Creating cell model of phenylketonuria disease by CRISPR-Cas9 mediated genome editing method

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Abstract

Phenylketonuria (PKU) is a monogenic disorder resulting from Phenylalanine hydroxylase (PAH) enzyme deficiency in liver hepatocytes. Untreated patients have clinical signs including growth retardation, microcephaly, short stature and low IQ. As the production of tyrosine from Phenylalanine is generally dependent on this enzyme, mutant PAH will result in accumulation of byproducts (liver cells), that is generated during deficient phenylalanine metabolism. This study aimed to produce a liver cell model harboring deletion in PAH, which could be applied to investigate mutations and their effect on cell metabolism as well as response to drugs and supplement administration. A large deletion in exon 6 of PAH was created by CRISPR/Cas9 system, using pSpCas9(BB)-2A-Puro (PX459) V2.0 and pSpCas9(BB)-2A-GFP (PX458) vectors. HPLC method was used to measure the level of phenylalanine and tyrosine in the cell with deleted PAH gene. Contrary to our expectation, a decrease in the phenylalanine level was observed in the culture medium and inside the cell, but the tyrosine level exhibited an anticipated decrease in the culture medium and inside the cell.

Background

Phenylketonuria (PKU) is a genetic disorder caused by a defect in the phenylalanine hydroxylase (PAH) enzyme. This enzyme is required for the conversion of L-phenylalanine (L-Phe) to L-tyrosine (L-Tyr)[1]. The phenylalanine hydroxylase gene (*PAH*) is located on the chromosome 12q23.2 and is about 79 kB in length, containing 13 exons[2]. The mutation in the PAH gene results in reduced activity of the phenylalanine hydroxylase enzyme in most forms of the disease. In the absence of treatment, the mutation causes unusual phenotypes such as growth retardation, microcephaly, short stature and low IQ. The main reason for these symptoms is the toxic accumulation of ingredients produced in the phenylalanine metabolism. In addition, the reduction or absence of PAH activity can lead to deficiencies in the production of tyrosine and its sub-products, such as melanin, thyroxine and catecholamine, neurotransmitters[3]. According to the phenotype, mutations can be either neutral or pathogenic due to disruption of structure and function of the enzyme. So far, more than 500 different alleles have been identified, which were mainly due to mutations in patients with phenylketonuria that has been documented in the PAH mutation database(<http://www.pahdb.mcgill.ca>). The basis for the treatment of the disease is to reduce the concentration of phenylalanine that leads to prevention of neurological and physiological disorders. Since these disorders transpire during the first month of birth, proper diet should be prescribed from birth[2].

Several treatments are now being considered, such as dietary restriction, enzyme replacement therapy and cell therapy. In the restricted diet method, a diet has to have low levels of phenylalanine in breast milk or commercially available foods, and in older children, the amount of protein intake should be calculated daily. Also, some foods like red meat, fish, milk, cheese, and other high protein foods must be eliminated from their daily meals[4].

Some PKU patients with severe classical forms do not respond to BH4 supplements due to lack of enzymatic activity; hence, such people use the enzyme replacement method. However, treatment with BH4 and enzyme replacement are not based on the PAH genotype. The replacement of enzyme can be facilitated by partial liver transplantation or normal liver cells[5]. One of the sub-methods in this area is to use the Phe Ammonia-lyse enzyme, which does not require a cofactor. This treatment has been effective in treating animal models (mice)[6]. Nowadays, another method that is being studied is the regeneration of the liver cell population with PAH expressing cells. The use of liver cells for transmission should have an advantage over the existing cells in order to be replaced[7]. Different systems are used to construct cell models with a desired mutation. These systems include TALEN, ZFN, and CRISPR-CAS9, which have advantages and disadvantages. The limitations of ZFN and TALEN methods include restriction in selecting the target location and application in multiplex method. Amongst the aforementioned methods, CRISPR-CAS9 method is considered as a novel method due to its ease of use. To change the target, it is only necessary to design a new guide sequence[8]. Nowadays, cellular models are strongly essential to examine the effect of a mutation on enzyme function and the effectiveness of drugs or supplements on the function of an enzyme.

In this study, we attempted to use CRISPR-CAS9 method to produce a HepG2 cell line with a deletion mutation in exon 6.

Methods

Cell culture

HepG2 cell line* was cultured in complete Dulbecco's modification of Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% Pen/Strep. Cultures were incubated in a humidified incubator at 37°C with 5% CO₂.

***Source: Shiraz university of medical sciences, Human Genetics Department.**

Primer and guide design

First genomic DNA was extracted from HepG2 cells (ThermoFisher, USA) based on the protocol provided by the manufacturer, and the target gene was sequenced to ensure lack of unintentional polymorphism, which might adversely affect the efficacy of the designed guide. Snapgene software was used for sequencing data analysis. Guides were designed for exon 6, using the online MIT tool (<http://crispr.mit.edu>). One guide on the sense (+) and the other guide on antisense (-) DNA strand with 141 bp distance, which contains the recognition site for Bbs1. VF and VR were forward and reverse primers, were used for sequencing the guides after cloning guides in Px459 and Px459 vectors. These primers were designed by alleleID 7.5 software. The sequences for guides and primers are represented in table 1.

Cloning and sequencing

Two strands from each guide were annealed as it was previously described by Ran et al.,2013. Then double-stranded guides were digested by Bbs1 and cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0 and pSpCas9(BB)-2A-GFP (PX458) vectors. Ligation and digestion protocol is presented in table2. Plasmids were transformed into the DH5α bacteria *via* CaCl₂ and heat shock method. Bacterial cells were grown on LB agar plate containing 50 mg/ml Ampicillin. Using a guide and one designed primer inside the vectors, colony PCR method was performed to detect positive colonies for the presence of recombinant plasmids that contain guides. One positive colony for each vector was grown in LB broth, and plasmids were extracted by VIVANTIS kit according to the manufacturer's protocol. PCR by VF/VR primers and Sanger sequencing were performed to confirm the presence of the desired guide sequences in PX459 and PX458 vectors.

Transfection and FACS analysis

Lipofectamine 2000 (Invitrogen Co.) was used to transfect HepG2 by PX459 and PX458. Briefly, HepG2 cells (confluency between 40-50 percent) were seeded in each well of a 6-well plate as it was described previously and grown to 80-90% confluency. Three hours before transfection, media were removed and refreshed with DMEM without antibiotics and FBS. Based on protocols provided by the Invitrogen company, HepG2 cells were transfected. PX459 and PX458 possesses antibiotic resistance and GFP, which were used as selectable markers. After 48 hours, transfected cells were inspected under an inverted fluorescent microscope to detect GFP positive cells that showed the transfection of PX458. Isolation of cells that carry PX458 vector was performed with fluorescence-activated cell sorting (FACS) method on GFP expressing cells, and then the sorted cells were cultured in puromycin medium. To select transfected cells by PX459, cells were grown in complete media containing 3.5 μL/mL puromycin.

Clonal isolation of cells

To isolate homozygote cells for deletion of exon 6 in both alleles, serial dilution for single cells cloning was performed based on Corning Incorporated Life Science protocol reported by Ryan et al.,2002. Briefly, cells were counted by hemocytometer method, and 2500 cells suspended in 100 μL medium in the first well of a 96 well plate. Serial dilution process was performed starting from the first well and diluted cells were seeded in the consequent wells. Wells seeded with single cells were incubated for four weeks and then grown in 6 well plates for another week. Some cells from each well were used for genomic DNA extraction. The extracted DNA samples were used in PCR for exon 6. Then, the PCR products were cloned in a TA vector (Vivantis Co.), transformed DH5α cells using the cloned vector, and the next day, plasmids were extracted from bacterial culture. PCR for exon 6 was performed on the extracted plasmids and the PCR product was used for Sanger sequencing.

Comparison of cell proliferation rate

In total, 55000 normal or mutated cell were separately cultured in three wells of a 6-cell plate. After 48 hours, the cells were harvested and counted again.

Comparison of phenylalanine and tyrosine levels in the used culture medium

The cells were cultured in equal numbers in the flasks to determine whether the amount of additional phenylalanine (overloaded by the mutation in enzyme) was introduced into the medium or not, and also to see if the mutated cells used more tyrosine from the medium. The used medium was picked up at 95% confluences from the flasks. The collected medium was centrifuged and the excess material was removed. Phenylalanine level was measured by the HPLC method.

Comparison of free phenylalanine and tyrosine in the normal and mutated cells

Equivalent numbers of normal and mutated cells were harvested and the level of free phenylalanine and tyrosine were measured by HPLC.

Results

PAH sequence and guide

Performing PCR on the exon 6 of the *PAH* gene and its sequencing showed that HepG2 cell lines possessed no mutation, and based on this sequence, the guides were designed. *PAH* gene PCR products are represented in figure **1a** and **1b**.

sgRNA cloning

sgRNA cloning into the destination vectors is shown by agarose gel electrophoresis (figure **2a**). Cloning PX459 and PX458 vectors were confirmed by Sanger sequencing. The guide sequences are highlighted in figures **2b** and **2c**.

Cell transfection

Transfection of HepG2 cells with PX458 were inspected by expressing GFP in the cytoplasm under fluorescent invert microscope (figure **3**). Sorted cells by FACS cultured in a selective medium containing puromycin (3.5 µg/ml) to purify HepG2 cells containing both PX458 and PX459, and then incubated for three days in a humidified incubator. The cells were harvested and DNA extraction was performed. PCR results of the target sequence deletion in the *PAH* gene is shown in figure **4**. Presence of two bands that resulted from non-homogenous cell population contained one or two mutated alleles.

Isolation of homozygote cells

Homozygote cells with both mutated alleles in their genome were obtained, using serial cell dilution. After culturing the singled cells in 96 well plates, they were allowed to propagate. Then, genomic DNA was isolated, *PAH* gene amplified and sequenced, showing deletion in the *PAH* gene (figure **5**).

TA cloning

TA cloning was used to obtain sequences of alleles after CRISPR/Cas9 deletion in the *PAH* gene. PCR for exon 6 showed cloning of the desired sequences into the TA vector (figure 6a). Sanger sequencing result is shown in figure 6b and 6c.

Comparing cell proliferation rate

Cell proliferation rate was performed *via* cell count method. The relative growth ratio of normal cells to mutated cells was about 2.3 times. In Figures 7a and 7b, the growth rate of cells can be seen simultaneously.

Comparing phenylalanine and tyrosine levels in the used medium

Phenylalanine and tyrosine assay were performed by HPCL method and confirmed by LC-MASS-MASS method (figure 8).

Comparing free phenylalanine and tyrosine levels in normal and mutated cells

The amount of phenylalanine and tyrosine within normal and mutated cells are presented in figure 9.

Conclusions

Amongst gene editing tools including ZFN, TALEN and CRISPR/Cas9, with their pros and cons, the latter takes more attention due to simple design of a 20 bp guide for this system in contrast to its counterparts that require the engineering of coding proteins to target specific sequence[9]. Another reason for the widespread use of CRISPR/Cas9 technology is its multiplexing application. In addition, CRISPR/Cas9 technology has exhibited more efficiency in mouse models by deletion in both alleles in the gene of interest[10], as well as correcting gene mutation through HDR DNA repair pathway[11]. Yi Pan et al., 2016 described the application of the CRISPR/Cas9 system for correcting one of the most prevalent mutations in PAH that restores the enzyme activity[12]. In 2018 Wattanapanich et al. investigated beta-thalassemia and corrected the corresponding gene through HDR, using CRISPR/Cas9 system that led to the production of mature HBB[13].

CRISPR/Cas9 system could be used for deletion by targeting two regions in the genomic sequences of the targeted gene. This tool has gained attention for gene therapy by creating a cell model of diseases that can help to explore new treatments[14]. For PKU it is very valuable to have a cell and animal model in order to investigate drugs and supplements effect on patients, which might have adverse effects with negative ramification[15].

Torres et al.,2014 used CRISPR/Cas9 to produce cell model that bears oncogenic translocation. In the current study, this technology was used to produce HepG2 cells bearing deletion in the *PAH* gene. HepG2 was used in this survey, as it originates from human hepatocyte liver cells that express the *PAH* gene. Based on the protein atlas database (<https://www.proteinatlas.org>) PAH expression in HepG2 is much higher than the other cell lines. Sequencing *PAH* gene after transfection by PX458, PX459 validated the

deletion of sequences between the targeted regions by guides. As NHEJ is the prevalent DNA repair pathway; hence, some denovo mutation was observed in both sides of the deleted region that led to indels.

Sanger sequencing analysis showed a 4bp deletion in upstream 5' region, as well as a cytosine insertion in the targeted regions, which exhibited the CRISPR/Cas9 system and the designed guides functionality for *PAH* gene knockout in HepG2. Results showed that cells containing two vectors were not necessarily homozygote for deletion in both alleles. This might indicate the proximity of targeted regions by two guides and asynchronous cut in both alleles, which demands more research. To create specific mutated cell lines and to determine the function and effect of mutations, the cutting location needs to be repaired by the HDR system.

It should be noted that the transfection process, mutation approval and sequencing were repeated experimentally and biologically, but the results were unpredictable. This meant that the mutation was exactly the same as the results before, which showed that failure created by the two CRISPR/Cas9 vectors were similarly repaired by NHEJ repair system. Hence, it can be assumed that by repairing the two strands breakage created by the compounds of the vectors, the mutation and repair is the same. To prove this, further studies are warranted.

Further, cell proliferation ratio in all wells was 2.3, indicating the negative effect of mutation on the growth of normal cells relative to mutated cells. This might also happen for cells in the body. Also, studying the amount of phenylalanine and tyrosine as the two essential amino acids in PAH pathway showed that the amount of phenylalanine in the culture medium not only did not increase, but a lot of phenylalanine was consumed. As expected, tyrosine in the medium was consumed and was reduced due to a defect in its synthesis route inside the cell. Contrary to expectation, the amount of phenylalanine in the cell decreased, which could be due to the increased growth of cancer cells that led to the intake of additional phenylalanine inside the cell.

Also, the reduction inside the intracellular phenylalanine might be due to its conversion to other metabolites such as phenyl ketones [16] that has to be assayed, because these metabolites are in the causative agent that create some phenotypes. The amount of tyrosine also significantly decreased in the cell, indicating that tyrosine was consumed from the medium and reduced its synthesis in the cell. With further studies on our cell model the level of phenyl ketones and precursor of tyrosine can be measured in order to find more reasons to reduce intracellular phenylalanine levels.

In summary, our study indicated that the cellular models obtained by the CRISPR-CAS9 method exhibited the effects of the mutation-induced in the *PAH* gene, so it is possible to insert known mutations in the PKU patients into the *PAH* gene to examine the effects of mutation on activity Enzyme and cells. It is also possible to measure the effect of drugs and supplements on these cellular models.

Abbreviations

CRISPR: *Clustered regularly interspaced short palindromic repeats*

PKU: Phenylketonuria

PAH: Phenylalanine hydroxylase

FBS: Fetal bovine serum

DMEM: Dulbecco's modification of Eagle medium

FACS: Fluorescence-activated cell sorting

PCR: Polymerase chain reaction

GFP: Green fluorescent protein

HPLC: High-performance liquid chromatography

HDR: Homology directed repair

NHEJ: Non-homologous end joining

Declarations

Acknowledgment

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Authors' contributions

BA, FF, SMBT designed the study, analyzed the data and wrote the primary manuscript. SM, JF Contributed to designing, interpretation of data and approving the final version of this manuscript for submission.

All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study did not use human or animal subjects

Consent for publication

The authors declare that they have no competing interests.

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Tables

Table 1. The sequences for guides and primers

CACCGCCCTCGAGTGGGAATACATGG	(+Forward (GF	G+) PX959)
AAACCCATGTATTCCACTCGAGGGC	(+Reverse (GR	(PURO)
CACCGCTGAGAAACGTCTTCCAGCT	(-Forward (GF	G-) PX458)
AAACAGCTGGAAGACGTTTCTCAGC	(-Reverse (GR	(GFP)
TTCTTGGGTAGTTTGCAGTTTTAA	Vector forward (VF)	
CACGCGCTAAAAACGGACTA	(Vector reverse (VR	

Table 2. ligation and digestion protocol

Mixture		
1 µl	Vector (backbone) (458 or 459)	
5 µl	Guide (annealed)	
2 µl	Tango buffer(10x)	
0.5 µl	T4 ligase	
0.5 µl	Enzyme Bbs1	
11 µl	Water (nuclease free)	
20 µl	Total volume	
Time and temperature		
mins 5	C° 37	
mins 5	C° 21	
µl 1	T4 buffer	Add To mixture
µl 1	Guide	
mins 30	21°C	
mins 5	C° 37	
mins 20	C° 65	

Figures

Figure 1

Exon 6 sequence examination as the normal control. a) The exon6 PCR product on gel electrophoresis (%1) showed the expected bands (402bps). In this step, the 1 kb ladder was used. b) Sanger sequencing data showed target sequence possessed no mutation.

Figure 2

Gel electrophoresis bands in colony PCR. A) Colony PCR was conducted on grown colonies. In this experiment VF and VR primers were used. The desired bands that were found indicated that the guide sequence was inserted into the vectors. B), C) The Sanger sequencing data showed that the guide sequences were entered into the vectors.

Figure 3

GFP positive cells. Twenty-four hours after transfection, the cells were observed by fluorescent invert microscope. Green cells showed that the vector px458 has a GFP selective marker, which was introduced into the cells.

Figure 4

The result of the desired gene proliferation in transfected cells by PCR. By proliferation of the target sequence, it was found that the cell population has two different alleles. A band of about 260, which indicate the removal of the desired sequence.

Figure 5

The proliferation of the mutated alleles in homozygous cells by PCR. The existence of a single-band of about 260bp is an indication that the cell population is homozygous for deletion mutation in the alleles.

Figure 6

TA cloning and sequencing results. a) PCR result of the mutated alleles in agarose gel electrophoresis. b) Sanger sequencing result of the mutated alleles. c) Deleted sequences is shown by yellow, cut regions by "/" sign and primer annealing region by green. Bolded region indicates PAM sequences. The presented alleles imply a 144bp deletion. A 4bp deletion in the upstream 5' region as well as a cytosine insertion in the region of deleted sequences, were observed.

Figure 7

Comparison of cell confluency. The confluency of the mutated and normal cells is shown in 7a and 7b.

Figure 8

Phenylalanine and tyrosine levels in the normal and mutated cells

Figure 9

The amount of phenylalanine and tyrosine within normal and mutated cells