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Fast clinical molecular diagnosis of hyperphenylalaninemia using next-generation sequencing-based on a custom AmpliSeq™ panel and Ion Torrent PGM sequencing

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ABSTRACT

Hyperphenylalaninemia (HPA) can be classified into phenylketonuria (PKU) and tetrahydrobiopterin deficiency (BH4D), according to the defect of enzyme activity, both of which vary substantially in severity, treatment, and prognosis of the disease. To set up a fast and comprehensive assay in order to achieve early etiological diagnosis and differential diagnosis for children with HPA, we designed a custom AmpliSeq™ panel for the sequencing of coding DNA sequence (CDS), flanking introns, 5′ untranslated region (UTR) and 3′ UTR from five HPA-causing genes (*PAH*, *PTS*, *QDPR*, *GCH1*, and *PCBD1*) using the Ion Torrent Personal Genome Machine (PGM) Sequencer. A standard group of 15 samples with previously known DNA sequences and a test group of 37 HPA patients with unknown mutations were used for assay validation and application, respectively. All variations were confirmed by Sanger sequencing. In the standard group, all the known mutations were detected and were consistent with the results of previous Sanger sequencing. In the test group, we identified mutations in 71 of 74 alleles, with a mutation detection rate of 95.9%. We also found a frame shift deletion p.Ile25Metfs*13 in *PAH* that was previously unreported. In addition, 1 of 37 in the test group was inconsistent with either the molecular diagnosis or clinical diagnosis by traditional differential methods. In conclusion, our comprehensive assay based on a custom AmpliSeq™ panel and Ion Torrent PGM sequencing has wider coverage, higher throughput, is much faster, and more efficient when compared with the traditional molecular detection method for HPA patients, which could meet the medical need for individualized diagnosis and treatment.

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1. Introduction

Hyperphenylalaninemia (HPA) is an autosomal recessive disorder due to a deficiency in a series of enzymes in the phenylalanine (Phe) metabolic pathway, leading to the accumulation of excessive Phe in the blood (>120 μmol/L). Not only the phenylalanine hydroxylase (PAH, EC 1.14.16.1), but also the PAH active cofactor tetrahydrobiopterin (BH4) participates in the normal metabolism of Phe. Moreover, the synthesis and regeneration of BH4 depend on GTP cyclohydrolase 1 (GTP-CH1, EC 3.5.4.16), 6-pyruvoyl tetrahydrobiopterin synthase (PTPS, EC 4.2.3.12), pterin-4-alpha-carbinolamine dehydratase (PCD, EC 4.2.2.96), and dihydropteridine reductase (DHPR, EC 1.6.99.7), which are encoded by *GCH1* (GenBank NG_008647.1), *PTS* (GenBank NG_008743.1), *PCBD1* (GenBank NG_008646.1), and *QDPR* (GenBank NG_008763.1), respectively [1]. Thus, a defect in the activity of any of the five aforementioned enzymes is likely to result in an abnormal elevation of plasma Phe levels. HPA is mainly classified into two types: the more common phenylketonuria (PKU) caused by mutations in *PAH* (GenBank NG_008690.1) and the less common BH4 deficiency (BH4D) caused by mutations in one of the other four genes. However, these types vary substantially in the severity, treatment, and prognosis of the disease. Patients suffering from BH4D present with progressive neurological dysfunction in addition to HPA and usually die in early childhood without treatment. Because of these consequences, an early and accurate differential diagnosis is essential to avoid irreversible damage to the nervous system. However, the traditional differential diagnosis methods are time-consuming and the results are easily affected by external factors. Therefore, rapid and accurate genetic diagnosis is crucial for a clear diagnosis of disease types, choosing the appropriate treatment in a timely manner, and for genetic counseling and prenatal diagnosis.

The Ion Torrent Personal Genome Machine (PGM), a bench-top sequencer based on semiconductor technology, can generate 10 Mb, 100 Mb, and even 1 G of sequence data per run using a 314™, 316™, and 318™ chip, respectively [2]. It has already been adapted for clinical molecular diagnosis, from specific genes such as *CFTR* [3], *BRCA1*, and *BRCA2* [4] to disease-causing gene panels such as inherited arrhythmia syndromes [5], long QT syndrome [6], and cardiomyopathies [7].

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In this study, we aimed to construct and evaluate the efficiency of a fast and comprehensive assay based on a custom AmpliSeq™ panel and the Ion Torrent PGM sequencer for the detection of mutations in HPA-related genes (*PAH*, *PTS*, *QDPR*, *GCH1*, and *PCBD1*). For this purpose, we analyzed 15 samples with previously known pathogenic mutations for Ion Torrent PGM validation and 37 samples with unknown mutations to test the efficacy of the analysis.

2. Materials and methods

2.1. Subjects

A total of 51 unrelated patients with HPA that had been diagnosed by conventional biochemical methods (blood Phe > 120 μmol/L) and one healthy control sample were enrolled in this study. Samples were divided into two groups: a standard group and a test group. A standard cohort of 14 patients and one healthy control sample had previously undergone genotype analysis of *PAH* or *PTS* using Sanger DNA sequencing in the Department of Medical Genetics at the Capital Institute of Pediatrics [8]. In order to verify the maximum flexibility of our assay, different types of pathogenic mutations included missense, nonsense, deletion, insertion, and splice-site mutations distributed across ten exons of *PAH* and four exons of *PTS*. Six mutations common in the Chinese population (p.Arg243Gln, p.Arg413Pro, p.Arg111Ter, p.Tyr356Ter, and p.Ex6-96A > G in *PAH* and p.Pro87Ser in *PTS*) were also included in this standard group. Sixteen polymorphisms located in different exons of *QDPR*, *GCH1* and *PCBD1* were considered as the positive references for Ion Torrent PGM validation.

The test group consisted of 37 patients clinically diagnosed with HPA but which the genotype was unknown. Thirty-two patients had undergone differential diagnosis via a BH4 loading test, urinary pterin analysis, or QDPR activity assay. In this group, 28 and 4 patients were diagnosed with PKU and BH4D, respectively. In addition, five patients were diagnosed with HPA by newborn screening. Informed consent was obtained from the minors' parents and the study protocol was approved by the Ethical Committee of the Capital Institute of Pediatrics.

2.2. DNA extraction and library construction

Genomic DNA was isolated from peripheral blood leukocytes using the proteinase K phenol/chloroform method. We designed a custom AmpliSeq™ panel using Ion AmpliSeq™ designer software (www.ampliseq.com) to target the coding DNA sequence (CDS), flanking introns, 5′ untranslated region (UTR) and 3′ UTR of *PAH*, *PTS*, *GCH1*, *PCBD1*, and *QDPR*. This panel consisted of two separate PCR primer pools and produced a total of 84 amplicons. An aliquot of each DNA sample (20 ng) was used for library construction by an Ion AmpliSeq™ Library Kit v2.0 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Various samples were distinguished by unique barcodes (Ion Xpress™ Barcode Adapters Kit, Life Technologies) and then pooled in equimolar concentrations.

2.3. Ion Torrent PGM™ sequencing

Pooled, bar-coded libraries were clonally amplified using the Ion OneTouch™ system (Ion OneTouch™ 200 Template kit v2DL, Life Technologies) according to the manufacturer's instructions. Ion sphere particles (ISPs) were enriched using the E/S module. The subsequently enriched template-positive ISPs were loaded onto an Ion 314™ or 316™ chip and sequenced on the PGM using the Ion PGM 200 sequencing kit.

2.4. Data analysis

Data from the PGM runs were processed using the Ion Torrent platform-specific pipeline software Torrent Suite v3.6.2 to generate sequence reads, split the reads according to the barcode, and align the sequences. Human genome reference sequence (hg19) was used for the reference. Coverage assessment was performed using the Coverage Analysis plugin. Variants were called using the variantCaller plugin with the Ampliseq™ and germline high-stringency workflow. The integrative genomics viewer (IGV) was used for visualization.

2.5. Mutation confirmation

Sanger sequencing was used to confirm the DNA variants derived from the PGM. Sequence variants were compared with db SNP (www.ncbi.nlm.nih.gov/projects/SNP), 1000 Genomes (www.1000genomes.org), PAH mutation database (www.pahdb.mcgill.ca), PAHvdb (www.biopku.org/home/pah.asp) and BIOMDB database (www.biopku.org/biomdb). Mutations not reported previously were screened in 100 chromosomes from healthy controls by PCR, restriction enzyme digestion, and agarose electrophoresis in order to rule out a single nucleotide polymorphism in the normal population. Mutation nomenclature followed the recommendation of the Human Genome Variation Society (www.hgvs.org/mutnomen). In addition, MutationTaster [9], MuPro (www.ics.uci.edu/~baldig/mutation.html), SIFT (sift.bii.a-star.edu.sg), and Polyphen2 (genetics.bwh.harvard.edu/pph2) were used to estimate evolutionary conservation and the effects of the amino acid substitution on the structure and function of the protein.

3. Results

3.1. Sequencing data

A custom AmpliSeq™ panel was designed to amplify the CDS, and 5′, and 3′ UTR regions of HPA-related genes (*PAH*, *PTS*, *QDPR*, *GCH1*, and *PCBD1*). Eighty-four amplicons were obtained from every sample using Ampliseq™ PCR, containing approximately 10-kb target regions. To ensure adequate depth of coverage for identification of variant sites, we pooled 10 bar-coded samples and 32 bar-coded samples on the 314™ chip and 316™ chip, respectively, for sequencing. Three separate runs on two 314™ chips and one 316™ chip were performed, containing 52 samples. Two 314™ chips generated an average of 54.59 Mb, with 80.36% and 81.33% aligning to the reference sequence at Q20 (=99% chance correct base called), respectively. Moreover, on average, 95.7% of reads mapped to the target regions and we obtained an average mean depth of coverage of 426× on these two 314™ chips. When 32 pooled samples were sequenced on a 316™ chip, the output was 473.07 Mb, resulting in 395.06 million Q20 bases. 98.4% of the reads mapped to the target regions, with an average mean depth of coverage of 1188× from the 316™ chip.

3.2. Identified mutations in the standard group by Ion Torrent PGM

Among the 52 samples both in the standard group and in the test group, 78/84 of the amplicons (92.9%) had a comparable amplification efficiency and six amplicons had a low amplification efficiency due to high GC content (Fig. 1). In the standard set, 55 variant sites were identified in the five targeted genes, which included 22 known pathogenic mutations, 27 single-nucleotide polymorphisms (SNPs), and one false positive call. For known mutations, we detected 10 missense, three nonsense, four splice site variants, one frameshift deletion, two frameshift insertions, and two non-frameshift deletions (Table 1). All of the pathogenic mutations and polymorphisms selected as the positive references detected by PGM were consistent with the results of previous Sanger sequencing (Table 1, Supplementary Table 1). For each mutation, the mean depth of coverage was 347×. Three patients carried homozygous mutations with an allele frequency of 100%, and nine patients carried heterozygous mutations with an allele frequency of 50.3 ± 3.59% (mean ± SD). No false positive calls were found in the control sample.

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