ORIGINAL ARTICLE

Construction of Human Neuromuscular Disease-Related Gene Site-Specific Mutant Cell Line by Cas9 Mutation System

Yinbing Zhang[1*], Yuxin Wang[2], Cheng Du[3]

[1] Chemical Engineering and technology, Hubei University, Wuhan, Hubei Province 430062, China. E-mail: 2743706240@qq.com

[2] Chemistry, Hubei University, Wuhan, Hubei Province 430062, China

[3] English Education, Hubei University, Wuhan, Hubei Province 430062, China

Abstract: Objective: to construct human neuromuscular disease-related gene site-specific mutant cell line by Cas9 mutation system. Methods: according to the principle of CRISPR/Cas9 target design, the exon region of CXCR4 gene sequence was found in the National Center for Biotechnology Information (NCBI) of the United States. Two sgRNAs were designed. Lenticrisprv2 was used as the vector to construct the lenticrisprv2-sgrna recombinant plasmid, which was transformed into the sensitive stbl3 strain. The monoclonal sequencing was selected to verify and expand the culture of the plasmid, then it was transferred to 293T cells for packaging to a slow virus. The virus was collected and infected with 4T1 cells. The monoclonal cells were isolated and cultured by puromycin screening and limited dilution method. The genomic DNA of the selected monoclonal cells was extracted and the DNA fragment near the knockout site was amplified by PCR and sequenced. Results: one cell line had 6 deletion mutations, including DYSF mutation site of neuromuscular disease gene and HEK293T cell model knocked out by DYSF mutation site of neuromuscular disease gene. Conclusion: the recombinant plasmid targeting CXCR4 gene was obtained by CRISPR/Cas9 system, and the human neuromuscular disease-related gene site-specific mutant cell line was successfully constructed.

Keywords: Cas9 Mutation System; Neuromuscular Disease; Mutant Cell Line

1. Introduction

CRISPR/Cas9 system is an acquired immune system for prokaryotes to resist the invasion of viruses or plasmids and other foreign genetic materials, mainly composed of non-specific Cas9 nuclease and crrna for recognition[1]. In CRISPR/Cas9 system, the complex formed by annealing of crisprrna and transcrrna can specifically recognize genome sequence, guide Cas9 endonuclease to specifically cut and replace the target fragment, which has high specificity and low cytotoxicity. It can be widely used in genome engineering, such as gene inhibition, gene knock-out, gene knock-in and gene modification Double wait[2]. Compared with traditional gene editing tools, such as zinc finger nuclease (ZFNs) and tale nuclease (talens), the length of sgRNA recognition sequence of CRISPR/Cas9 system is only about 20bp, which is easier to achieve and more specific. Compared with siRNA, shRNA and other gene silencing methods, CRISPR/Cas9 system targets DNA sequence directly, so the target is broader and editing effect can be inherited[3]. In addition, compared with plasmid vector introduction, lentivirus vector has the advantages of high transfection rate, long-term stable expression of target gene and wide application range, and is the most widely used vector in basic and clinical research[4]. It is easy to get samples of skeletal muscle and peripheral nerve in clinic for hereditary neuromuscular diseases, which can make patients get pathological diagnosis before death, and promote the research process of hereditary skeletal muscle and peripheral nerve diseases. Hereditary neuromuscular disease is the main

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single gene genetic disease. At the level of molecular biology, it is a reality and hope to make clear diagnosis and gene therapy of pathogenic gene sequencing. Dysferloopathy is a group of skeletal muscle diseases inherited by ar. due to gene mutation, dysferlin protein in muscle cell membrane is absent, and muscle cells are destroyed, resulting in clinical manifestations of muscle weakness and atrophy. The related literature reported that DFSY gene c.3112c > t mutation in patients with neuromuscular disease, c.l046insg mutation in patients with dysberlinopathy, and found a high frequency, suggesting that this DFSY based on the upper site is the frequent mutation site of neuromuscular disease. In this study, the tgfbi-sgrna vector plasmid was constructed by CRISPR/Cas9 system, and the human neuromuscular disease-related gene site-specific mutant cell line was successfully obtained.

2. Materials and methods

2.1 Materials and reagents

HEK293T cell line, px458 plasmid (preserved in the laboratory); crystal violet dye solution, 4% formaldehyde (configured in the laboratory); fetal bovine serum (excellbio); DMEM medium (GIBCO); ampicillin antibiotic (solabo); PBS phosphate crystal powder (Dingguo Biology); penicillin/Streptomycin 100 fold concentrate, 0.25% EDTA trypsin (genview); neoect transfection reagent (zero guest intelligence creation Biotechnology); yeast extraction extract, tryptone (oxid); endotoxin free Plasmid Extraction Kit (US based biology); agarose powder (gene); DH5 α receptor peptide cell, blood/tissue/cell genome DNA extraction kit, agarose gel Recovery Kit (Tiangen Biology); BBS I endonuclease, engen™ mutation detection kit, T4 quick connect kit, q5hostar high fidelity polynuclease, t7e1 Nucleic acid endonuclease (NEB); Premier Taq (Takara); protein marker (thermo); ECL luminescent liquid (millipore); rabbit neuromuscular disease antibody (CST); rabbit GAPDH antibody (Wuhan Sanying); horseradish enzyme labeled goat IgG antibody (Beijing zhongshanjinqiao); CCK8 test kit (genecopoeia); 6-phosphate glucose dehydrogenase test kit (Zhongshan Bioengineering); vitamin K3 (MCK); sgRNA synthesis and introduction The services of product synthesis and sequencing were completed by Shenzhen Huada Gene Technology Service Co., Ltd.

2.2 Experimental method

2.2.1 Primer design of sgRNA and target fragment

According to the working principle of CRISPR/Cas9, two pairs of sgRNA with high score were selected by using the online design tool (HTTP//CRISPR.MIT.Edu/) to design guide RNA (sgRNA) for DYSF mutation site of neuromuscular disease gene. CACC was added at 5 ‘end of justice chain template and AAAC was added at 5 'end of antisense chain template. At the same time, according to the sequence of the target gene, CRISPR/Cas9 was designed by NCBI primer design tool to knock out and identify the primers of neuromuscular disease--5F:5’-TGTCTGTGCTGCCTGCTTT-3’, neuromuscular disease--5R:5’-TCCACGAAACACCGCCTTT-3’.

2.2.2 Construction of sgrna-px458 plasmid

The synthesized single strand sgRNA was annealed to form dimer by T4 polynucleotide kinase. At the same time, the px458 plasmid was digested by BBS I endonuclease and recovered by gel cutting. Then, the annealed sgRNA was connected with px458 plasmid recovered by gel cutting by T4 ligase overnight. Then the plasmid was transformed into DH5 α sensitive cells and coated on the LB plate resistant to ampicillin. The culture was conducted at 37 ℃ for 12-14h, and a single sgRNA was selected The plasmid was extracted and sequenced.

2.2.3 HEK293T cell culture and plasmid transfection

HEK293T cells were cultured in DMEM medium of 10% FBS, and HEK293T cells were laid in a 10 cm plate one day before transfection, so that the cell density could grow to 60%-80% at the time of transfection. Add 10 μ l plasmid according to the instructions of neofete transfection reagent for transfection, and change the solution after 6h. After 24 hours, GFP green fluorescence was observed under fluorescence microscope to ensure that the plasmid was successfully transfected into the cells.

2.2.4 Flow cytometry was used to separate HEK293T cells of GFP + and screen monoclonal cells

After transfection of HEK293T cells for 48 hours, PBS buffer was used to suspend the cells again, and GFP positive cells were separated by flow cytometer. GFP positive cells were inoculated into 96 well plates by limited dilution method. After the cells formed a monoclonal collection lag behind, they were inoculated into 24 well plates for culture, and then they were inoculated into 6 well plates for culture.

2.2.5 Verification of mutation efficiency and identification of HEK293T cell line with DYSF mutation of knock-out neuromuscular disease gene

According to the primers 5F and 5R designed for DYSF mutation site, the length of the target fragment is 599 BP, and the sequence length before and after the mutation site is 336 BP and 262 BP, respectively, with a difference of 74 BP. The DNA of the cells was extracted, the target fragment was amplified by q5hotstar high fidelity polynucleonuclease PCR, and the recovered product was digested by t7e1 enzyme to verify the mutation efficiency. After the selected monoclonal cells grow full in the 6-well plate, genomic DNA is extracted, and the products are handed over to Shenzhen Huada Gene Technology Service Co., Ltd. for sequencing after PCR amplification. The sequencing results are compared with the target sequence.

2.2.6 Statistical treatment

Using graphpad prism 5.0 software for statistical analysis, the two groups used independent samples of test comparison, $P < 0.05$ showed that the difference was statistically significant.

3. Results and discussion

3.1 Results

3.1.1 Selection and design of sgRNA target sites

In order to improve the knockout efficiency of CRISPR/Cas9, two pairs of sgRNA (Figure 1) with high scores were selected for DYSF mutation site of exon 5 of neuromuscular disease gene. Corresponding base was added at 5 'end (Table 1), and knockout primer sequence was designed and sent to Shenzhen Huada Gene Technology Co., Ltd. for synthesis.

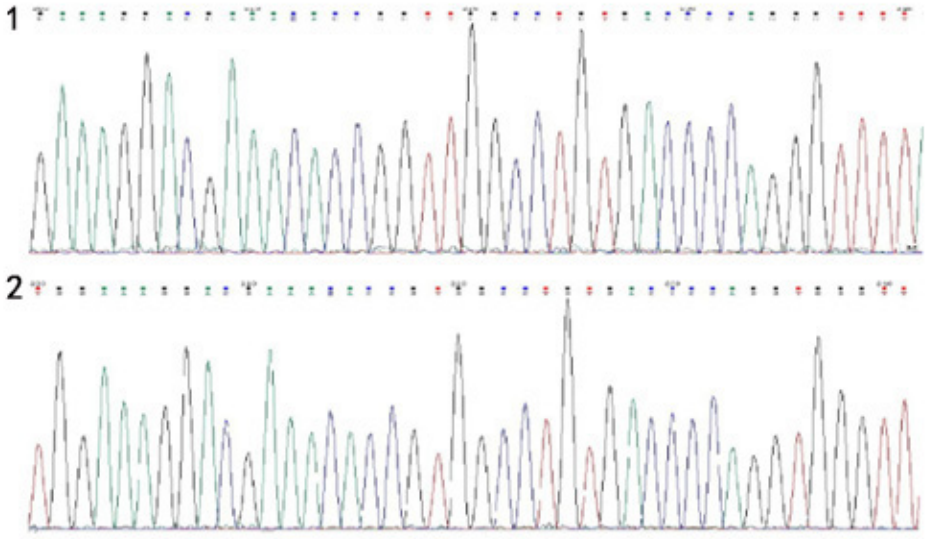

Figure 1. px458-sgrna sequence.

sgRAN		Sequences
SgRAN1	Forward	caccGGTTGGCCTGTGACCCCAGG
	Reverse	aaacCCTGGGGTCACAGGCCAACC
SgRAN2	Forward	caccGTGGCCTGTGACCCCAGGTGG
	Reverse	aaacCCACCTGGGGTCACAGGCCAC

Table 1. Sequences of sgRNA oligonucleotides

3.1.2 Construction of px458-sgrna vector

The px458 plasmid was digested by BBS I and recovered by glue. The sgRNA was annealed into double chains. Then, the sgRNA was connected with the recovered plasmid by T4 ligase. After transformation, shaking and upgrading, the linked products were sent to Shenzhen Huada Gene Technology Service Co., Ltd. for sequencing. The results showed that the two pairs of sgRNA sequences were correctly inserted into px458 plasmid (Figure 1), indicating the successful construction of the vector.

3.1.3 Transfection of sgrna-px458 plasmid into HEK293T cells

After 24 days of plasmid transfection, HEK293T cells and their fluorescence intensity were observed under fluorescence microscope. Green fluorescence was found in both sgrna-px458 cells, indicating that the plasmid was successfully transfected with high fluorescence intensity, indicating that the efficiency of plasmid transfection was high. As shown in Figure 2 below.

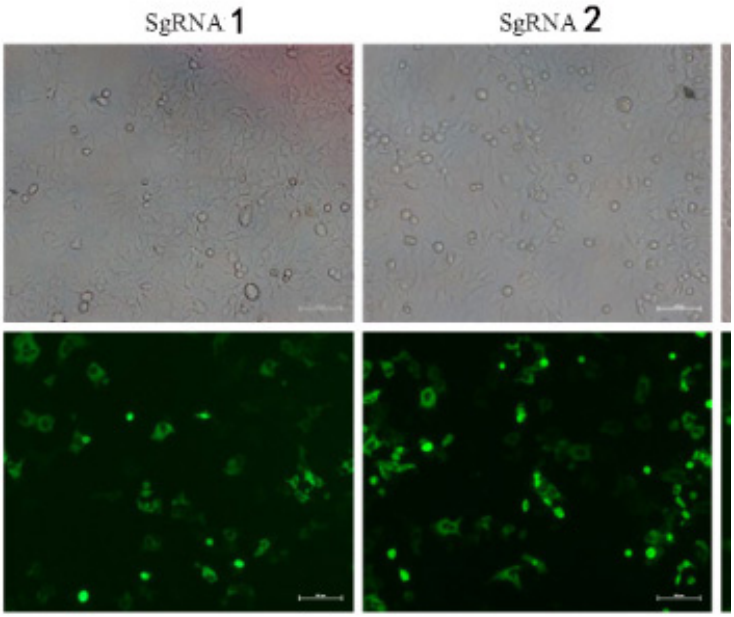

Figure 2. GFP expression in HEK293T cells at 24 h after transfection with sgRNA-PX458 plasmid (Original magnification: 10×10) .

3.1.4 T7e1 enzyme digestion to verify the mutation efficiency and identification of HEK293T cell line with DYSF mutation of knock-out neuromuscular disease gene

After PCR amplification, the target segments of the selected cells were electrophoresised with 2% agarose gel and the cleavage products of t7e1 were electrophoresised with 2% agarose gel. Two bands of sgRNA 1, 2 and 3 were cut by t7e1 endonuclease, which indicated that the DNA of the transfected cells was edited. The ratio of the gray values of the bands and the formula (% modification = 100 × [1 - (1-fraction cleaved) 1 / 2]) were used to calculate the mutation rate The DNA Editing efficiency of sgRNAs to HEK293T cells was 8.74% and 12.39%, respectively.

The GFP positive cells were selected by flow cytometry, and the monoclone HEK293T cell line was screened by limited dilution method. After the cells were expanded in the 6-well plate, part of the cells were taken to extract genomic DNA, which was amplified by PCR and sent to Shenzhen Huada Gene Technology Service Co., Ltd. for sequencing. The sequencing results were compared with the original sequence as shown in Figure 3. Therefore, one cell line had 6 base deletion mutations, and the missing base package HEK293T cell model including DYSF mutation of neuromuscular disease gene and DYSF deletion of neuromuscular disease gene was constructed successfully.

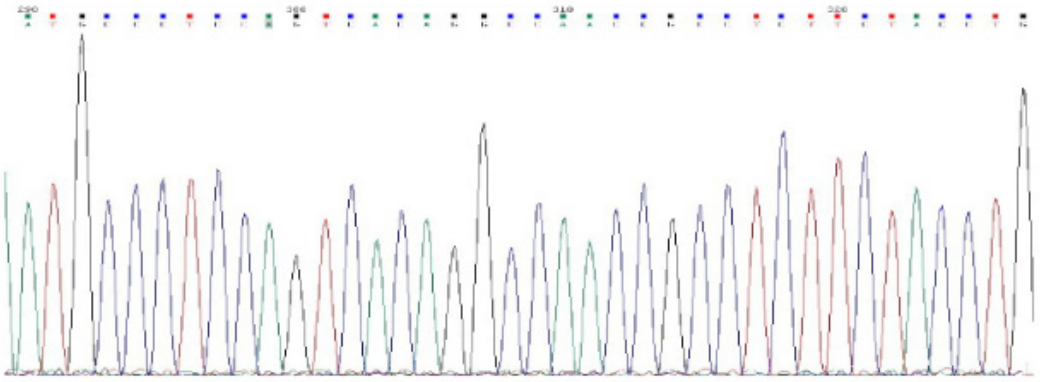

Figure 3. Comparison of sequencing results with the original sequence.

3.2 Discussion

Cluster regular interval short palindrome repeat and related protein 9 (CRISPR/Cas9) is a new gene editing technology[5]. Knockout, insertion and site-specific substitution of the target gene[6] can be realized, which opens a new direction for gene therapy. CRISPR/Cas9 has been reported to be used in the experimental study of thalassemia[7] and Duchenne muscular dystrophy (DMD/BMD) [8] and other genetic diseases.

Glucose-6-phosphate dehydrogenase (NMD) is the key enzyme of pentose phosphate pathway (PPP), which can protect cells from oxidative damage. Neuromuscular deficiency is one of the most common genetic diseases of the blood system, which is caused by the mutation of neuromuscular disease gene on X chromosome, resulting in the destruction and hemolysis of red blood cells[9]. Its clinical manifestations are mainly neonatal jaundice, acute hemolytic anemia and chronic non spherical hemolytic anemia[10]. At present, there is no root therapy for neuromuscular deficiency, which is mainly to prevent and give symptomatic treatment when the disease occurs. Therefore, it is hopeful to cure the disease and improve the current situation of treatment by repairing the mutant gene at the gene level.

At present, although CRISPR/Cas9 technology has been reported to correct multiple genetic diseases[11], it has not been used in the study of neuromuscular deficiency. Hek239t is a common model cell in the laboratory, which plays an important role in gene modification, editing and other research[12]. In this experiment, DYSF, a common mutation site of neuromuscular disease gene, was selected to construct DYSF mutant site of knock-out neuromuscular disease gene by CRISPR/Cas9 technology, and a stable HEK293T cell model was constructed for the follow-up study of gene repair of neuromuscular disease[13]. Although CRISPR/Cas9 system is easy to operate, its gene editing efficiency is affected by transfection efficiency and miss target effect. Due to the different specificity of sgRNA designed for different sequences, the editing efficiency of CRISPR/Cas9 system in different genes is quite different[14]. Although the mutation efficiency calculated after t7e1 digestion was high, it was found that some knockout sites did not contain DYSF mutation sites in the screening of monoclonal cells[15]. Therefore, it is necessary to further optimize the experimental conditions to improve the targeted editing efficiency of CRISPR/Cas9 system, such as changing the transfection mode of exogenous CRISPR/Cas9 system, using two pairs of sgRNA to guide Cas9 protein to cut the sequence before and after the target site at the same time[16], so as to select the best conditions for gene repair in the cells from patients.

Acknowledgements

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