaryotic protein expression. Mammalian cells are the most advanced eukaryotic cells in nature, and can be used as recipient cells for gene expression, and can be used for complete post-translational modification of the expression products, including disulfide bond formation, glycosylation, phosphorylation, The formation of the polymer, etc. The protein obtained by mammalian cell expression has better natural biological activity. Cao Shouchun et al. [12] successfully established a stable CHO cell line expressing rabies virus glycoprotein. In order to detect the expression of M protein, this study also synthesized peptide fragment with linear epitope (CSDMSLQTQRSEEDKD) by bioinformatical analysis of virus M protein of CTN strain, and obtained anti-M protein rabbit polyclonal antibody. The results showed that the polyclonal antibody could recognize the rabies virus, but did not cross-react with other viruses such as hemorrhagic fever and JE virus.

Based on this work, we cloned the gene from the CTN-1V strain of rabies virus with independent intellectual property rights, and successfully constructed CHO mammalian cell line stably expressing the matrix protein, which laid the foundation for further study of rabies virus matrix protein function. In the future, will also use the cell line as a tool to further explore the rabies virus matrix protein function.

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