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MOLECULAR GENETICS OF PHENYLKETONURIA AND THE DEVELOPMENT OF A NEW THERAPEUTIC APPROACH: FROM PHENOMICS TO THERAPY

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Phenylketonuria (PKU) is an inborn error of amino acid metabolism caused by deficiency of phenylalanine hydroxylase (PAH). PAH is a hepatic enzyme that catalyzes the hydroxylation of phenylalanine (Phe) to tyrosine (Tyr). The cofactor tetrahydrobiopterin (BH_4) is required for this process. A defect of PAH leads to elevated Phe levels in blood and brain, resulting in neurological and neuropsychological defects. Dietary Phe restriction is the main treatment for PKU to prevent neuronal damage. Supplementation with BH_4, glycomacropeptide and/or large neutral amino acids (LNAAs) can also alleviate symptoms in some PKU patients. However, poor compliance, economic burden and the limited therapeutic effects of these treatments have rendered new improved therapeutic approaches to PKU desirable.

PKU is an inherited autosomal recessive disorder, and more than 75% of patients are compound heterozygous. Previous studies have shown that the metabolic phenotypes in PKU are highly correlated with the *PAH* genotypes. However, because only hundreds of PKU patients were investigated in the past studies, the correlation between *PAH* genotypes and phenotypes has not been accurate. There are also unresolved issues in the current definition of the *PAH* allelic phenotype values (APV) for PKU phenotype prediction. In this work, we aimed to reinvestigate the current APV and to develop a new system of genotypic phenotype values (GPV) in order to develop better predictors of PKU phenotypes and obtain additional information about genotype-phenotype correlations in PKU patients. The data of a total of 955 *PAH* variants from the locus-specific database *PAH*vdb and genotypes of 10,625 PKU patients from the BIOPKU database were used in this study. Based on these data, we redefined the APV in accordance with the average blood Phe levels and estimated the GPV of the most common genotypes in PKU patients. The new APV and GPV are likely to become highly beneficial for predicting biological and clinical phenotypes in

PKU patients.

The combination of two *PAH* alleles theoretically determines the residual enzyme activity *in vivo* and *in vitro*. Inconsistencies in genotype-phenotype correlations have been observed in compound heterozygous patients, and a particular combination of two *PAH* alleles may produce a phenotype *in vitro* that is different from the expected phenotype possibly due to interallelic complementation. The transient co-expression of two *PAH* variants from previously selected genotypes in mammalian cells was used in this work. PAH activity was measured by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), and protein expression determined by western blot. The actual PAH residual activities of these genotypes were compared with the predicted PAH activities from *PAH*vdb and phenotypes from the BIOPKU database. The co-expression of two distinct *PAH* variants revealed possible dominance effects (positive or negative) by one allele affected the residual activity in *PAH* compound heterozygotes variants.

Furthermore, the above-mentioned challenges of the current PKU treatment require an approach to genetically repair the *PAH* variant with efficacy. The CRISPR/Cas9 system is a recently developed genome editing technique that can be used to correct site-specific mutations in target genes. However, high chances of off-target effects and low homologous recombination repair (HDR) efficiency have limited the application of the CRISPR/Cas9 system in precision medicine. In this work, the *PAH*_c.1222C>T COS-7 cell line was generated as a PKU model *in vitro*, and the CRISPR RNA-guided *Fok*I Nuclease system (FokI-dCas9 system), which can decrease off-target effects and increase HDR efficiency, was used. RS-1 as an HDR enhancer was also used to increase the efficiency of gene therapy. After treatment, both the PAH activity and protein expression of *PAH*_c.1222C>T COS-7 cells were notably rescued. This study suggests that the *Fok*I-dCas9 system is a promising genome editing technique to treat PKU and also has tremendous potential as a precise medicine for other inherited disorders.

In summary, this work provides new predictors of PKU phenotypes based on genotypes, a possible explanation for the inconsistency in PKU genotype-phenotype, and a promising approach for genetic repair of *PAH* variants. All of these will hopefully benefit the diagnosis and treatment of PKU.