Phenylketonuria: Genes in phenylketonuria, diagnosis, and treatments

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Abstract

Introduction: Phenylketonuria (PKU) is a rare autosomal-recessive disorder inherited in accordance with the law of segregation. Detection tools for people with PKU can include Sanger Sequencing (SS) and Next Generation Sequencing (NGS). Diet therapy, Large Neutral Amino Acids (LNAA), and Specific Nutrient Combination (SNC) can help alleviate people with PKU. In the future, genetic manipulation techniques can help to eliminate PKU. Objectives: In this review, the author describes the progress in a study that focused on detection tools such as SS and NGS, the phenylalanine hydroxylase (PAH) gene and mutations in the PAH gene, use of drugs for PKU, and genetic manipulation techniques such as Adeno-associated virus (AAV) vectors and clustered regularly interspaced short palindromic repeats (CRISPR) RNA-guided FokI nuclease system (FokI-dCas9 system). Methods: The author searched the PubMed Database at National Center for Biotechnology Information (NCBI) for articles on PKU disorder. These articles were published between 2014 and 2019. Articles were open access and in English. Searches were also done at Google and ScienceDirect. Results: PKU derives from mutations in the PAH gene. Features of PKU may include ataxia, intellectual ability, and seizures. MassARRAY method, minisequencing method, SS and NGS can detect PKU on humans. Diet therapy, BH₄, LNAA, SNC, enzyme therapy can help patients with PKU. However, these drugs cannot treat PKU permanently. In the future, genetic manipulation techniques can be used. AAV vectors and FokI-dCas9 system can be useful to eliminate PKU disorder. Conclusion: Guthrie method, MassARRAY method, minisequencing method, SS and NGS are tools for detecting PKU. Treatments with such as diet therapy, LNAA, SNC, and enzyme therapy are useful for PKU disorder. AAV vectors and FokI-dCas9 system are methods that can be useful for eliminating PKU in the future. Keywords: phenylalanine hydroxylase (PAH), Phenylalaninase, PKU, PKU1

1. Introduction

Phenylketonuria (PKU) is a genetic disorder that can affect intellectual disability and other medical problems. Other names for this disorder are deficiency disease or, Folling disease, Folling’s disease, and phenylalanine hydroxylase (PAH) deficiency disease. Mutations in the PAH gene result in PKU that cause reduction of the


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activity of PAH. This enzyme catalyzes hydroxylation of phenylalanine to tyroxine. This disorder affects about 1 in 2,500 (Wang et al., 2019) to 100,000 births (Muntau et al., 2019). The carrier frequency is 1 in 50 (Tolve et al., 2018). PKU has a high prevalence in Turkey, Ireland, and the United Kingdom. The prevalence PKU is 1 in 2,600 in Turkey, 1 in 4,500 in Ireland, 1 in 10,000 in the United Kingdom, 1 in 11,000 in China, and 1 in 15,000 in the United States. PKU is quite rare in Japan: 1 in 100,000 (Muntau et al., 2019) and Thailand 1 in 212,535 births (Chaiyasap et al., 2017). This disorder is a rare autosomal recessive and is inherited in accordance with Mendel’s first principle. Both male and female have the same chances of inheriting PKU.

PKU, if undiagnosed, can lead to ataxia (Wang et al., 2019), eczema (Van Wegberg, 2017) intellectual disability (Van Wegberg, 2017), microcephaly (Van Wegberg, 2017), neurological and psychosocial problems (Wang et al., 2019), and seizures (Wang et al., 2019; and Van Wegberg, 2017). Less than 5% of PKU patients have a deficiency in tetrahydrobiopterin (BH4) synthesis, and essential cofactor for hydroxylation of phenylalanine to tyroxine (Wang et al., 2019). Reduction of PAH leads to increase of phenylalanine in blood and brain (Van Wegberg, 2017). Four classification of PKU (Neto et al., 2019) severe (higher than 1,200 µM), moderate (between 900 and 1,200 µM), mild (between 600 and 900 µM), and non-PKU mild (lower than 600 µM) (Neto et al., 2019).

Screening for PKU in newborns or older is an important method to diagnose this disorder (Muntau et al., 2019). Several techniques i.e., Sanger Sequencing (SS) and Next Generation Sequencing (NGS) are available for screening this disorder. Drugs are available to treat PKU are BH4, Large Neutral Amino Acids (LNAAs) (Daly et al., 2019; and Taslimifar et al., 2019), Specific Nutrient Combination (SNC) (Bruinenberg et al., 2019), and gene therapy (Durrer et al., 2017; and Pan et al., 2016). Use of these drugs can help patients with PKU to live as normal people. In the future, gene therapy can be a useful tool for eliminating PKU disorder. Gene therapy includes Adeno-associated virus (AAV) vectors and Fold-dCas9 system.

In this review article, the author describes the progress in a study of PKU focused on genetic aspects, diagnosis, treatments including such as SNC and gene therapy. Genetic aspects include the PAH gene and mutations in the PAH gene, while diagnosis could include SS and NGS. Finally, treatment of PKU is comprised of drugs and gene therapy.

2. Methodology

The author searched the PubMed Database at National Center for Biotechnology Information (NCBI) for articles on PKU. Searches included the PAH gene, mutations in the PAH gene, diagnosis tools, drugs, and gene therapy for PKU. All articles were open access and in English published between 2014 and 2019. The author references were included in this review. Other relevant publications were also included in the searches.

2.1. Genes in phenylketonuria

The PAH gene supplies directives for making PAH. Mutations can arise in gene and cause damage of protein (Neilan, 2018; and Neilan, 2017a, b and c) of PKU patients. These mutations cause permanent changes in DNA in the PAH gene. Mutations in the PAH gene result in four classes for PKU: severe, moderate, mild, and non-PKU mild hyperphenylalaninemia (Table 1) (Van Wegberg, 2017).

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<th>Table 1: Classifications in PKU</th>
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Note: HPA = hyperphenylalaninemia; and PKU = phenylketonuria.

3 Online Mendelian Inheritance in Man, https://omim.org/ entry/ 621600
2.2. The PAH gene

Official name of the PAH gene is phenylalanine hydroxylase. Other names include L-Phenylalanine, tetrahydrobipterin: oxygen oxidoreductase (4-hydroxylating), PH4H_HUMAN, Phenylalaninase, Phenylalanine-4-Hydroxylase, Phenylalanine-4-Monooxygenase, and PKU1. The human PAH gene occupies chromosome 12q at position 23.2, 12q23.2. The PAH gene spans 90 kb, contains 13 exons, and includes base pairs 102,836,885 to 102,958,410. The PAH gene encodes an aromatic amino acid hydroxylase protein family. (Chaiyasap et al., 2017). The encoded PAH enzyme converts phenylalanine to tyrosine.

2.3. Mutations in the PAH gene

Neto et al. (2019) found that mutations in the PAH gene can include deletion with frame shift, in-frame deletion, large deletion, missense, nonsense, and splicing sites (Table 2) (Wang et al., 2017). There have been identified PAH 1101 variants in PAHvdb Database about 50% are missense mutations. Gu et al. (2014) suggested that mutations in the PAH gene consist of 13.5% deletions, 1.8% insertions, 5% nonsense, 60.5% missense, and 11% splicing site. (Pecimonova et al., 2019) The p.Arg408Trp is the most common mutation in many populations, Gundorova et al. (2019) suggested that the pathogenic variant p.Arg408Trp is widespread in Europe. The p.Arg408Trp is not frequently found in Asia. 80 to 85% of the p.Arg408Trp mutation in the world is in the Baltic countries: Estonia, Latvia, and Lithuania. 50% is in the East European countries: Czech Republic, Hungary, Russia, and Slovakia. In Balkans, the frequency was 30% to 40%. In Western and Middle Europe, the pathogenic variant p.Arg408Trp mutation is 5% to 25%. Other pathogenic variants may include p.Arg261Gln, p.Tyr414Cys, and p.Ser349Pro (Gundorova et al., 2019).

2.4. Diagnosis tools for phenylketonuria

Newborn screening for PKU diagnosis depends on diagnosis tools such as Guthrie method, fluorometric method SS and NGS. SS is the method of choice to sequence short pieces of DNA and causative mutations. NGS is a tool for diagnosing newborns with inborn errors of metabolisms (IEMs) (Wang et al., 2019) and to sequence whole exome and genome levels. In addition to SS and NGS, tandem mass spectrometry (MS/MS) is a useful tool for detecting IEMs such as phenylalanine and tyrosine (Gu et al., 2014). Single-gene testing or

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multiple gene panel can also be a useful tool for diagnosing PKU disorder (Regier and Greene, 2000). New methods such as MassARRAY and minisequencing have also been developed.

The newborns’ blood samples were collected by heel prick on filter paper that was subsequently tested using Guthrie method and/or the fluorometric method. The positive cases underwent plasma amino acid analysis using high performance liquid chromatography to determine phenylalanine levels. Determination of urinary pterin levels can be used to screen BH$_2$-deficiency (Chaiyasap et al., 2017).

Chaiyasap et al. (2017) used NGS method for diagnosing PKU and BH$_2$-deficiency. The authors took three milliliters of peripheral blood from each patient and extracted genomic DNA by using Gentra Puregene Blood kit (Qiagen, Hilden, Germany). The extraction process was according to the manufacturer’s protocol. Genomic DNA was for exome sequencing using service from Macrogen, Inc (Seoul, South Korea). The preparations of samples were according to Agilent SureSelect Target Enrichment kit (Agilent Technologies, Santa Clara, CA, USA) preparation guide. Sequencing for libraries was with HiSeq2000 or HiSeq2500 sequencer. Aligned for the result sequences were according to the human genome sequence using Burrows-Wheeler Alignment. The authors used Picard software for making and removing duplicate sequences. For data quality assessment, genotyping, and variant calling, the authors used Genome Analysis Toolkit (GATK3.v4). For variant annotation, SnpEff_v.4.1 was used. With this method, found mutations in the PAH gene (Chaiyasap et al., 2017). It shows that NGS is an appropriate molecular genetics technique for diagnosing mutations in the PAH gene.

The Life Technologies can help for the sample preparation and SS. The BigDye Terminator v.1.1 Cycle Sequencing kit (Gu et al., 2014) and by an ABI PRISM 3130XL (Thermo Fisher Scientific) (Tolve et al., 2018) can perform SS. An ABI 3730 can run sequencing after ethanol purification.$^8$ A total of 42 exons of PKU, BH$_2$-deficiency associated genes are needed to be analyzed.$^7$ MS/MS testing of blood spots can be performed using the NeoBase$^TM$ non-derivatized MS/MS kit (PerkinElmer, MA, USA) (Wang et al., 2019). The NGS procedures can also use Exeter Rapid Capture Enrichment Reference Guide. Runs can use Miseq DX platform and data analysis can use BaseSpace Variant Interpreter (Illumina, San Diego, CA, USA) (Tolve et al., 2018). NGS is useful for the clinical genetic analysis of IEMs diagnosis (Chaiyasap et al., 2017).

In newborn screening, blood taken from heel stick was spotted on Whatman 903 filter paper and dried for screening need. The time of sampling was between the 3rd and 10th day of life. Dried blood spots were pre-processed following the instruction of NeoBase$^TM$ none-derivatized MS/MS kit (PerkinElmer, MA, USA), and then they were analyzed by using TQD MS/MS system (Waters, MA, USA) and NeoBase$^TM$ non-derivatized system. The analyses can include phenylalanine, tyrosine, and acylcarnitines. The normal ranges and cut-off values of analytes in the manual of the NeoBase$^TM$ non-derivatized MS/MS kit (Cat. 3040-001Z, PerkinElmer, MA, USA) and from the worldwide collaborative project can be applied. Suspected positive cases were recalled for the repeated test by MS/MS (Guo et al., 2018).

Sarker et al. (2019) validated the method of liquid chromatography tandem mass spectrometry (LC-MS/MS) for diagnosis of IEMs with the target for three different age groups. The authors used a second-tier test, gas chromatography-mass spectrometry, to confirm patients with PKU. There were seven patients older than one year in test of LC-MS/MS. Gas chromatography-mass spectrometry confirmed three patients with phenylketonuria. About 43% of IEM came from consanguineous family across the globe. Sarker et al. (2019) collected blood specimens by heel prick method. The authors spotted about 75 µL of blood on Whatman$^TM$ Generic Multipart filter paper (GE Healthcare Westborough, MA, USA) to prepare a dried blood spot card for LC-MS/MS analysis. The authors collected about 80% specimens between 24 hours and 72 hours after birth and collected about 20% specimens between day 4 and day 7 after birth. The collections of blood specimens for older children were after 4-hour fasting with standard venipuncture method. Then prepared a dried blood spot card by spotting about 75 µL blood on Whatman$^TM$ 903 filter paper (Sarker et al., 2019). In addition, 5 mL fasting urine specimens also collected for urinary metabolic screening tests. Tests include ferric chloride test, 2,4-Dinitrophenylhydrazine test, Cyanide nitroprusside test, and test for urine reducing sugar and ketone bodies. Drying of the dried blood specimen cards were as long as four hours at room temperature and stored at −70°C in plastic ziplock bags with desiccants until analysis was done. After metabolic screening tests, about 2 mL urine specimens were stored at −70°C for second-tier tests such as metabolic profiling using gas chromatography-mass spectrometry (Surna1y and Mijamammni, 2017).

Tolve et al. (2018) suggested a procedure for diagnosing PKU: minisequencing. The authors have optimized the detection of the 24 probes in four multiplex PCR followed by four single-nucleotide extension reactions
and four electropherograms. The method showed 100% sensitivity and 100% specificity. Tolve et al. (2018) successfully tested the method on dried blood spots. This genotyping assay needs 48 hours to do the test and is able to characterize both alleles in more than 50% of the PKU subjects (Tolve et al., 2018). This method is a useful tool for detecting PKU disorder.

SS and MassARRAY are techniques for detecting genetic variants in IEMs. SS, which is applied for the known gene locus in genetic analysis, is a golden standard to determine the DNA sequence. Both NGS and MassARRAY, which can be used for genetic analysis of spectrum of gene, are high throughput sequencing methods. Moreover, MassARRAY has been used more widely and longer to detect known mutations. In addition, it is reliable and the cost is less (Guo et al., 2018).

Regier and Greena (2000) suggested single-gene testing or multiplegene panel for diagnosing PKU disorder. For single-gene testing, sequence analysis of PAH is performed first followed by gene-targeted deletion/duplication analysis if only one or no pathogenic variant is found. In addition, a multiplegene panel that includes PAH and other genes of interest may also be considered (Regier and Greene, 2000).

The establishment of the diagnosis of PAH deficiency in a proband is based on two criteria. First, it is a plasma phenylalanine concentration persistently above 120 µmol/ L (2mg/dL) and altered ratio of phenylalanine to tyrosine in the untreated state with normal BH₄ cofactor metabolism. Second, it is based on the finding of biallelic pathogenic variants in the PAH gene by molecular genetics testing (Regier and Greene, 2000). The follow-up testing commences for the second time positive cases, including biochemical tests or genetic analysis. The recall and follow-up protocol in the guidelines “Follow-Up Testing for Metabolic Disease Using Tandem Mass Spectrometry” may be used. Specialists based on the clinical symptom, screening test, and biochemical and genetic analysis make definitive diagnosis. The parents of all cases with definitive diagnosis are informed, and they are referred to specialists for the treatment (Guo et al., 2018).

2.5. Treatments
Treatment of PKU may be performed with such as dietary therapy, sapropterin, LNAA, and gene therapy (Table 3). Dietary restriction of phenylalanine is predominantly treatment for PKU. Sapropterin has been successful treatment in BH₄ responsive patients. However, sapropterin can only be beneficial about 30% of all PKU patients. LNAA has been explored and still needs long-term studies to understand the full effect of this treatment. Gene therapy may be a useful tool for treating PKU in the future. Research has been conducted to get the right tool for treating PKU patients with genetic manipulation techniques.

Dietary therapy is associated with a risk for nutritional deficiencies. For example, growth retardation and early-onset osteoporosis can be caused by deficiencies in specific substances, such as minerals or vitamins, in a phenylalanine-restricted diet (Guo et al., 2018). A diet therapy limits food with high phenylalanine; dairy products, eggs, meat, and fish. However, huge social and economic burdens are imposed by the diet therapy. In addition, compared with age-matched individual without PKU, patients with PKU who consume a phenylalanine-restricted diet may not achieve their full neurodevelopment potential. Accordingly, various alternative therapeutic strategies have been proposed for PKU treatment (Guo et al., 2018). It could experience deficits in cognitive functioning, for instance in processing speed, attention, working memory, and social-cognitive functioning (Bruinenberg et al., 2019). Dietary therapy is the basis of PKU management. It consist of three parts: natural protein restriction, Phe-free-L-amino acid supplements, and low protein food. Treatment should be commenced before the age of 10 days. Many countries will require change in timing of national NBS, logistical and diagnostic procedures (Van Wegberg, 2017). However, treatment with dietary therapy is ineffective in patients with PKU exhibiting BH₄ deficiencies (Guo et al., 2018).

BH₄ can help stabilize misfolded mutant enzymes and prevent their proteolysis, and increase enzyme activity was demonstrated by changing the dose and formulation of BH₄ (Guo et al., 2018). BH₄ is also involved in catecholamine, serotonin, and nitric oxide biosynthesis (Sarker et al., 2019). BH₄ is a cofactor in neurotransmitter synthesis that include dopamine, epinephrine, norepinephrine, and serotonin. Patients with high residual activity of the PAH enzyme have a greater probability of BH₄ response, but a minority of patients with classical PKU also may benefit from BH₄ therapy. Efficacy and safety of BH₄ therapy has been proved in children with <4 years old (Van Wegberg, 2017). Synthetic BH₄ is commercially available. In 2007, the US approved sapropterin dihydrochloride as an adjuvant therapy for PKU. Furthermore, when administering at doses of 5-25 mg/kg/day for up to 22 weeks, BH₄ caused a decrease in the plasma phenylalanine concentration in approximately 32-50% of treated participants, as illustrated by analyzing
the effects of cofactor treatment in clinical trials (Guo et al., 2018). It is important to give an early and precise diagnosis for PKU patients to provide a proper treatment to the patients (Chaiyasap et al., 2017).

LNAA may decrease plasma phenylalanine concentration in affected adolescents and adults. However, women of childbearing age should not take LNAA (Sarker et al., 2019). LNAA can be useful to adult patients with PKU that cannot tolerate a phenylalanine-restricted diet. Four L-type amino acid transporters are present in the transport system through which certain molecules, such as LNAA, are transported to the brain. These transporters transport LNAA, including the branched-chain amino acids valine, leucine, and isoleucine; the aromatic amino acids tyrosine, triptophan, and phenylalanine. These also include some other amino acids, such as threonine, methionine, and histidine. Thus, to cross the blood-brain barrier, these molecules compete with each other to bind the transporters based on their plasma concentrations. High LNAA supplementation thus inhibits plasma phenylalanine transport and reduces brain phenylalanine concentrations. Additional supplementation of LNAA was of limited value, but it may be of benefit in those unable to adhere to their phenylalanine-free L-amino acid supplements. Although some centers routinely administer phenylalanine-free LNAA supplements to older patients who are unable to adhere to dietary treatment. These supplements remained untested in children under the age of 11 years, and not reports in use in pregnant women. In addition, further research is required to ascertain the ideal dosages and amount of each specific L-amino acid within the LNAA supplement (Guo et al., 2018).

Bruinenberg et al. (2019) suggested that a SNC was postulated to improve brain function in PKU. The authors examined at three-time points: 3, 6, and 9 months for memory and motor function. In the Novel Object Recognition (NOR) test, Bruinenberg et al. (2019) found that PKU mice, despite being subjected to high phenylalanine conditions, could master the task on all three time points when supplemented with SNC. Under low phenylalanine conditions, PKU mice on control diet could master the NOR at all three time points, while PKU mice on the SNC supplemented diet could master the task at time points 6 and 9 months. SNC supplementation did not consistently influence the performance in the open field (OF), spatial object recognition (SOR) test or balance beam (BB) in PKU mice. The low phenylalanine diet was able to normalize concentrations of norepinephrine and serotonin. However, these neurotransmitters were not influenced by SNC supplementation. This study demonstrates that both a long-lasting low phenylalanine diet, the diet enriched with SNC, as well as the combined diet was able to ameliorate some, but not all of these PKU-induced abnormalities. These diets seem to improve some, but not other domains that are impaired in the BTBR PKU mouse model. The authors demonstrate that a long-term intervention study in BTBR PKU mice improves NOR, while a long-term intervention with a low phenylalanine diet nearly normalizes serotonin and norepinephrine levels. Bruinenberg et al. (2019) suggest that future research should be aimed at developing an optimal nutritional intervention to target brain function in PKU patients (Bruinenberg et al., 2019).

Enzyme therapy for enzyme substitution for PKU patients has been available. Replacement with PAH fusion proteins is effective in mouse studies. However, PAL-replacement therapy appears to be a more promising approach for treating PKU. In addition, this therapy also converts excess phenylalanine into readily excreted and less toxic products. However, mouse models of PKU showed that injection administering or oral version of the PAL enzyme has some limitations. For example, injection of PAL activates immune responses, whereas oral administration of PAL is associated with enzyme degradation. It reduces the effectiveness of the therapy. Notably, conjugation of PAL with polyethylene glycol (PEG) decreased PAL-induced immune responses. Clinical trials on PKU patients are promising. Phase I and II trials using injectable recombinant PAL conjugated with PEG has shown a reduction in the phenylalanine levels (Guo et al., 2018).

2.6 Genetic manipulation

Genetic manipulation techniques are promising techniques for treating PKU disorder. Multiple methods are available. These techniques can include such as adenoviral vectors, AAV, and clustered regularly interspaced palindromic repeats (CRISPR/Cas9) system (Nelwan, 2019).

Restriction of dietary phenylalanine is for preventing neuronal damage in PKU patients. Deprived compliance by PKU patients and the heavy economic burden confront the dietary therapy. Although dietary therapy recovers cognitive performance, other neuropsychological parameters such as attention, and executive functions are impaired compared to healthy controls. Diet therapy, BH4, LNAA, SNC, and enzyme therapy may be beneficial for PKU patients. However, the ideal method would be to edit variants in the PAH gene. Gene therapy may help for editing the mutated gene (Pan et al., 2016).
PKU gene therapy in animal models has been developed for more than two decades (Pan et al., 2016). Adeno virus, AAV, and lentivirus are viral vectors that have been developed for treating PKU disorder (Guo et al., 2018). However, the temporary recovery a very low gene transfer rate limited this approach. Adenovirus, AAV, and lentivirus still need modification to be used for gene therapy in PKU patients.

Pan et al. (2016) used a modified CRISPR system which employs the fusion of inactive Cas9 (dCas9) and the FokI endonuclease (FokI-dCas9) to correct p.Arg408Trp in the PAH gene. This technique can correct allele frequency 21.4%. This correction could rescue residual PAH activity and restore normal function. Co-expression of a single guide RNA plasmid, and the presence of a single stranded oligodeoxynucleotide in PAH- c.1222>T COS-7 cells –an in vitro model for PKU—corrected the PAH variant and restored PAH activity (Nelwan, 2019). It seems that the FokI-dCas9 system is a promising tool for treating PKU disorder.

3. Conclusion
PKU has an autosomal recessive inheritance pattern in accordance with the law of segregation. Mutations in the PAH gene result in PKU disorder: severe, moderate, mild, and non-PKU mild HPA. To control PKU, the diagnosis has a significant role. Diagnosis techniques for PKU include SS, NGS, MS/ MS. Dietary and enzyme therapies can help people with PKU. Drugs for PKU patient include BH₄, LNAA, and SNC. Genetic manipulation techniques are potential and useful tools to control PKU in the future. Adenovirus vectors, AAV vectors, and FokI-dCas9 system may be useful for treating PKU disorder.

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Conflicts of interest
The author has no conflicts of interests regarding the content of this article.

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