Original Article

Construction and Detection of a Novel Type of Recombinant Human rAAV2/2-ND4

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Abstract

Purpose: To construct a novel AAV-mediated gene delivery of the human ND4 complex I subunit and to detect its expression level in mitochondria for potential application in gene therapy for Leber's hereditary optic neuropathy (LHON).

Methods: A novel type of normal human ND4 gene was synthesized artificially to contain a mitochondrial targeting sequence that induces the translocation of this gene into mitochondria. This recombinant adeno-associated virus type 2/serotype 2 (rAAV2/2)-mediated NADH dehydrogenase subunit 4 (ND4) gene was constructed, purified, condensed, and amplified by PCR. The physical titer of rAAV2/2-ND4 was determined by slot-blot hybridization using a digoxigenin-labeled H1 probe. Expression of ND4 in mitochondria was evaluated by immunofluorescence.

Results: The constructed rAAV2/2-ND4 specifically amplified the target gene band of ND4 and the physical titer of ND4 gene was 1.0×10^{11} vg/mL, confirming that the recombinant adenovirus vector contained the ND4 target gene. Expression of the ND4 gene was detected in mitochondria by immunofluorescence.

Conclusion: A new type of rAAV2/2-ND4 was successfully constructed and may have potential in gene therapy for LHON. (***Eye Science 2013; 28:55-59***)

Keywords: Leber's hereditary optic neuropathy; NADH dehydrogenase subunit 4; recombinant adeno-associated virus; mitochondrial targeting sequence

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Introduction

Leber's hereditary optic neuropathy (LHON), is a maternally inherited disease that results in optic nerve degeneration due to dysfunction of mitochondrial oxidative phosphorylation induced by a mitochondrial DNA point mutation. Complex I in the respiration chain contains 7 mitochondrial DNA-encoded subunits and 35 nuclear DNA-encoded subunits[1]. Among the 7 mitochondrial subunits, ND1, ND4, and ND6 are responsible for 90% of LHON[2]. Mutation of ND4 is the most frequently encountered and causes the most severe symptoms that seldom can be alleviated. Therefore, research on gene replacement at this site is of clinical significance[1].

We successfully constructed RAAV2/2-ND4 in a previous study, but transfection with this foreign gene led to transfer to the nucleus rather than to the mitochondria. The present study describes the construction of a novel type of AAV2/2-ND4 that is targeted to the mitochondrial membrane and therefore can potentially play a role in gene therapy for LHON.

Materials and methods

Synthesis of target gene

The gene sequences of ND4 and cox10 were designed based on the method proposed by Crystel Bonnet's research team and the GenBank database. The gene sequence had two parts: the mitochondrial targeting sequence (MTS) of cox10 and the coding sequence of ND4. The discrepancy in genetic codons between mitochondrial and nuclear DNA was taken into account by modifying the gene sequence based on the amino acid sequence of the ND4 protein. The amino acid sequence of the translation product in the

cytoplasm was exactly the same as that in the mitochondria indicated by the gene sequence from the GenBank database. Newly-synthesized ND4 protein was transferred into mitochondria, guided by the MTS. The 3'non-coding region of cox10 had a full length of 2889 bp. The open reading frame was located from 1bp to 1461bp and encoded 487 amino acids. The 5'non-coding region, with an ATG start codon and 3'non-coding region, was 1425 bp in length and had a polynucleotide polyadenylation signal and a poly(A) tail (Figure 1). The plasmid used was N0548-1, containing the clone vector P2-gene, an enzyme cutting site at KpnI/SalI and the recombinant pSNaV/ rAAV2/2-ND4.

Main reagents

Reagents including 10× PCR Buffer, dNTP mixture, rTaq, DNA polymerase, DNA marker, 6 × loading buffer were purchased from TAKARA. The DNA extraction kit was bought from QIAGEN. Other materials included DNase I (Sigma), NBT/BCIP (Roche), NBT (LOT:13552020); BCIP (LOT: 12329021), primary antibody, Anti-monoclonal antibody (1:200), and ND4 antibody (1:100) (Santa Cruz Biotechnology, Inc.). Materials for secondary antibody staining included goat anti-mouse im munoglobulin (1:300) and goat anti-rabbit immunoglobulin (1:300), a quantum dot kit (QK605MD) (Wuhan Jiayuan Quantum Dots Co., Ltd) and an OlympusBX51fluorescence microscope (CCD DP72 Olympus, Japan).

Construction of rAAV2/2-ND4

At 1 d before transfection, 293T cells were inoculated in a 225 ml flask at a density of 3.0×10^7 cells/ml. The culture medium was DMEM containing 10% fetal calf serum and incubation was carried out at 37℃ in 5% CO_2 overnight. The culture solution was replaced by DMEM culture medium containing fresh 10% fetal calf serum on the day of transfection for subsequent inoculation. The cells were harvested and transfected with PlasmidTrans II kit (VGTC) when cell growth reached 80-90% confluence, as follows: 1.Equal amounts of pAdHelper, pAAV-r2c5, and pSNaV-ND4 plasmids were mixed with DMEM + PlasmidTrans Ⅱ (VGTC) in a 1.5 ml-Eppendorf tube and left to stand for 10 min at room temperature; this was labeled as reagent A. 2. Reagent A was homogenously mixed with 30ml of DMEM+10% fetal calf serum and labeled as reagent B. 3. B was added to cell culture flasks, inoculated, and incubated at 37℃ in 5% CO_2. 4.The culture medium was replaced by complete culture medium (DMEM+10% fetal calf serum) at 16h after transfection. The cells were harvested at 48h after transfection, suspended in PBS, and treated with 3 cycles of freezing and thawing.

Purification and condensation of the rAAV2/2-ND4 virus

The separation, condensation, and purification of rAAV2/2-ND4 were performed by chloroform treatment, PEG/NaCl precipitation, and chloroform extraction, respectively. The overall recycle rate was calculated as the number of virus particles in the final products/the number of virus particles in the initial products. The sample was separated by 10% SDS-PAGE, and stained with Coomassie brilliant blue until low background and distinct bands were observed.

PCR analysis of target genes of rAAV2/2-ND4

A 10 μl rAAV2/2-ND4 sample was collected, boiled for 5 min, and cooled in an ice bath. The ND4 target gene was specifically amplified by PCR using an ND4 primer, confirming that the recombinant virus carried the target genes. Subsequent analysis was performed according to the manufacturer´s instructions (TIANGEN).

Specific primer:
1F: 5'-ATCTCCGCACACTCTCTCCTCA- 3'
1F:5'-ATCTCCGCACACTCTCTCCTCA-3'
1R: 5'-GAGGAAAACCCGGTAATGATGTC-3'
1R:5'-GAGGAAAACCCGGTAATGATGTC-3'

Measurement of rAAV2/2-ND4 titer

The physical titer of rAAV2/2-ND4 was determined by slot-blot hybridization using a digoxigenin-labeled H1 probe (expressed as vg/ml)[4-6]. The plasmid pSNAV rAAV2/2-ND4 was quantitatively measured, diluted by dilution buffer solution at a gradient concentration, and dripped onto a nylon membrane. The rAAV2/2-ND4 sample was digested by DNase I and RNase at 37℃ for 1h (final concentration: 1μg/ml). The rAAV2/2-ND4 virus genome was extracted[4-6], placed in boiling water for 5 min, and then cooled in an ice bath. The sample was serially diluted and dripped onto a nylon membrane.

Subsequent analysis was carried out according to the manufacturer's instructions. The physical titer of rAAV2/2-ND4 was calculated as copy number of rAAV2/2-ND4 × the dilution rate of virus sample that gave an equivalent hybridization signal intensity.

Immunofluorescence

The 293T cells were inoculated at a density of 5× 104 cells in a 6-well plate and incubated in 5% CO_2 for 24 h, until the cells had adhered to the plate wells, grew the cells for 24 hours, then replaced the growth medium with PBS containing rAAV2-ND4, followed by further incubation. The following procedures were conducted according to manufacturer's instructions: PBS wash, added by 0.1% Triton-X 100, inoculated for 10~20 min, PBS wash, 2% BSA at 37℃ for 15 min, supplemented by diluted primary antibody mixture and inoculated for 2 h. PBS wash, added by diluted secondary antibody, inoculated for 45 min; PBS wash, added by the mixture of QDs-SA and QDs-IgG conjugates, inoculated for 1~2 h; PBS wash and eventually observed under fluorescence microscope. All incubations were performed at 37℃.

Results

Newly-synthesized target genes

The 5'-terminal structure was the MTS of cox10, followed by the newly-encoded ND4 sequence and the 3'non-coding region of cox10(Figure1).

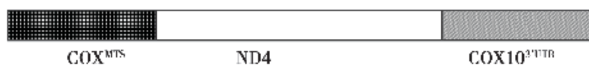

Figure 1 Target gene

Detection of recombinant adeno-associated virus condensation

The separation, condensation, and purification of rAAV2/2-ND4 were performed by chloroform treatment, PEG/NaCl precipitation, and chloroform extraction, respectively. SDS-PAGE showed a single protein band, indicating that the recombinant protein was the same protein(Figure 2).

Figure 2 The purified protein was analyzed by SDS–PAGE electrophoresis and stained by Coomassic brilliant blue: only one single protein band was seen.

PCR analysis of target genes (ND4 gene)

The PCR amplification product was 2889 bp in length using rAAV2/2-ND4 vector as template. Two bands appeared below the marker band at 2500 bp and 5000 bp. A target band of approximately 2889 bp was obtained, consistent with the positive control and in accordance to the default band of 2889 bp (Figure 3).

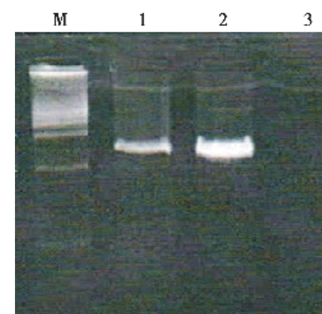

Figure 3 Results of electrophoresis show that the position of rAAV2/2–ND4 was closed to DL2800, and have the same position with the positive control according with the initiatory hypothesis of 2889bp.(M:Marker; 1:rAAV2/2–ND4; 2: pSNaV/ rAAV2/2–ND4 positive control; 3:negative control.

Measurement of rAAV2/2-ND4 titer (Figure 4)

The physical titer of rAAV2/2-ND4 was 1.0×10^{11} vg/ml, as shown in Figure 3.

Immunofluorescence(Figure 5)

The fluorescence intensity was slightly higher in Figure 5G than in Figure 5C. In the control group, normal cells contained the ND4 gene in their mitochondria. Therefore, the number of ND4 genes transferred into mitochondria did not differ from that shown in Figure 5C. In Figures 5D and 5H, the nucleus was stained blue, mitochondria were red, and ND4 was green and had the same locations in cyto-

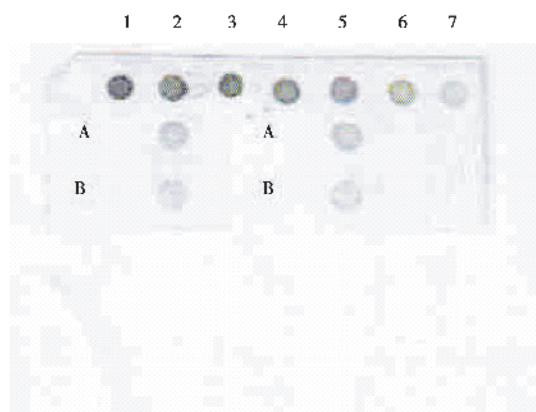

Figure 4 From 1 to 7 were the order of different copies(vg · mL^{-1})of pSNAV/rAAV2/2–ND4 (1×10^{12} vg · mL^{-1}、0.5×10^{12} vg · mL^{-1}, 0.25×10^{12} vg · mL^{-1}, 0.125×10^{12} vg · mL^{-1}, 0.0625×10^{12} vg · mL^{-1}, 0.03125×10^{12} vg · mL^{-1}, 0.0156×1012 vg · mL^{-1}), A: Twice diluted original solution, B: Fourfold diluted original solution.

plasm. The cytoplasm was yellow-brown due to double staining. The yellow-brown fluorescence was stronger in Figure 5H than in Figure 5D, indicating that Figure H has a higher level of expression of the ND4 gene and that newly-synthesized ND4 can be transferred to the mitochondria.

Discussion

At present, no effective treatment exists for LHON despite rapid progress in gene therapy. Constructing a normal ND4 gene is the key for gene therapy for this disease. In 2002, two groups successfully transferred the ND4 gene into cells and enhanced the in vitro oxidation-reduction reaction in mitochondria[2,3]. In preliminary studies, we constructed a recombinant AAV2-ND4 virus and detected its expression in the rabbit retina[4,8]. However, the foreign ND4 gene was only delivered to the nucleus and not to the mitochondria, probably due to the super-hydrophobicity of the NDA-encoding proteins in the mitochondria, which prevented the binding of ectopically expressed proteins to the mitochondria[2,3]. Therefore, a method to transfer mitochondrial DNA into mitochondria is required.

Previous experiments indicated that the transfer of mRNA-encoding mitochondrial proteins onto the surface of mitochondria depends on the presence of a MTS and the 3'non-coding region (3'-UTR)[3]. In vitro experiments demonstrated that injection of normal ND4 genes containing MTS maintained cellular oxidation and reduction functions and completely repaired mitochondrial defects[2,9]. Animal experiments also showed that rat eyes injected with recombinant rAAV2/2-ND4 containing MTS were significantly alleviated compared with the fellow eyes[9-11], hinting that MTS plays a role in accurate mitochondrial localization of the normal ND4 gene. However, the sequence used in previous studies also contained a green fluorescent protein (GFP) sequence, which is not permissible in human research. Therefore, we

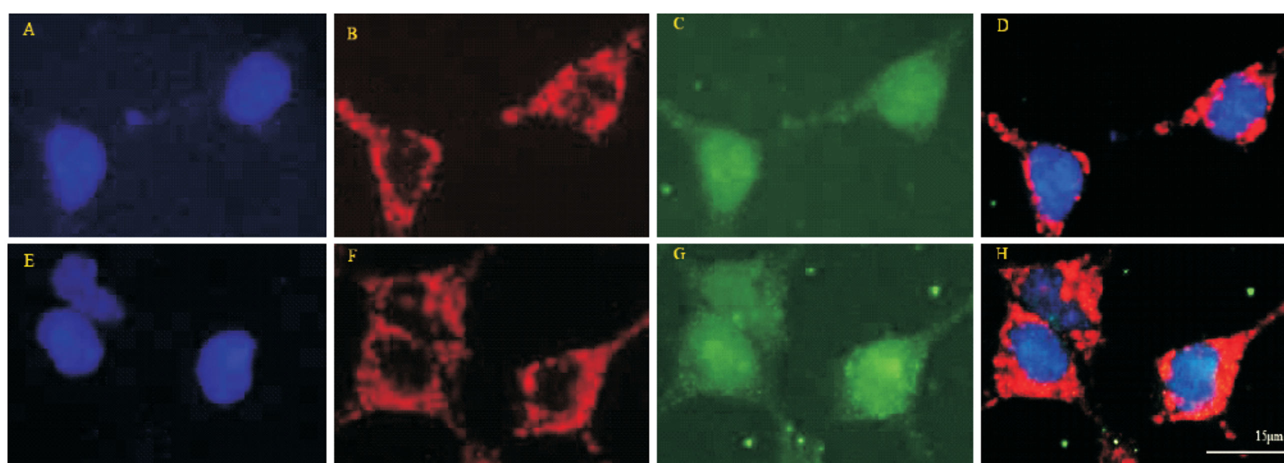

Figure 5 Immunofluorescence photography of 293 cells. Contract groups (A, B, C, D); AAV2–ND4 groups(E, F, G, H). A and E showed 293 cell nuclei were blue which were counterstained with DAPI; B and F showed Mitochondria were marked red with mitotracker; C and G showed ND4 were dyed green; D and H were merged pictures, orange was the result of ND4 overlaps with mitochondria.

constructed a novel virus vector that did not contain the GFP sequence.

The novel target gene was synthesized according to the approach by Bonnet C[3,9]. The normal ND4 DNA sequence was designed (nucleotide position 11778 was involved with encoding amino acid at 340 position of ND4), as shown in Figure 1. The obtained ND4 sequence was transfected into the nucleus via adeno-associated virus, which enabled encoding and synthesis of proteins within nucleus that were precisely the same as ND4-encoded proteins in the mitochondria. Meanwhile, an 84 bp DNA sequence from Cox10 was added in front of the sequence, to encode the MTS required to transfer ND4 proteins produced in the nucleus into the mitochondria. The purpose of adding the full-length 3'noncoding region of cox10, the polynucleotide polyadenylation signal, and the poly (A)-tail was to prevent the enzymatic hydrolysis of the nucleotide in the cytoplasm and to guarantee the transferring accuracy. Consequently, the encoded ND4 gene was recombined with an adeno-associated virus, transfected into retinal cells, delivered to the nucleus, and expressed as normal ND4 protein. The protein contained an N-terminal signal peptide, which directed its transfer into the mitochondria. The mature ND4 protein then functioned within the mitochondria after enzymatic hydrolysis.

In this study, new target genes of ND4 were syn thesized, transfected with AAV2, rAAV2/2-ND4 was successfully constructed, and ND4 target genes were amplified by PCR with a relatively higher titer of 1.0×10^{11} vg/ml. ND4 fluorescence intensity in the experimental group was somewhat stronger than in the control group, suggesting that the newly-synthesized target genes are translocated into the mitochondria. However, there was no striking difference between two groups because the normal cells used in the control group already contained a certain amount of ND4 gene. Further studies should be conducted to validate this conclusion. The construction of this new virus vector should provide further impetus for animal and clinical research that could lead to gene therapy for LHON.

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