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MOLECULAR ANALYSES OF PHENYLKETONURIA IN THE INTELLECTUALLY DISABLED CHILDREN FROM FAISALABAD, PUNJAB, PAKISTAN

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ABSTRACT

Background and Objective:
Phenylketonuria (PKU) is a rare inherited metabolic disorder, caused by mutations in the phenylalanine hydroxylase (PAH). It is a treatable disorder if diagnosed earlier in life. The objective was to identify PKU patient(s) amongst the intellectually disabled children.

Methods:
Blood samples (n=100) were collected from intellectually disabled children from Faisalabad, Pakistan. Screening was performed on plasma samples through High Performance Liquid Chromatography (HPLC), and DNA samples were examined for mutation analysis of PAH through direct PCR and SSCP analyses.

Results:
In the current study, 85% consanguinity rate was observed, with the average BMI (16.15 kg/m2) and head circumference(50.21 cm) was observed and the age range of the patients were 8-14 years. Moreover, through biochemical and genetic analyses, not a single PKU patient was identified.

Conclusion:
Based on just one previous report and our small dataset it is concluded that either mutations are not common in the hotspot regions or chances of occurrence of PKU might be rare in Pakistan. Moreover, there is a need of more research on large scale to find the incidence of PKU in Pakistan.

Key Words: Inborn Errors of Metabolism (IEMs), Phenylketonuria (PKU), Phenylalanine hydroxylase (PAH), Pakistan

INTRODUCTION

Phenylketonuria (PKU; OMIM 261600) is a common inborn error of amino acid metabolism causing intellectual disability (ID) in children. It is one of the treatable aminoacidopathies if diagnosed earlier in life. Classical PKU is caused by the deficiency of phenylalanine hydroxylase (PAH; EC number: 1.14.16.1) enzyme due to mutations in the *PAH* (OMIM; 612349). It is an autosomal recessive genetic disorder caused due to disturbance in the metabolism of phenylalanine (Phe), ultimately leads to ID and spectrum of other health related disorders. Deficit or complete failure of *PAH* leads to inactivity of enzyme to convert phenylalanine (Phe) to tyrosine (Tyr). Depending upon the levels of Phe in patients; severity of PKU varies from mild hyperphenylalaninemia (HPA; 120-600 μmol L^{-1}), mild PKU (600-1200 μmol L^{-1}), moderate PKU (900-1200 μmol L^{-1}) to classic PKU (more than 1200 μmol L^{-1}).[1-3] Symptoms of PKU include development of permanent intellectual impairment, behavioral and psychological problems, growth retardation, seizures, epilepsy, eczema, skin rashes and fairer skin color.[4,5]

Cytogenetic location of *PAH* is 12q23.2 and has 13 exons which encodes a protein of 452 amino acids.

According to PAH database (http://www.pahdb.mcgill.ca/), more than 567 mutations have been reported, and overall to date 950 have been identified.[6] Severity of this disorder is associated with nature and type of mutations which alter the structure and function of the enzyme.[7-9]

Overall, the incidence of PKU is 1 out of 10,000 among Caucasians. But it varies in Asian populations such as; Japan 1 out of 70,000,[10] Korea 1 out of 41,000,[11] China 1 out of 11,144, India 1 out of 18,300,[12] Turkey 1 out of 4370,[13] Ireland and Jordan 1 out of 4000,[14] and Iran 1 out of 3627.[15] However, till now there is no report available in Pakistan about the incidence of PKU. Most importantly, PKU is a treatable disorder if diagnosed earlier in life and special diet therapies are available.[16,17] In most of the developed and some of the developing countries, Newborn screening (NBS) programs are available for the screening of PKU by measuring the levels of Phe and Tyr through high performance liquid chromatography (HPLC) or mass spectrometry (MS).[3]

However, in Pakistan there is no NBS program available for the screening of different IEMs. Thereby, the purpose of this study was to investigate PKU patients from suspected intellectually disabled children. For this purpose, amino acid profiling was performed by HPLC, and exon 6 and 7 of PAH (hotspot regions) examined in all the collected n=100 intellectually disabled children through PCR-SSCP analyses.

METHODS

Study Design: Prospective cross-sectional observational study.

Place and duration of study: This study was performed at National Institute of Biotechnology and Genetic Engineering (NIBGE)from 2016-2018 and was approved by the institutional (NIBGE) ethics committee.

Sample size: One-hundred intellectually disabled children.

Sampling technique: Non-probability consecutive sampling.

Data Collection: Informed written consent was obtained from the parents and each head of the special education centers. Clinically and bio-demographically important information was collected like; family history (disease history and consanguinity), clinical features (IQ level, delayed developmental milestones, eye examination), body weight, height, and head circumference.

Blood samples from the intellectually disabled children were collected in EDTA coated vacutainer tubes. Blood plasma was separated by centrifugation the whole blood at 13,000 rpm for 5 minutes, and genomic DNA was extracted by organic (Phenol:Chloroform:Isoamyl alcohol) DNA extraction method, and saved at -20 ºC. Prior to amino acid profiling by HPLC (Thermo Fisher), amino acid extraction was performed (100 μl plasma + 500 μl absolute ethanol) by centrifugation at 14,000 rpm for 10 minutes, supernatant was taken for analysis on HPLC. Prior to PCR, DNA was quantified by agarose gel electrophoresis and NanoDrop (ND 1000) spectrophotometer (ThermoFisher). Genetic screening of exon 6 and 7 of PAH was directly carried out by PCR and SSCP analyses. Primer's sequences for the amplifications of exons 6 and 7 are listed in Table 1

Table1: Primer's sequences of exon 6 and 7

Primers	Primer sequence 5'– 3'	T_m	Amplicon
PKU6F	GTAGAAAAACCAAGTGATTTCCCG	61.2	706 bp (for exon 6)
PKU6R	GAAAGTGGATAAACACAGAGGG	61.0	
PKU7F	TTATGCAGATGAGGAAACTGAGGC	62.9	610 bp (for exon 7)
PKU7R	ATTGGGATCTCTCATTCTCACTGC	62.9	

PCR was carried out for both exons using(Bio-Rad T100 ™) Thermal Cycler. PCR conditions were 95°C for five minutes followed by consecutive 34 cycles at 94°C for 30 sec: 56°C (for exon 6), 58°C (for exon 7) for 45 sec, 72°C for 45 sec and final extension at72°C for 10 minutes.

For SSCP analysis, 6 μl of PCR product was taken, added 12 μl of formamide dye solution in PCR tubes and the products were heated at 95°C for 5 minutes. Tubes were immediately transferred on the ice and were kept at -20°C for 10 minutes then electrophoresed on 8% polyacrylamide gel.

Data analysis: The results were analyzed by using SPSS version 20.

Ethics statement: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards

RESULT

All the intellectually disabled children were less than 14 years of age, 68 were males, 32 were females and interestingly 85% consanguinity rate was observed in the collected samples. Average BMI and head circumference was 16.15 kg/m2 and 50.21 cm respectively. All the intellectually disabled children showed delayed developmental milestones while most of them showed hyperactive and aggressive behavior. HPLC was performed to find the levels of amino acids especially Phe and Tyr. After the amino acid analysis through HPLC not a single PKU patient was found, one of the representative chromatograms is shown in Figure 1

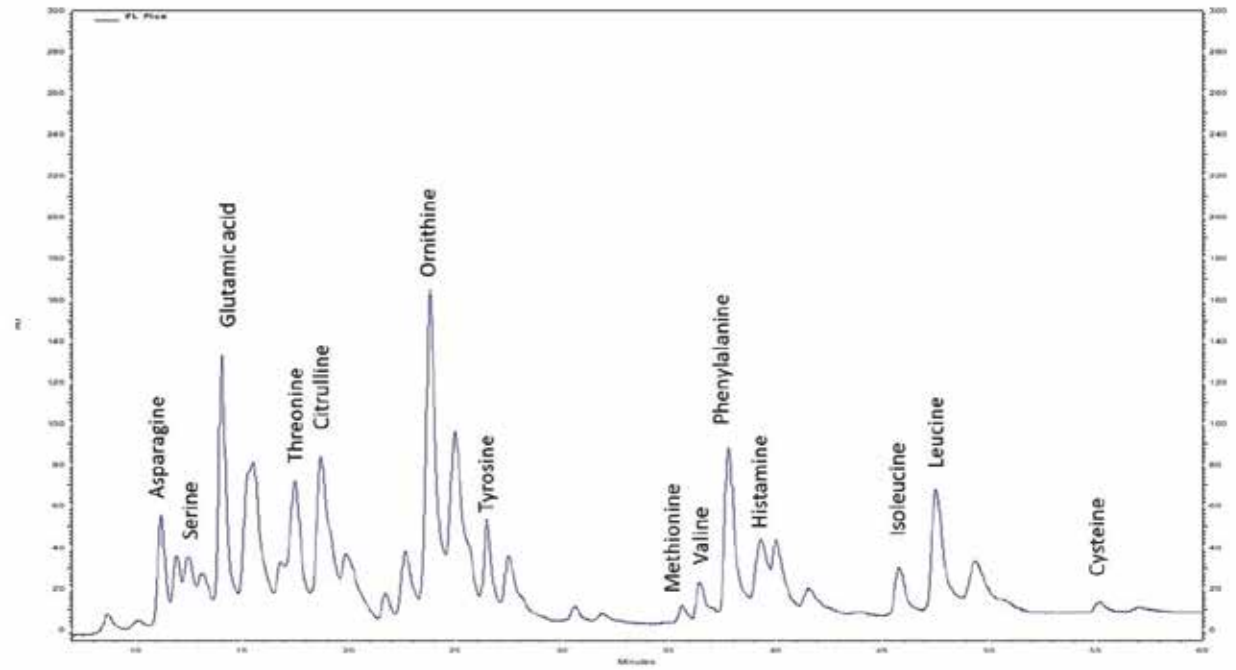

Figure 1: HPLC chromatogram of intellectually disabled patient show normal profile

For more confirmation, PCR was performed on all the collected samples and some results of random samples of both exons are shown in Figure 2

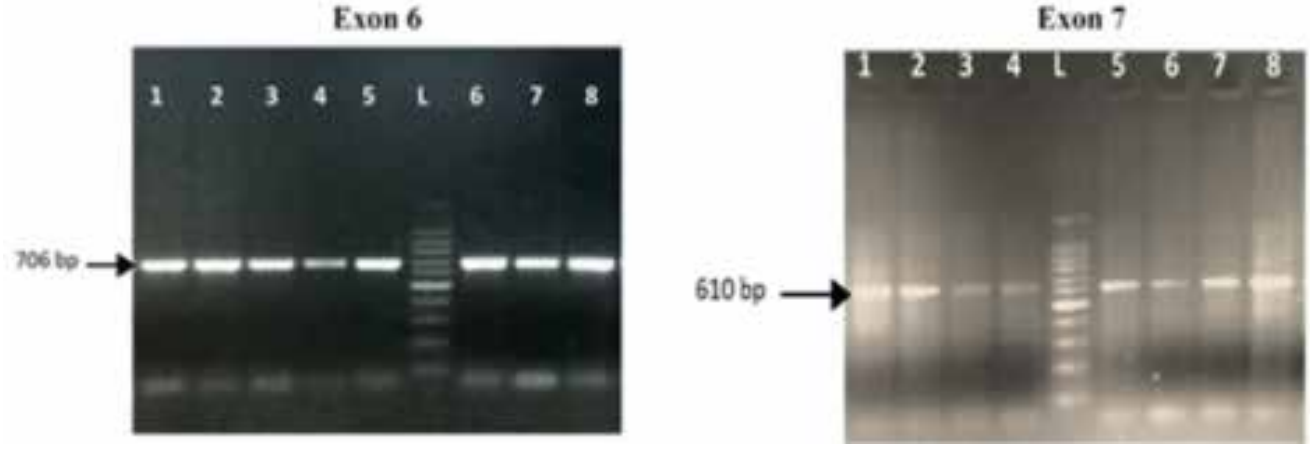

Figure 2:Amplification of exon 6 and 7 of PAH with 100 bp ladder (L)

SSCP analysis was also carried out for all the samples and single stranded products were analyzed on 8% polyacrylamide gel electrophoresis (PAGE) at 170 V for 4-5 hour to observe any change in band pattern of conformers. No change in band pattern was observed in any of the intellectually disabled samples. SSCP results of random samples of both exons are shown in Figure 3.

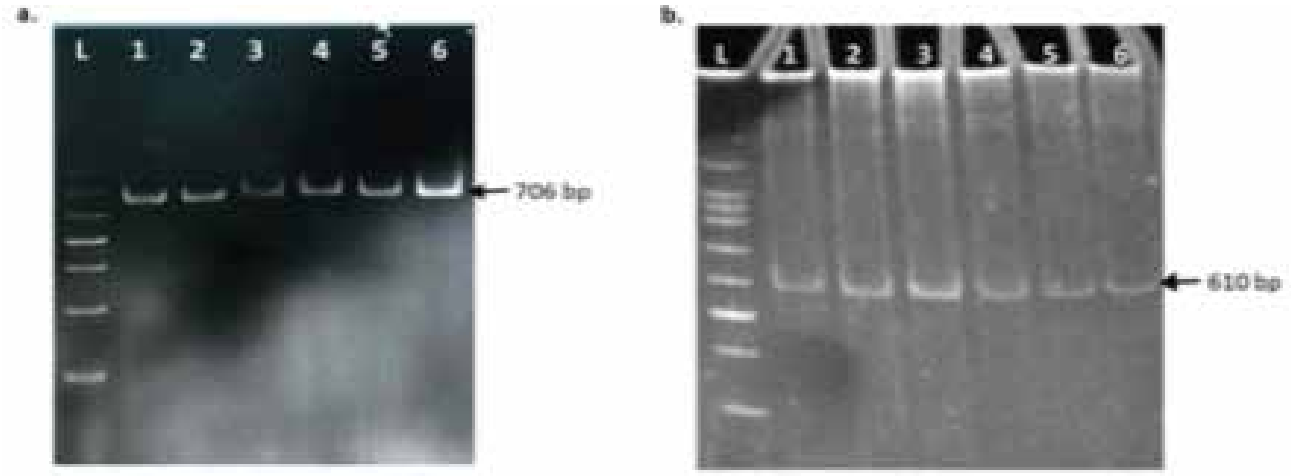

Figure 3: SSCP analysis of both exons; (a.) exon 6 (b.) exon 7 with 100 bp ladder (L)

DISCUSSION

PKU is a prevalent IEM disorder that causes intellectual disability in children. All over the world its prevalence increases in the countries with the high rate of consanguinity. As it is reported that the rate of first cousin marriages in Iran is 17% and PKU prevalence is also higher i.e. 1/3627, and 50% of this PKU prevalence is because of consanguineous marriages.[18] In Jordan cousin marriages accounts for 20-30% of all marriages,and PKU incidence rate is also higher i.e. 1/4000.[19] Similarly in Turkey PKU incidence rate is 1/4370,[13] and 49% of these reported incidences accounts for consanguinity which accounts for total of 22% marriages.[20] In Pakistan consanguinity is a common custom, about 60-70% of marriages and out of this 17-38% of marriages occurs among first-cousins.[21,22] As PKU frequency increase due to consanguinity so we expected that PKU might also be frequent in Pakistan.

However, in the current study previously undiagnosed 100 intellectually disabled children were selected for biochemical and genetic screening of PKU. All the intellectually disabled children had developmental delayed milestones while most of them also showed psychological problem and 85% of subject's parents had cousin-marriages.

As amino acid profiling through HPLC is a main screening analysis for PKU and for the other amino acid related disorders, however, all the collected intellectually disabled children showed normal profiles of amino acids, so not found high level of phenylalanine as it is reported in the case of PKU.[23] Genetic analysis of two hotspot regions of PAH i.e. exon 6 and 7 was performed to find causative mutations. As it has been reported in most of the Asian countries these two regions (exon 6 and 7) have the highest rate of mutations that causes PKU in children, and 73% of mutations have been reported in Iranian population in the same regions.[15] In China, a total of 76.3% exons mutations have been reported, 33.4% were located in exon 7 and 10.5% were present in exon 6, being the hotspot regions for *PAH*.[24] In Indian families, it also has been reported that exon 6 and 7 has the higher rate of mutations in the *PAH*.[25] Similarly, in PAH database most of the mutations are reported in exon 6 and 7 of gene and their flanking introns. Overall, in PAH database 78 (13.76%) mutations have been reported in exon 6 while 87 (15.34%) mutations in exon 7 from all over the world. Thereby, it would be logical to first scan these two regions for mutation detection and if no mutations found then scan all the other exons and adjacent introns.

Therefore, in the current study, all the samples were analyzed for exon 6 and 7 (two hotspot regions). After the HPLC, PCR and SSCP analyses, PKU patient was not found in all collected samples. This suggests that in Pakistani population PKU might be less prevalent as compared to other countries. Moreover, all the samples were taken only from Faisalabad region of Punjab Pakistan, which might be one possibility that PKU not be frequent in this region but could be more frequent in other regions of Pakistan.

Limitations

Less number of samples are used in this study, large scale study is required with massive number of samples. Moreover, in the current study, only 2 exons explored, but there is a need to explore all the exons and adjacent introns for mutation scanning, after the amino acid profiling through HPLC.

CONCLUSION

Results of current study are slightly dissimilar from other consanguineous populations, because no PKU patient was detected from the collected patient samples. However, conclusion is difficult to draw for whole population based on collected dataset. However, to our best knowledge this is the first study from Pakistan attempting to diagnose intellectually disabled children for PKU. However, after the screening analyses, the diagnosed patients could be benefitted from the available treatments and in such a way we can reduce the burden of intellectual disability from the population.

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Author's contribution:
Habiba Hussain; concept, data collection, data analysis, manuscript writing, manuscript review
Muhammad Wasim; concept, data collection, data analysis, manuscript writing, manuscript review
Haq Nawaz Khan; concept, data analysis, manuscript review
Hina Ayesha; data analysis, manuscript writing, manuscript review
Fazli Rabbi Awan; concept, data analysis, manuscript review