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Massive parallel sequencing as a new diagnostic approach for phenylketonuria and tetrahydrobiopterin-deficiency in Thailand

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Abstract

Background: Hyperphenylalaninemia (HPA) can be classified into phenylketonuria (PKU) which is caused by mutations in the phenylalanine hydroxylase (*PAH*) gene, and BH4 deficiency caused by alterations in genes involved in tetrahydrobiopterin (BH4) biosynthesis pathway. Dietary restriction of phenylalanine is considered to be the main treatment of PKU to prevent irreversible intellectual disability. However, the same dietary intervention in BH4 deficiency patients is not as effective, as BH4 is also a cofactor in many neurotransmitter syntheses.

Method: We utilized next generation sequencing (NGS) technique to investigate four unrelated Thai patients with hyperphenylalaninemia.

Result: We successfully identified all eight mutant alleles in PKU or BH4-deficiency associated genes including three novel mutations, one in *PAH* and two in *PTS*, thus giving a definite diagnosis to these patients. Appropriate management can then be provided.

Conclusion: This study identified three novel mutations in either the *PAH* or *PTS* gene and supported the use of NGS as an alternative molecular genetic approach for definite diagnosis of hyperphenylalaninemia, thus leading to proper management of these patients in Thailand.

Keywords: Next generation sequencing, Exome, Hyperphenylalaninemia, Phenylketonuria, Tetrahydrobiopterin deficiency, Newborn screening

Background

Phenylketonuria (PKU) is an autosomal recessive metabolic disorder, characterized by progressive intellectual disability, mousy odor, autism, seizures, eczema and motor deficits [1]. The incidence of PKU in Caucasians is approximately 1:10,000 births [2]. Early diagnosis of the disease is beneficial since intellectual disability can be prevented by certain therapeutic intervention [3]. Generally, measuring blood phenylalanine levels provides a reliable and practical diagnosis for PKU. If the blood levels of phenylalanine exceed

120 $\mu\text{mol/l}$ (2 mg/dl), the individual is considered to be hyperphenylalaninemia (HPA) and needs further diagnosis [4]. The majority of Caucasian patients with excessive phenylalanine levels are classified as classic PKU (MIM#261600), which is caused by loss-of-function mutations in the phenylalanine-4-hydroxylase (*PAH*) gene. About 2% of Caucasian cases are BH4-deficient [5], caused by mutations in the genes involved in BH4 biosynthesis consisting of 6-pyruvoyl-tetrahydropterin synthase (*PTS*), GTP cyclohydrolase I (*GCHI*), quinoid-dihydropteridine reductase (*QDPR*), pterin-4- α -carbinolamine dehydratase 1 (*PCBD1*), and sepiapterin reductase (*SPR*) [5–7]. Interestingly, the incidence of PKU in Thailand is much less with a report of 1:212,535 in

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newborns [8] and the proportion of Thai patients with HPA who have BH4 deficiency remains unknown.

Although both PKU and BH4-deficiency patients show high blood phenylalanine levels and progressive intellectual disability, they respond to the treatment differently. Patients with PKU need additional supplement and are required to follow a diet that limits food with high phenylalanine such as dairy products, eggs, meat and fish [9]. This diet therapy is highly recommended to continue throughout their life to prevent behavioral disorders, cognitive, and emotional dysfunction [10]. On the other hand, patients with BH4 deficiency require BH4 supplement, generally 2–20 mg/kg/day [11, 12], as BH4 is also a cofactor in neurotransmitter synthesis, such as dopamine, serotonin, norepinephrine and epinephrine. Thus, deficiency of BH4 leads to not only elevated phenylalanine levels, but also deficiencies of these neurotransmitters [11]. Therefore, it is important for physicians to give an early and precise diagnosis, whether HPA patients are PKU or BH4-deficiency, to provide a proper treatment to the patients.

Neonatal screening for PKU in Thailand was started in 1996 by Department of Medical Sciences, Ministry of Public Health, aiming to early detect and provide treatment to PKU patients through a screening program [8]. The newborns' blood samples were collected by heel prick on filter paper which was subsequently tested using the Guthrie method and/or the fluorometric method. The positive cases underwent plasma amino acid analysis using high performance liquid chromatography to determine phenylalanine levels [13]. Determination of urinary pterin levels can be used to screen BH4-deficiency. Unfortunately, it is not an integral part of newborn screening in Thailand, which may lead to late diagnosis in some cases [13].

Conventional genetic diagnosis for PKU and BH4 deficiency relies on Sanger sequencing of the entire coding regions of the responsible genes. A total of 42 exons of PKU, BH4-deficiency associated genes are needed to be analyzed. This approach is time consuming and labor intensive [14]. In contrast to Sanger sequencing, next-generation sequencing (NGS) can provide high throughput information from massive parallel sequencing from a single procedure [15] which is faster and more efficient. This technology has been widely used for research purpose but increasingly applied for genetic diagnosis, including PKU and BH4-deficiency [14, 16–19].

In this study, we apply next generation sequencing technique to investigate four unrelated Thai patients with hyperphenylalaninemia. The disease-causing variants were identified in all patients.

Methods

Patients

Patients I, II, III and IV are children from four unrelated non-consanguineous families. Their neonatal phenylalanine

screening levels were 21.4, 53.11, 37.9, and 42.6 mg/dl, respectively (normal <20 mg/dl). After the diagnosis of hyperphenylalaninemia, all patients were treated with low-phenylalanine diet. For those diagnosed with BH4 deficiency, BH4 supplement was given. More clinical details are in Table 1.

Written informed consent for genetic analysis was obtained from the parents of the patients.

Exome sequencing

Three milliliters of peripheral blood were taken from each patient after informed consent. Genomic DNA was extracted by using Genra Puregene Blood Kit, Qiagen (Qiagen, Hilden, Germany). The extraction process was done according to the manufacturer's protocol. Genomic DNA from four patients were sent for exome sequencing using service from Macrogen, Inc. (Seoul, South Korea). The samples were prepared according to Agilent SureSelect Target Enrichment Kit (Agilent Technologies, Santa Clara, CA) preparation guide. The captured libraries were sequenced with Illumina HiSeq 2000 or HiSeq2500 Sequencer. The result sequences were aligned to the human genome reference sequence (UCSC hg19) using Burrows-Wheeler Alignment (bwa-0.7.10, <http://bio-bwa.sourceforge.net/>) [20]. Picard software (picard-tools-1.118, <http://broadinstitute.github.io/picard/>) was used for marking and removing duplicated sequences. Genome Analysis Toolkit (GATK3.v4, <https://www.broadinstitute.org/gatk/>) [21] was used for data quality assessment, genotyping and variant calling. Finally, SnpEff_v.4.1 (<http://snpeff.sourceforge.net/>) [22] was used for variant annotation.

Genotype analysis

All homozygous and compound heterozygous variants with <1% allele frequency in global population and located on genes associated with hyperphenylalaninemia (*PAH*, *PTS*, *GCHI*, *QDPR*, *PCBD1*, *SPR* and *GCHFR*) were included. The variants with >1% of the 165 unrelated Thai exomes, our in-house database, were excluded. The novel variants were confirmed by Sanger sequencing. The patients' genomic DNA was amplified by polymerase chain reaction (PCR), using primers specific to the site of the mutation (Table 2). The PCR products were then sent for Sanger sequencing by Macrogen Inc. (Seoul, South Korea). The sequences were analyzed by Gene Codes Sequencer software (v.5.4.1) (Gene Codes Corporation, MI). The sequencing data were compared to unaffected control sequences. ClustalX 2.1 (<http://www.clustal.org/clustal2/>) [23] was used for amino acid conservative analysis. ExAC Browser: Exome Aggregation Consortium Database (<http://exac.broadinstitute.org/>) was used to confirm the novelty of the variants [24].

Table 1 Clinical and molecular findings

Patient	I	II	III	IV			
Sex	F	M	F	F			
Age at definite diagnosis (mo)	1	21	6	1			
Age at last visit (mo)	51	36	16	5			
Development	normal	delayed	normal	normal			
Mutated gene	<i>PAH</i>	<i>PTS</i>	<i>PTS</i>	<i>PTS</i>	<i>PTS</i>	<i>PAH</i>	<i>PAH</i>
Genomic position (hg19)	12:103,248,934	11:112,099,388	11:112,101,362	11:112,103,916	11:112,104,166	12:103,234,294	12:103,249,009
dbSNP ID	novel	rs104894275	rs370340361	novel	novel	known [18]	rs62514927 [30]
Zygoty	homozygous	compound het	compound het				
Reference allele (REF)	–	A	C	A	A	C	T
Alternate allele (ALT)	T	G	T	C	G	G	C
No. reads with REF in proband	0	25	55	21	14	114	55
No. reads with ALT in proband	51	31	46	16	5	101	33
Mutation type	frameshift ins	missense	missense	missense	missense	splice acceptor	exonic splicing enhancer
cDNA change	c.686_687insA	c.155A > G	c.200C > T	c.274A > C	c.326A > G	c.1200-1G > C	c.611A > G
Protein change	p.Asp229Glufs*54	p.Asn52Ser	p.Thr67Met	p.Asn92His	p.Asn109Ser	N/A	p.Tyr204Cys
Prediction SIFT	N/A	damaging	damaging	damaging	damaging	N/A	damaging
Prediction PROVEAN	N/A	deleterious	deleterious	deleterious	deleterious	N/A	neutral

Results

All four patients showed approximately 20,000 DNA variants across the exome (Table 3). Mutation analysis successfully identified all eight mutant alleles in PKU or BH4-deficiency associated genes. Patients I and IV had mutations in the *PAH* gene, while Patients II and III had mutations in the *PTS* gene. The detailed characteristics of the mutations are summarized in Table 1. Sanger sequencing of the novel mutations confirmed the presence of the mutations found by exome sequencing (Fig. 1a and b). The amino acid alignment from ClustalX revealed that the two novel missense mutations in the *PTS* gene identified in Patient III were highly conserved (Fig. 1c).

Discussion

Newborn screening program for HPA is important since severe intellectual disability from PKU or BH4 deficiency is preventable with proper treatment. Although Thailand

has established Neonatal Screening Program for PKU for many years, screening positive patients do not undergo a test for BH4-deficiency due to the unavailability of pterin analysis in Thailand [25]. In addition, BH4 loading test cannot distinguish patients with BH4 deficiency and BH4-responsive PKU [5]. The conventional Sanger sequencing has been the gold standard test for a molecular approach in the genetic diagnosis of inherited disorders. This direct approach is suitable for sequencing hot-spot point mutations or small genes [26]. However, it is rather costly and time consuming for diagnosis of genetic disorders which involve large genes or multiple genes, including genes associated with PKU and BH4-deficiency. The costs associated with NGS are rapidly decreasing. Therefore, we utilize NGS as a diagnostic tool in our study as it has been demonstrated to be effective in several previous reports [14, 16–19].

All eight mutant alleles were successfully identified. Of these, three have never been previously reported. Patient I has a novel homozygous frameshift mutation (c.686_687insA) in the *PAH* gene. Patient II has two compound heterozygous missense mutations (c.155A > G and c.200C > T) in the *PTS* gene. The c.155A > G mutation has been previously reported in Chinese patients [27] and the c.200C > T in Italian patients [28]. Patient III has two novel compound heterozygous missense mutations (c.274A > C and c.326A > G) in the *PTS* gene. Both mutations are predicted to be damaging and deleterious from

Table 2 Primer sequences for Sanger sequencing of novel mutations

Primer name	Sequence	Tm (°C)
<i>PAH_E6_F</i>	5'-GAT GGC AGC TCA CAG GTT CT-3'	60.5
<i>PAH_E6_R</i>	5'-CTT GTC TTC CCC TTC CCT CT-3'	60.5
<i>PTS_E5-6_F</i>	5'-TGA TAA GGT GAG GTT TAG AGG C-3'	60.1
<i>PTS_E5-6_R</i>	5'-CTC CAG AGC ACA ATG TGT ACG-3'	61.2

development and occasional abnormal movement (orofacial dyskinesia and chorea). This might be due to the age of Patient II when diagnosed, which was one year and nine months old. On the other hand, Patient III was given BH4 supplement at the age of 6 months after the diagnosis was made. The patient showed normal development at her last visit when she was one year and four months old. This demonstrated the importance of providing early and precise diagnosis to HPA patients.

Only 2% of Caucasian patients with HPA are BH4 deficient. Interestingly, of our four consecutive patients, two are BH4 deficient. The total number of patients are very small. However, since the treatment to prevent intellectual deficit is different between both types, it is warrant to determine the proportion of PAH and BH4 deficiency among Thai patients with HPA. If a significant percentage of Thai patients with HPA have BH4 deficiency, it should be justified to perform genetic testing using NGS as soon as HPA is identified.

Conclusion

This report represents the first study in Thailand to successfully use next-generation sequencing to detect causative mutations in PKU and BH4-deficiency cases, which allow physicians to provide a precise diagnosis and proper effective treatment to the patients. This approach is ready to substitute conventional sequencing for genetic diagnosis of patients with hyperphenylalaninemia.

Abbreviations

BH4: tetrahydrobiopterin; HPA: hyperphenylalaninemia; NGS: next generation sequencing; PAH: phenylalanine hydroxylase; PKU: phenylketonuria

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Availability of data and materials

The data sets supporting the results of this article are available in the NCBI SRA database, which can be accessed via NCBI BioProject repository (accession number: PRJNA401212, <https://www.ncbi.nlm.nih.gov/bioproject/401212>).

BioSample accession numbers: SAMN07599868, SAMN07599869, SAMN07599870, SAMN07599871.

Authors' contributions

PC, KS and VS conceived and designed the experiments. Clinical data were collected by KS and VS. PC, CS and CI performed genetic analysis. Data analysis and interpretation were performed by PC, CI, AS, KS and VS. PC, CS and AS were responsible for drafting the manuscript. KS and VS critically revised the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the institutional review board, Faculty of Medicine, Chulalongkorn University. The written informed consent for genetic analysis was obtained from the parents of the patients.

Consent for publication

The parents gave permission for the publication of the patient's clinical details and images.

Competing interests

The authors declare that they have no competing interests.

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