

Original Article

Novel Pathogenic Mutation Mapping of *ASPM* Gene in Consanguineous Pakistani Families with Primary Microcephaly

Novo mapeamento de mutações patogênicas do gene *ASPM* em famílias consanguíneas com microcefalia primária do Paquistão

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Abstract

Autosomal recessive primary microcephaly (MCPH) is a neurodevelopmental disorder characterized by a congenitally reduced head circumference (-3 to -5 SD) and non-progressive intellectual disability. The objective of the study was to evaluate pathogenic mutations in the *ASPM* gene to understand etiology and molecular mechanism of primary microcephaly. Blood samples were collected from various families across different remote areas of Pakistan from February 2017 to May 2019 who were identified to be affected with primary microcephaly. DNA extraction was performed using the salting-out method; the quality and quantity of DNA were evaluated using spectrophotometry and 1% agarose gel electrophoresis, respectively in University of the Punjab. Mutation analysis was performed by whole exome sequencing from the Cologne Center for Genomics, University of Cologne. Sanger sequencing was done in University of the Punjab to confirm the pathogenic nature of mutation. A novel 4-bp deletion mutation c.3877_3880delGAGA was detected in exon 17 of the *ASPM* gene in two primary microcephaly affected families (A and B), which resulted in a frame shift mutation in the gene followed by truncated protein synthesis (p.Glu1293Lysfs*10), as well as the loss of the calmodulin-binding IQ domain and the Armadillo-like domain in the *ASPM* protein. Using the in-silico tools Mutation Taster, PROVEAN, and PolyPhen, the pathogenic effect of this novel mutation was tested; it was predicted to be “disease causing,” with high pathogenicity scores. One previously reported mutation in exon 24 (c.9730C>T) of the *ASPM* gene resulting in protein truncation (p.Arg3244*) was also observed in family C. Mutations in the *ASPM* gene are the most common cause of MCPH in most cases. Therefore, enrolling additional affected families from remote areas of Pakistan would help in identifying or mapping novel mutations in the *ASPM* gene of primary microcephaly.

Keywords: Primary microcephaly, *ASPM* gene, Whole-exome sequencing, Novel mutation.

Resumo

Microcefalia primária autossômica recessiva (MCPH) é um distúrbio do neurodesenvolvimento caracterizado por uma redução congênita do perímetro cefálico (-3 a -5 DP) e deficiência intelectual não progressiva. O objetivo do estudo foi avaliar mutações patogênicas no gene *ASPM* a fim de compreender a etiologia e o mecanismo molecular da microcefalia primária. Amostras de sangue foram coletadas de várias famílias em diferentes áreas remotas do Paquistão de fevereiro de 2017 a maio de 2019, que foram identificadas como afetadas com microcefalia primária. A extração do DNA foi realizada pelo método salting-out; a qualidade e a quantidade de DNA foram avaliadas por espectrofotometria e eletroforese em gel de agarose a 1%, respectivamente, na Universidade de Punjab. A análise de mutação foi realizada por sequenciamento completo do exoma do Cologne Center for Genomics, University of Cologne. O sequenciamento de Sanger foi feito na Universidade do Punjab para confirmar a natureza patogênica da mutação. Uma nova mutação de deleção de 4 bp c.3877_3880delGAGA foi detectada no exon 17 do gene *ASPM* em duas famílias afetadas por microcefalia primária (A e B), que resultou em uma mutação de *frame shift* no gene seguida por síntese de proteína truncada (pGlu1293Lysfs * 10), bem como a perda do domínio IQ de ligação à calmodulina e o domínio do tipo Armadillo na proteína *ASPM*. Usando as ferramentas in-silico Mutation Taster, PROVEAN e PolyPhen, o efeito patogênico dessa nova mutação foi testado; foi previsto ser “causador de doenças”, com altos escores de patogenicidade. Uma mutação relatada anteriormente no exon 24 (c.9730C > T) do gene *ASPM*, resultando em truncamento de proteína (p.Arg3244*) também foi observada na família C. Mutações no gene *ASPM* são a causa mais comum de MCPH na maioria dos casos. Portanto, a inscrição de famílias afetadas adicionais de áreas remotas do Paquistão ajudaria a identificar ou mapear novas mutações no gene *ASPM* da microcefalia primária.

Palavras-chave: Microcefalia primária, gene *ASPM*, sequenciamento de exoma completo, Nova mutação.

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1. Introduction

Autosomal recessive primary microcephaly (MCPH) is a rare, neurodevelopmental defect that invariably leads to smaller brain size and decreased cognitive functions. It is characterized by reduced head circumference (-3 to -5 SD), based on age and sex (Naveed et al., 2018; Wollnik, 2010). MCPH patients present a variable degree of intellectual disability; typically, a smaller brain size with simplified gyral patterns and pituitary anomalies in the cerebral cortex, as well as a significant decrease in the size of the architecturally normal brain (Woods and Parker, 2013; Thornton and Woods, 2009). Mutations in genes that are involved in brain growth and development (such as proliferation or differentiation of progenitor cells) or cell apoptosis can lead to MCPH (Tischfield et al., 2011; Roberts et al., 2002; Jayaraman et al., 2018).

To date, 25 MCPH loci have been mapped (MCPH1 to MCPH25) and have the following causative genes: Microcephalin, *WDR62*, *CDK5RAP2*, *CASC5*, *ASPM*, *CENPJ*, *STIL*, *CEP135*, *CEP152*, *ZNF335*, *PHC1*, *CDK6*, *CENPE*, *SASS6*, *MFSD2A*, *ANKLE2*, *CIT* and *WDFY3*, *COPB2*, *KIF14*, *NCAPD2*, *NCAPD3*, *NCAPH*, *NUP37* and *MAP11*. These molecular basis studies clarify our understanding of microcephaly genetic disorder (Perez et al., 2019; Bond et al., 2002). The *ASPM* gene (MIM 605481) at the MCPH5 locus has 28 exons and spans 62.54 kb of genomic DNA, with a 10,434 bp long open reading frame (ORF) that encodes a protein 3477 amino acids in length. The *ASPM* protein has four main regions: a putative N-terminal microtubule-binding domain, a calponin-homology domain (CH), 81 calmodulin-binding IQ repeats, and a C-terminal region. Most IQ motifs are organized into higher order trimer repeats (HOR) containing two 23-amino acid residue units and one 27-amino acid residue unit (Hina et al., 2019; Li et al., 2016).

The incidence of MCPH is relatively high (approximately 1/10,000) in regions where consanguineous marriages are common, such as in Pakistan. So far in Pakistani population, *ASPM* gene mutations are almost 50% of all MCPH cases reported, including 94 nonsense and missense mutations, 88 small deletion mutations, 16 small insertion mutations, 2 gross deletions, 18 splicing mutations, and 1 mutation of complex rearrangement from a total of 219 pathogenic *ASPM* gene mutations (HGMD, Professional 2017.4, <http://www.hgmd.org/>). The *ASPM* gene encodes the protein involved in brain development and cell division. It is also important for maintaining neural progenitor cells (early brain cells maintained by the *ASPM* protein) that ultimately develop into neurons (mature brain cells) (Bibi et al., 2018).

The overall brain size and the total number of neurons are determined by *ASPM* protein involvement. *ASPM* protein mutations are likely the probable cause of microcephaly (Bunday, 1997).

Microcephaly is a significant disorder having genotypic and phenotypic heterogeneity. During searching, identification, and blood sampling of microcephaly families, we would be capable to properly counsel these affected families and their relatives. Well guidance and awareness of this disorder and to avoid marriages within families may eradicate this disorder. With novel mutation analysis may lead to identification of new protein involved

in causes of primary microcephaly. Through this project a new molecular spectrum of microcephaly may be opened.

2. Materials and Methods

Microcephaly-affected Pakistani families were located and identified from February 2017 to May 2019 and were selected for participating in this research. The study was approved by the ethics committee of the University of the Punjab, Lahore Pakistan. A complete family history was obtained from the guardians of these families. For clinical assessment, all patients were examined by genetic consultants and medical experts. Consanguineous sib ship was confirmed through multiple interviews. Relevant information for these families was gathered by completing questionnaires.

2.1. Sample Collection and Extraction

Peripheral blood samples were collected in EDTA vacutainers from all family members, including parents, normal siblings, and affected individuals. DNA extraction was done in University of the Punjab, by using the salting-out method (Helms, 1990) using Falcon 50 ml tubes. DNA quality and quantity were estimated by spectrophotometry (Nanodrop method) and 1% Agarose gel electrophoresis, respectively. For DNA staining, fluorescent dye ethidium bromide was used. Bands of DNA were visualized on a UV gel transilluminator documentation system (imaging system by SynGene).

2.2. Mutation Detection Using Whole-Exome Sequencing

Whole-exome sequencing is a powerful diagnostic tool for evaluation of highly heterogeneous neurodevelopmental disorders. Whole-exome sequencing (WES) in affected individuals (V-3 of Family A; V-1 of Family B; IV-1 of Family C) from the affected Pakistani families was performed in the Cologne Center for Genomics, University of Cologne by using the Agilent (Santa Clara, CA) version 6 enrichment kit and the Illumina HiSeq 4000 sequencing system (paired-end reads, 2375 bp). The experimental procedure and data handling were performed as reported previously (Hussain et al., 2012; Hussain et al., 2013). The VARBANK database (<http://varbank.ccg.uni-koeln.de>) and analysis tool kit were used for the filtration and prioritization of variants (Szczepanski et al., 2016).

2.3. Sanger Sequencing

Sanger sequencing was performed to confirm the pathogenic mutation discovered by WES. The Ensemble genome browser (<http://asia.ensembl.org/index.html>) was used to download the 10877 bp genomic sequence of the *ASPM* gene (ENST00000367409.8). The following primers were designed using the primer3 software (<http://frodo.wi.mit.edu/primer3/>): F5'-TTCCAAAGATGAACACAAGAAG-3' and R5'-ACCATAACGAGCTTTTCAGG-3' for exon 17, and F5'-GATGTAGATATAAATAGAAAACATTGG-3' and R5'-CAGGGGCATATTTTGTGAC-3' for exon 24. The PCR products were sequenced bidirectionally using a Big Dye Terminator v1.1 cycle sequencing kit on an ABI3730xl

automated DNA Sequencer. Sequences were analyzed with DNASTAR (Lasergene) and Mutation Surveyor (Soft Genetics).

3. Results

Consanguineous Pakistani families with inherited autosomal recessive primary microcephaly with at least two living affected members born to phenotypically normal parents were recruited for this study. Photographs of affected members and pedigrees of these affected families were constructed and are represented in Figure 1. Clinical information of the MCPH patients was described, including their head circumference (Standard deviation), age, sex,

age at which they started walking, mental retardation, severity in behavior, and the presence of any other disorders, such as epilepsy and speech delay in Table 1. Parents in these families showed normal intelligence scores and normal head circumference. A novel homozygous deletion mutation c.3877_3880del GAGA was detected in exon 17 of the ASPM gene in two Pakistani families (A and B) with MCPH. Electropherograms of exon 17 for the ASPM gene showed the variation c.3877_3880delGAGA in V: 3 and V: 1, represented in upper traces of family A and B, respectively. Electropherograms IV: 5 and IV: 2 represented carriers (parents) of family A and B in medium traces. Electropherograms of wild type DNA represented in lower traces. Black arrows indicate the position of the mutations (Figure 2a). Sanger electropherograms of exon 24 for the ASPM gene showed the variation c.9730C>T in IV: 1 affected sibling of family C and wild type DNA. Black arrows indicated the position of the mutation (Figure 2b).

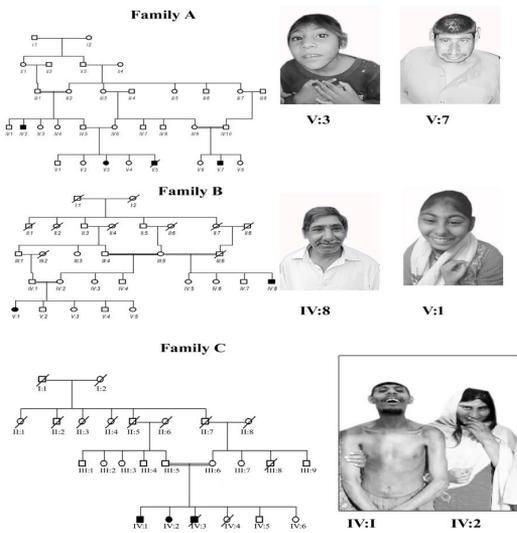


Figure 1. Pedigrees and photographs of affected members of MCPH families with sloping forehead and variable head circumference. Squares represent males while circles represent females; the shaded symbols are for affected individuals, while the double line between individuals represents consanguinity.

4. Discussion

The ASPM protein is essential for normal mitotic spindle function in embryonic neuroblasts. By promoting the division of neural progenitor cells during early brain development, ASPM gene helps to determine the total number of neurons and overall brain size (Geoffrey et al., 2005). Defects in the ASPM protein are associated with autosomal recessive microcephaly and mild to severe mental retardation (Hashmi et al., 2016). The ASPM gene mutation c.3877_3880delGAGA, which truncates the protein (p. Glu1293Lysfs*10) and causes a loss of the calmodulin-binding IQ domain and Armadillo-like domain of the ASPM protein, was detected in exon 17 in two Pakistani families (A and B) with MCPH (Figure 3a). Spindle formation during cell division, which is regulated by calmodulin, is associated with the IQ motif of the ASPM protein. During interphase, calmodulin is in the cytoplasm and is associated with mitotic spindles during mitosis. Inhibition of cytokinesis and abnormal spindle formation takes place due to mutations in the calmodulin-binding

Table 1. Clinical information of the MCPH patients included in this study.

Family ID	Patient ID sex	Age (year)	OFC (SD)	Mental retardation	Age at walking years	Behavior Pattern	Others anomaly
A	IV:2 (M)	22	-9.4.0	Severe	10	Very aggressive	Epilepsy, reduced height, speech delay
	V:3 (F)	7	-12.0	Moderate	2	Friendly	Speech delay, hear impairment
B	V:1 (F)	17	-8.9	Moderate	2	Friendly, active, school going	Hormonal Disturbance
	IV:8 (M)	48	-8.9	Moderate	3	Friendly and active	reduced height, speech delay
C	1V:1 (M)		-9.7	Severe	3	Violently react	Seizures, general weakness
	1V:2 (F)	22	-6.3	Severe	2	Aggressively	Speech delay

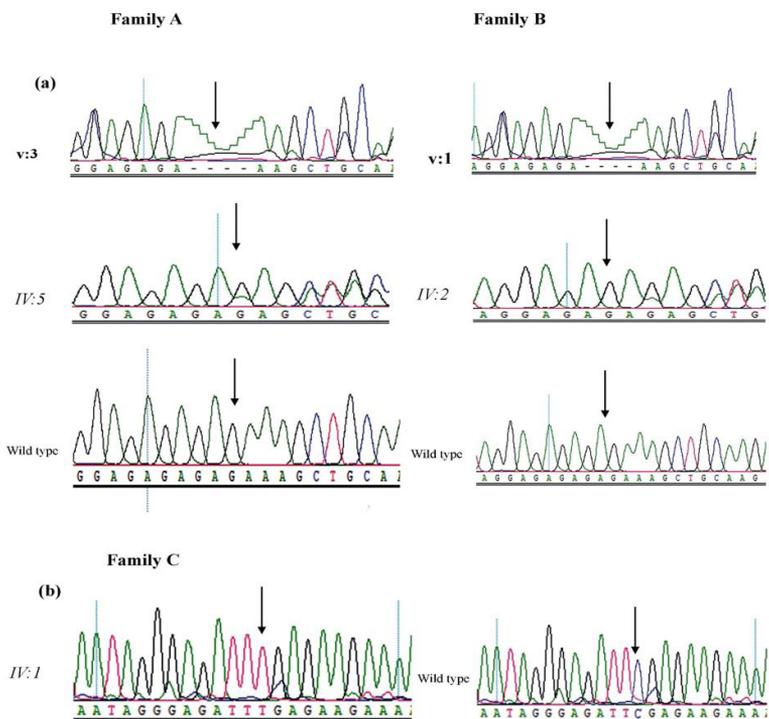


Figure 2. a) Electropherogram of novel mutation in exon 17 of the *ASPM* gene; homozygous deletion mutation c.3877_3880delGAGA of microcephaly affected individual in Family A and B. b) Reported mutation of exon 24 (c.9730C>T) of the *ASPM* gene of microcephaly affected individual in Family C

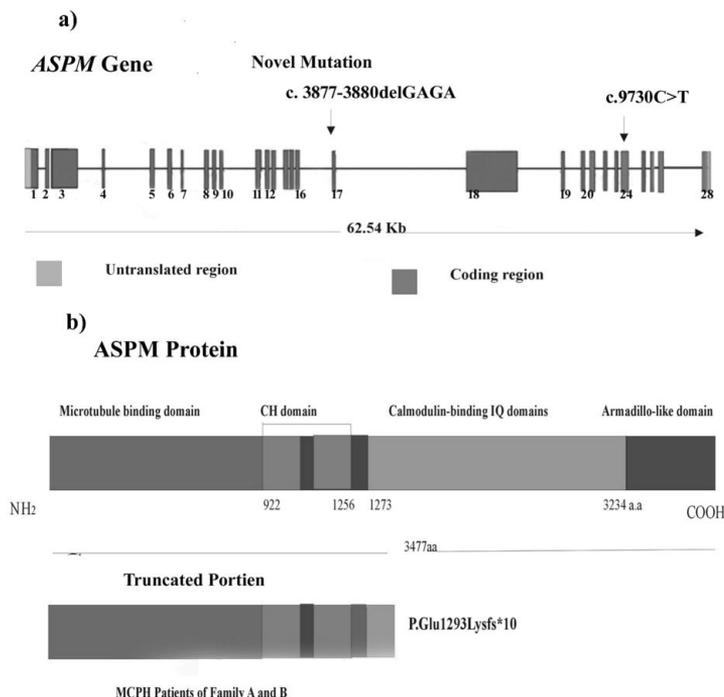


Figure 3. (a) *ASPM* gene structure with 28 exons, Novel mutation identified in exon 17 in Family A and Family B; previously reported mutation finding in exon 24 in Family C. (b) *ASPM* gene codes for the 3477-amino acid protein with four protein domains; the nonsense mutation p. Glu1293Lysfs*10 in patients from families A and B leads to a truncated protein that is missing a large part of the calmodulin-binding IQ domain and the Armadillo-like domain of *ASPM* protein.

Table 2. Pathogenicity score of novel mutations detected using in silico tools.

S. No	Online tools	Pathogenicity score
1	MutationTaster (http://www.Mutationtaster.org/)	Disease Causing
2	Polyphen-2 " http://genetics.Bwh.harvard.edu/pph2/)	0.010 (sensitivity: 0.96; specificity: 0.77)
3	PROVEAN	6.910 Deleterious
4	MUTATION ASSESSOR (http://mutationassessor.org)	1.04 F Functional impact low

Table 3. Gene (exon), Ref. Seq. ID, cDNA mutations, amino acid changes, Genome AD frequency and family ID are elaborated for novel and reported mutations.

Gene (Exon)	Ref. Seq. ID	cDNA mutation	Amino acid change	GnomAD Frequency	Family ID	Reference
<i>ASPM</i> 17	NM_018136.4 " http://www.ncbi.nlm.nih.gov/nuccore	c.3877_3880delGAGA	p. Glu1293Lysfs*10	0.000004000	A	Novel mutation
<i>ASPM</i> 17	NM_018136.4 " http://www.ncbi.nlm.nih.gov/nuccore	c.3877_3880delGAGA	p. Glu1293Lysfs*10	0.000004000	B	Novel mutation
<i>ASPM</i> 24	NM_018136.4	c.9730C>T	p. Arg3244*	0.000003990	C	Reported

IQ domain of the *ASPM* protein (Khan et al., 2017; Willingham et al., 1983). Novelty of this mutation in the Pakistani population also confirmed by visiting <https://pakmutation.kust.edu.pk/>. The pathogenic effect of this novel mutation was tested using in silico tools, including MutationTaster, PolyPhen, and PROVEAN; the mutation was predicted to be "disease causing," characterized by high pathogenicity scores while for functional impact used Mutation Assessor elaborated in Table 2. Deletion of glutamic acid causes a frameshift mutation in the *ASPM* protein (p. Glu1293Lysfs*10), leading to protein truncation due to premature stop codon; untranslated calmodulin-binding IQ and armadillo-binding domains (Figure 3b). Genome AD frequency, Ref. Seq. ID, cDNA mutation, and amino acid change are elaborated (Table 3). We also identified one previously reported mutation (c.9730C>T) in the 24th exon of the *ASPM* gene in one family (family C) showing clinical signs of primary microcephaly (Welsh et al., 1979). The MCPH5 locus shows varied incidence in different ethnic populations. For instance, this rate is in the range of 43–86% in the Pakistani population, 33.5% in the Indian population, and 13.3% in the Iranian population (Darvish et al., 2010; Nicholas et al., 2009; Paramasivam et al., 2007).

5. Conclusion

The *ASPM* gene (MCPH5) occupies the most prominent locus in the disease etiology of primary microcephaly, owing to its mutation rate which is as high as 50 to 90%. Deletion of glutamic acid causing frameshift mutation, insertion of premature stop codon leading to protein truncation and untranslated calmodulin-binding IQ and armadillo-binding

domains involved in cytokinesis and spindle formation. *ASPM* is very crucial gene involve in brain functioning and determine the size of brain. Identification of this novel mutation has increased the mutational spectra of *ASPM* gene, but further functional exploration is required to develop a phenotype genotype correlation.

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