Application of CRISPR Technology in the Treatment of Phenylketonuria

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Abstract. Phenylketonuria (PKU) is an autosomal recessive disorder caused by a pathogenic variant of the phenylalanine hydroxylase (PAH) gene. The pathogenic mechanism involves mutations in the PAH gene leading to the accumulation of phenylalanine (Phe) in the blood to neurotoxic levels, preventing patients from metabolizing phenylalanine normally and thereby affecting the development of the nervous system. Traditional treatment methods primarily consist of adhering to a strict low-phenylalanine diet to reduce the intake of phenylalanine from food, or employing gene therapy techniques to repair or replace the defective PAH gene. The emergence of CRISPR technology in recent years has opened new possibilities for treating genetic diseases, including PKU, especially in gene correction and disease model creation. Gene editing techniques, exemplified by CRISPR-Cas9, theoretically enable the direct modification or repair of defective PAH genes at the DNA level, and this approach holds promise for achieving more precise and permanent genetic repairs. This research begins by introducing the pathogenic mechanism of PKU from a genetic perspective, from the structure to the function of the PAH gene, analyzing the defects caused by its mutations, then discusses the potential of gene therapy using CRISPR technology to correct PAH gene mutations, highlighting new techniques such as prime editing and base editing. This research aims to summarize the current state of CRISPR technology applications in treating PKU, cataloging and evaluating various methods. It introduces new considerations based on existing research and looks forward to future research directions, thereby offering new potential treatments for PKU.

Keywords: CRISPR-Cas9, Genome editing, Phenylketonuria, Genetic therapy, Therapeutic strategies.

1. Introduction

Phenylketonuria (PKU) is a genetic disorder resulting from mutations in the phenylalanine hydroxylase (PAH) gene, which encodes the enzyme, essential for converting phenylalanine to tyrosine [1]. The primary site of PAH is the liver, where hyperphenylalaninemia's (HPA) neurotoxic effects can be avoided by eliminating excess L-Phe [2]. The patient's hyperphenylalaninemia phenotype is determined by the PAH enzyme's activity, which is influenced by the position and type of the mutation [3]. The classic PKU phenotype is caused by little or no enzyme activity [3]. Certain mutations reduce but do not entirely eliminate the activity of the phenylalanine hydroxylase enzyme, leading to a less severe form of PKU or mild hyperphenylalaninemia [3].

In individuals with PKU, due to the PAH gene mutations, PAH activity is reduced or absent, leading to an accumulation of phenylalanine in the blood. High levels of phenylalanine are toxic to the brain, causing intellectual disability and other neurological issues if not managed through diet or other treatments. Without treatment, individuals with PKU can experience profound intellectual disabilities, seizures, along with behavioral, psychiatric, and motor disturbances [4]. Additionally, a distinctive musty body odor, lighter pigmentation of the skin, hair, and eyes, as well as possible cortical blindness and eczema, may manifest in some cases [1].

Globally, the prevalence differs, averaging around 1 in 10,000 newborns [5]. This highlights the essential role of prompt diagnosis and early intervention. PKU has a significant impact on patients' quality of life and dietary restrictions, requiring lifelong management. Traditional PKU treatment methods primarily focus on dietary management to limit phenylalanine intake, often through a phenylalanine-restricted diet and the use of phenylalanine-free medical foods. While effective in managing phenylalanine levels, these dietary restrictions pose significant challenges in terms of

patient compliance, social inclusion, and the risk of nutritional deficiencies. During adolescence and adulthood, dietary therapy becomes less and less effective. Only a small percentage of treated individuals are able to maintain desired blood phenylalanine (Phe) concentrations solely through a diet [6]. Additionally, such treatments do not address the underlying genetic cause of PKU, limiting their ability to offer a permanent cure.

Gene therapy represents a cutting-edge approach aimed at correcting or substituting faulty genes within cells to address genetic disorders, primarily discussed in the context of human health, and it offers promising avenues for treating diseases stemming from genetic mutations or undesirable gene expressions. With a wide array of genetic conditions potentially curable through this method, gene therapy is increasingly gaining interest among scientific and pharmaceutical circles. With the potential to precisely edit DNA to correct genetic defects, clustered regularly interspaced short palindromic repeats (CRISPR) technology has emerged as a ground-breaking tool in the field of genetic engineering. The emergence and swift advancement of CRISPR/Cas technology are transforming the landscape of gene therapy, enabling highly adaptable treatments for a variety of human genetic disorders. By harnessing the CRISPR-Cas9 system, researchers can target specific genetic mutations responsible for diseases like PKU, offering the possibility of a one-time treatment that addresses the root cause of the condition. The application of CRISPR technology in genetic diseases represents a significant leap forward from traditional treatments, promising a future where inherited disorders like PKU could be permanently corrected.

Several innovative therapies for PKU are currently in various preclinical stages, and this research focuses on gene therapy aimed at restoring liver PAH expression through the use of gene editing technologies. The ensuing sections will traverse the landscape of CRISPR technology's role in PKU treatment, from elucidating its genome editing mechanisms and the genetic foundations of PKU to evaluating HDR-mediated gene correction strategies, current research, future directions, and navigating the challenges of CRISPR application, offering a comprehensive narrative on the potential and hurdles of employing CRISPR-Cas9 in combating PKU.

2. Genome editing and CRISPR-Cas9 mechanism

Genome editing is a method that allows scientists to change an organism's DNA precisely and efficiently. This technique enables the addition, removal, or alteration of genetic material at particular locations in the genome. Genome editing utilizes engineered, precision-targeted nucleases that combine sequence-specific DNA-binding domains with non-specific DNA cleavage domains, facilitating site-specific genomic alterations through cellular DNA repair processes, leading to insertions, deletions, or substitutions at the intended loci [7].

Currently, the most widely used gene editing technology in humans is the CRISPR-Cas9 system. This technology has gained widespread application in the scientific community due to its high precision, ease of operation, and relatively low cost, including research and treatment of genetic diseases, agricultural improvement, and biological research across various fields. The CRISPR-Cas system achieves targeted gene modification by using a guide RNA (gRNA) that is specifically designed to match the target gene sequence. This gRNA binds to the Cas9 enzyme, directing it to the exact location of the target DNA. Once there, Cas9 creates a double-stranded break in the DNA at the target site. The cell then repairs this break, and during the repair process, modifications such as insertions, deletions, or substitutions can be introduced into the genome at the target location. The Cas9 nuclease system is distinguished in the field of gene editing for several key advantages: its precision and efficiency in targeting specific genomic sequences, the simplicity of gRNA design which enables versatility across different genetic targets, and its adaptability to a wide array of organisms and cell types. The CRISPR/Cas system has been successfully used to edit genes in a diverse range of cells, including human somatic cells, various types of stem cells, as well as a variety of plant and animal cells [8].

3. The genetic basis of phenylketonuria

The PAH gene is located on chromosome 12, has 13 exons and a length of 90 kb [9]. The pathogenic mutations in the PAH gene predominantly occur in the exon regions, with a significant concentration found in exons 3, 6, 7, and 11 [10]. The mammalian PAH enzyme is tetrameric, consisting of 52 kDa subunits [11]. Each subunit comprises three main domains: a regulatory domain (RD) with a flexible N-terminal tail at the start, a catalytic domain (CD) central to its enzymatic function, and an oligomerization domain (OD) that facilitates the enzyme's dimerization and tetramerization, crucial for its activity [11]. The catalytic domain is key for iron, cofactor, and substrate interaction, facilitating the enzyme's hydroxylating action, while the oligomerization domain enables the enzyme's tetrameric structure through domain swapping and coiled-coil formation [12].

Blau et al. have conducted research on summary of variables, the catalytic domains contained the majority of the variations (61.2%), followed by the regulatory (16.8%) and oligomerization (5.2%) domains [10]. Variants in both alleles of the PAH gene lead to the production of a variant PAH mRNA [10]. This, in turn, results in the creation of a PAH protein that may be unstable, exhibit reduced activity, or be entirely inactive, compromising the liver's capacity to convert Phe to tyrosine (Tyr) [10].

Given the complex nature of PKU's genetic foundation, where mutations in the PAH gene lead to the production of a deficient enzyme, disrupting the metabolic conversion of phenylalanine to tyrosine, the pursuit of innovative therapeutic strategies is critical. The next chapter introduces the cutting-edge realm of CRISPR technology, elucidating how this gene-editing tool rectifies the underlying genetic errors in PKU by targeting and correcting the specific mutations within the PAH gene.

4. CRISPR-Cas advances in genetic therapy

The advancements in CRISPR-Cas technology, particularly through base editing (BE), prime editing (PE), and homology-directed repair (HDR), offer groundbreaking approaches for precise genetic corrections without double-strand breaks, minimizing risks of indels and off-target effects. These methods enable targeted modifications including base-to-base conversions and the repair of damaged DNA with high fidelity, promising a significant leap forward in treating genetic disorders like PKU by directly correcting mutations at their source. This evolution in genome editing not only enhances the precision and safety of gene therapy but also broadens its applicability in research and therapeutic contexts, marking a pivotal shift towards more effective and permanent treatments for inherited diseases.

Base editing, a CRISPR-Cas technology evolution, offers improved safety and precision by enabling direct C-G to T-A or A-T to G-C conversions without double-strand breaks (DSBs), thus minimizing indel formation risks [13]. Fig. 1 elucidates the mechanism of BE technology. This method, which includes cytosine (CBE) and adenine (ABE) base editing, leverages a Cas9-sgRNA complex and a guide RNA scaffold with a specific sequence near a genomic PAM site, maintaining the system's targeting efficiency while enhancing its application safety [13]. DNA base editors combine a Cas enzyme for DNA binding with a single-stranded DNA modifying enzyme for precise nucleotide changes. They are categorized into cytosine and adenine base editors, allowing for all four transition mutations (C to T, T to C, A to G, G to A) using CRISPR/Cas-based editing systems [14]. This technology enables targeted genetic alterations without requiring double-stranded DNA breaks, offering a potent tool for genome editing with broad applicability in research and therapy. To correct mutated PAH genes through Base Editing, one would specifically target the PAH gene mutations responsible for PKU, using cytosine or adenine base editors to directly convert the mutated base without introducing double-strand breaks, thus minimizing off-target effects and unwanted indels.

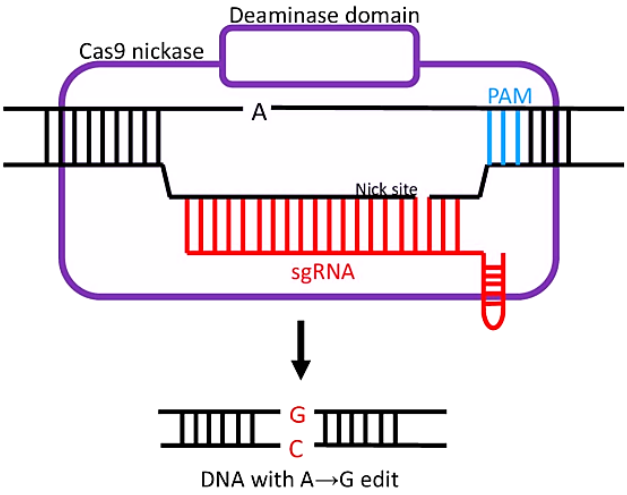


Figure 1. The mechanism of BE [13]

In the prime editing system, the Cas9 nuclease is fused with reverse transcriptase to form a fusion protein, guided to specific genetic sites by a prime editing guide RNA (pegRNA). Fig. 2 illustrates the operational mechanism underlying PE technology. The pegRNA's primer binding site hybridizes with the DNA's non-target strand, enabling the reverse transcriptase domain to synthesize a new strand on the DNA, incorporating the desired edits [15]. This newly synthesized strand then replaces the original DNA and integrates into the genome, facilitating precise genetic modifications. This allows for the introduction of specific edits (insertions, deletions, and all types of base-to-base conversions) directly into the target site of the PAH gene, potentially correcting the mutation at its source with high fidelity and without significant DNA damage.

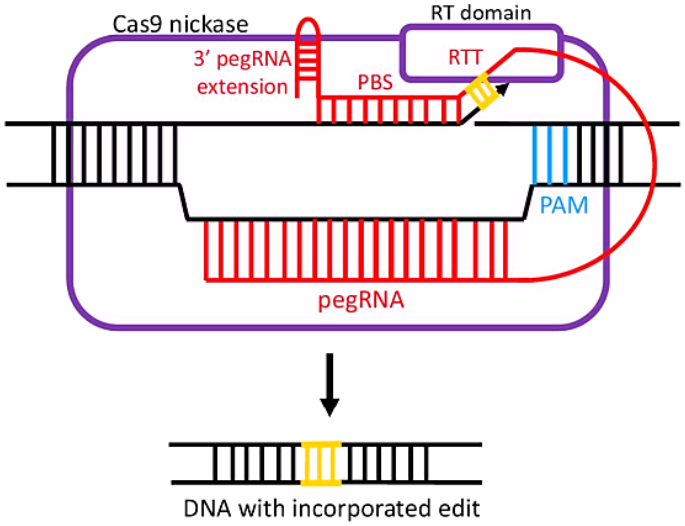


Figure 2. The mechanism of PE [13]

Within the HDR mechanism, the homologous chromosome DNA acts as a template for the repair of damaged DNA, ensuring the restoration process is carried out without errors [16]. The process involves designing a gRNA that targets the specific genomic location of the mutation within the PAH gene. Upon introduction to the cell, the Cas9 enzyme, guided by this RNA, precisely cuts the DNA at the desired spot. Then, a donor DNA template, which carries the correct sequence, is introduced into the cell. This template serves as a blueprint for the cell's HDR machinery to repair the DSB, incorporating the correct sequence into the genome.

5. Current research and future directions

Researchers have successfully applied advanced gene editing techniques in the livers of mice, utilizing both prime editing and base editing to effectively correct the c.1222C>T mutation in the PAH gene of a PKU mouse model [17]. This innovative treatment method significantly reduced the Phe levels in mice, maintaining them well below the threshold of 360μmol/L without harming liver function. The research team utilized a lipid nanoparticle (LNP) delivery system to efficiently transport base editors and gRNA into mice, restoring normal Phe levels within 48 hours in a PKU mouse model. Long-term observations indicated that the therapeutic effects lasted for a year. This breakthrough not only demonstrates the feasibility of permanently correcting PKU-related gene mutations using gene editing technology but also offers new hope for improving the quality of life for PKU patients.

The Brooks team utilized CRISPR/Cas9 technology to create a homozygous PAH gene knockout (KO) mouse model in the C57BL/6J strain by converting the 7th codon of the PAH gene into a stop codon [18]. The study focused on 2-6-month-old male Hom mice, conducting behavioral, biochemical analyses, MRI, and histopathological evaluations. Hom mice exhibited comprehensive PAH enzyme deficiency, elevated phenylalanine levels in blood and brain, reduced tyrosine and neurotransmitters, decreased myelin content, and significant behavioral deficits, mirroring symptoms observed in PKU patients. Additionally, adenine base editing (ABE) was applied to correct the common PAH P281L mutation in PKU, significantly reducing blood phenylalanine levels within 48 hours using lipid nanoparticle delivery, offering new hope for PKU treatment. The study includes in vitro studies to validate the efficiency and specificity of the ABE approach, off-target assessment to ensure minimal unintended editing, and in vivo studies to demonstrate the therapeutic potential of this approach in correcting the PKU-causing P281L variant in mice. The findings indicate that ABE can rapidly and definitively normalize blood phenylalanine levels in PKU mice, suggesting a promising therapeutic strategy for PKU treatment. This research represents a significant advancement in genetic editing therapies, particularly for diseases like PKU where traditional treatments have limitations. The use of ABE to precisely correct specific mutations without the need for double-strand breaks reduces the risk of off-target effects and offers a potentially safer and more effective treatment option.

The Richards team utilized CRISPR-Cas9 and HDR in newborn mice, employing dual rAAV8 vectors for Cas9 nuclease and targeted guide RNA to address PAH deficiency in $Pah^{enu2/enu2}$ mice [19]. The study involved delivering CRISPR/Cas9 via liver-targeted recombinant AAV2/8 vectors to correct the Pah^{enu2} allele in PKU mice. The addition of the NHEJ inhibitor coumarin enhanced HDR, leading to permanent allelic correction in some liver cells, partial restoration of liver PAH activity, and significant reduction in blood phenylalanine. The impact of this study on the prospects of CRISPR technology in treating PKU is profound. It demonstrates a viable pathway for correcting the genetic mutation responsible for PKU directly in vivo, offering a potential one-time treatment option that could mitigate or eliminate the disease's metabolic consequences. This approach could revolutionize PKU treatment, moving beyond dietary management to direct genetic correction, thus improving patients' quality of life and reducing long-term health risks associated with high phenylalanine levels. This approach also mitigated maternal PKU effects during reproduction, showcasing the potential for permanent PKU gene correction with CRISPR/Cas9.

6. Challenges in CRISPR application

The three studies offer innovative CRISPR-based approaches for treating PKU. The first research focuses on BE and PE as precise genome editing tools that avoid double-strand breaks, highlighting their potential for correcting genetic mutations in diseases. The second research employs ABE to correct the PKU-causing mutation in a mouse model, demonstrating its efficacy in normalizing blood phenylalanine levels. The third research utilizes AAV-mediated CRISPR/Cas9 for direct gene correction in murine liver cells, showing significant therapeutic benefits in PKU mice. These studies collectively underscore CRISPR's versatility in addressing genetic diseases, with each technique

offering a unique approach to gene editing. However, they also face challenges, such as ensuring specificity and efficiency of editing, minimizing off-target effects, and developing safe, effective delivery methods for clinical applications. The ongoing refinement of CRISPR technologies and their application methodologies will be crucial in overcoming these hurdles and harnessing their full therapeutic potential.

From the discussion, it is clear that CRISPR technology, particularly PE and BE, holds significant promise for treating PKU. Successful applications in mouse models have notably reduced phenylalanine levels, guiding future clinical treatments. However, the application in humans must overcome challenges of accuracy and safety, with precision and security of gene editing being paramount. Current research focuses on animal models, with clinical applications in early stages. Future efforts will involve deeper research into the effects and safety in humans, designing rigorous clinical trials to monitor long-term outcomes and potential risks, improving gene editing efficiency and precision, and developing comprehensive treatment strategies for all PKU patients.

7. Conclusion

While still in the research phase, gene therapy based on CRISPR technology is rapidly advancing, paving new pathways for the treatment of PKU. This study provides a brief overview of the genetic basis, clinical manifestations, and epidemiology of PKU, and briefly describes the strategies of PKU gene therapy utilizing CRISPR technology.

Current research on PKU gene therapy using CRISPR technology faces several challenges, including ensuring the precise targeting and editing of the PAH gene without off-target effects, which can lead to unintended genetic alterations. Another major concern is the efficient delivery of CRISPR components to the liver cells where PAH enzyme activity is critical. Additionally, the potential immune response against CRISPR components and the long-term stability and safety of the edited genes remain significant hurdles. These limitations underscore the need for further development and validation of CRISPR-based therapies for PKU in clinical settings.

Future research directions to address the shortcomings in CRISPR technology for PKU gene therapy include developing more sophisticated gene editing tools with higher specificity to minimize off-target effects. Efforts are also underway to innovate safer and more efficient delivery systems, such as nanoparticle-based carriers, to target liver cells more effectively. Furthermore, strategies to mitigate potential immune responses against CRISPR components are being explored. Additionally, long-term studies are needed to assess the stability and safety of gene edits. Advancements in these areas are crucial for translating CRISPR-based PKU therapies from the laboratory to clinical application.

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