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Full Length Research Paper

A nonsense (c.3978G>A) abnormal spindle-like, microcephaly associated (ASPM) gene mutation is a major cause of primary microcephaly in Pashtoon ethnic group of Pakistan

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Primary microcephaly (MCPH) is an autosomal-recessive congenital disorder characterized by smaller-than-normal brain size and mental retardation. MCPH is genetically heterogeneous with six known loci: MCPH1 to MCPH7. The abnormal spindle-like, microcephaly associated (ASPM) gene at MCPH5 locus, which accounts for 37 to 54% of MCPH, appears to be the most common cause of microcephaly. More than 50% of the MCPH families genetically analyzed in Pakistan were mapped to MCPH5 locus including both families in this study. On mutation screening of ASPM gene by PCR amplification and direct DNA sequencing, a common c.3978G>A transition was identified in exon 17 of ASPM gene to be responsible for diseased phenotype in both families. This change results to the substitution of an amino acid residue at position 1326 from tryptophan to a stop codon (p.Trp1326Stop). The same mutation was also identified in several other families of Pakistani origin. Since the disease is both clinically and genetically heterogeneous, the diagnosis of MCPH1–7 is based on clinical findings; brain imaging that shows reduced brain volume with grossly normal architecture, family history consistent with autosomal recessive inheritance and molecular genetic testing when available. The mapping of large number of families to MCPH5 locus and identification of a common mutation, that is, c. 3978A>G of ASPM gene will enable us to formulate future strategies to control and prevent the disease by genetic counseling, prenatal/postnatal diagnosis and carrier testing.

Key words: Primary microcephaly (MCPH), abnormal spindle-like, microcephaly associated (ASPM) mutations, microcephaly, Pakistani families.

INTRODUCTION

Autosomal recessive primary microcephaly (MCPH, MIM 251200) is clinically described as a congenital neurological disorder in which the affected individual have head circumference at least three standard deviations (SDs) below the expected mean for age and sex and mild-to-severe mental retardation. The incidence of MCPH is approximately 1 in 10,000 individuals in Pakistan and 1 in 1,000,000 in the Caucasian population (Woods et al., 2005). MCPH is more common in populations where consanguineous are practiced more frequently (Nicholas et al., 2009; Kousar et al., 2010).

MCPH is a genetically heterogeneous disorder and seven loci (MCPH1–MCPH7) have been mapped to date. Six loci out of seven reported (MCPH1 to MCPH3, MCPH5 and MCPH6) have been identified in families of northern Pakistani origin (Woods et al., 2005) and only a single locus, MCPH4 was reported to be identified in the

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Table 1. A summary of clinical findings of affected members of MCPH families.

Pedigree code	Age (year)	Head circumference (cm)	Clinical finding
1MIC003	19	44	Slopping forehead; Mild mental retardation
1MIC006	15	44.5	Slopping forehead, rare seizures; Moderate mental retardation
1MIC008	08	42	Narrow forehead; Moderate s mental retardation
1MIC010	03	43.5	Slopping forehead; Mild mental retardation
2MIC004	29	42.5	Normal
2MIC006	26	46	Slopping forehead; Moderate mental retardation

Figure 1. Photographs of few primary microcephaly patients of MCPH Pakistani families.

Moroccan population. Five genes for the 7 known loci are reported to date and they include MICROCEPHALIN at MCPH1, CDK5RAP2 at MCPH3, ASPM (abnormal spindle-like, microcephaly associated) at MCPH5, CENPJ at MCPH6 and STIL at MCPH7. In the Pakistani population, autosomal recessive primary microcephaly is quite frequent and mutations of ASPM gene seem to be the most common cause of autosomal recessive primary microcephaly. About 71 distinct MCPH associated ASPM mutations are reported to date (Nicholas et al., 2009; Kousar et al., 2010). In families of northern Pakistan with primary microcephaly, 43% of them had mutations in ASPM gene (Roberts et al., 1999, 2002). The identification of a large number of recessive ASPM gene mutations in Pakistani families with MCPH probably reflects the effects of consanguinity.

In this study, the MCPH5 locus was mapped in two families with primary microcephaly from Khyber Paktoonkhwa, Pakistan. A common c.3978A>G mutation was identified in exon 17 of ASPM gene to be responsible for diseased phenotype in the both families. This change results to the substitution of amino acid residue at position 1326 from tryptophan to a stop codon (p.Trp1326Stop). This mutation was found to segregate within both MCPH families. The mutation identified in these families was also observed in several other families to be responsible for MCPH in northern region of Pakistan (Nicholas et al., 2009). The mapping of large number of families to MCPH5 locus and identification of a common mutation, that is, c. 3978A>G of ASPM gene will enable us to formulate future strategies to control and prevent the disease by genetic counseling, prenatal/postnatal diagnosis and carrier testing.

MATERIALS AND METHODS

Sample collection and DNA preparation

Blood samples from affected individuals, their parents and clinically normal siblings of both families were collected with informed consent. Before sample collection, pedigree was drawn and detailed clinical examination was performed as summarized in Table 1. It was observed that microcephaly is present at birth in all affected individuals (Figure 1). Affected individuals have mild to moderate degree of mental retardation. However, no other neurological findings were observed in any of the affected individual. Genomic DNA was extracted from peripheral blood by following the standard phenol-chloroform extraction procedure (Maniatis et al., 1982).

Table 2. Known loci and list of STR markers used for genotyping in Pakistani families.

Locus name	Chromosome	Gene	STR marker
MCPH1	8p22-pter	Microcephaly	D8S264, D8S1099, D8S277 and D8S1130
MCPH2	19q13.1-13.2	Unknown	D19S433, D19S178, D19S246, D19S589 and D19S254
MCPH3	9q34	CDK5RAP2	D9S934, D9S282 and D9S915
MCPH4	15q15-q21	Unknown	ACTC, D15S659 and D15S643
MCPH5	1q31	ASPM	D1S518, D1S1660, D1S1678, D1S1663, D1S2141 and D1S549
MCPH6	13q12.2	CENPG	D13S787 and D13S1493
MCPH7	1p32.3-p33	STIL	D1S2134, D1S1661 and D1S2652

Genotyping and linkage analysis

For identification of locus responsible for the MCHP phenotype in both families, genomic DNA from each individual was genotyped using microsatellite marker for the known microcephaly loci (Table 2). The microsatellite markers for each locus were amplified by polymerase chain reaction (PCR). Each PCR reaction was performed in a 10 µl volume, containing 1.5 mM $MgCl_2$, 0.6 µM of each forward and reverse primer, 0.2 mM dNTPs, 1U Taq DNA polymerase and PCR buffer [16 mM (NH_4) $2SO_4$, 67 mM Tris-HCI (pH 8.8), and 0.01% of the nonionic detergent Tween-20] (Bio-line, London, UK). Amplification was performed with an initial denaturation for 4 min at 94°C, followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 55°C for 35 s, extension at 72°C for 35 s and a final extension at 72°C for 7 min. The PCR products were separated on 10% non-denaturing polyacrylamide gels (Protogel; National Diagnostics, Edinburgh, Scotland, UK). The gel was stained with ethidium bromide and photographed under UV illumination. Alleles were assigned to individuals and genotypic data was used to find genotypes of all individuals of both family members. The phenotype was analyzed as an autosomal recessive trait.

ASPM gene mutation screening

Polymerase chain reaction (PCR) amplification of twenty eight (28) exons of ASPM gene was performed with intronic forward and reverse primers spanning the whole exonic regions. PCR amplification was performed in a 50 µl reaction volume containing 250 ng of genomic DNA, amplification buffer containing 600 nM of each primer, 1.5 mM $MgCl_2$, 200 mM of dNTPs and 2.5 U of Taq DNA polymerase (Applied Biosystems, Warrington, U.K.) in an PxE thermal cycler (Hybaid, Basingstoke, U.K.). The amplification conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, primer specific annealing temperature (55 to 65°C) for 45 s and 72°C for 45 s. Aliquots (5 µl) of the PCR products were analyzed by 2 to 2.5% agarose gel. PCR products were then purified using QIAquick PCR Purification Kit (Qiagen, Crawley, U.K.) and sequenced directly using Big Dye® Terminator v3.1 cycle sequencing kit in an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA, USA). Potential mutations were confirmed by bi-directional sequencing and analyzed by using CLC viewer software (www.clcbio.com).

RESULTS AND DISCUSSION

Both Pakistani families with MCPH were mapped to MCPH5 locus on chromosome 1q31 (Figure 2A and B), which harbors ASPM gene. We have screened both families for mutations of the ASPM gene. A common homozygous G>A pathogenic mutation (c.3978G>A) in exon 17 of the ASPM gene was found in all the affected individuals of the both families (Figure 3). The change was identified in homozygous condition only in the patients and the normal parents were carrier for the change. The CLC sequence viewer software predicted the substitution of amino acid residue at position 1326 of ASPM gene protein product from tryptophan to a stop codon (p.Trp1326Stop). It resulted to a truncated protein product of 1325 amino acids, instead of normal 3477 amino acid protein.

The genetic disorders that are strongly associated with consanguinity are inherited as an autosomal recessive trait (Hamamy et al., 2007). In Pakistan, consanguineous marriages are common and 60% of marriages are reported to be within families and approximately 50% of marriages are practiced between first cousins (Hussain and Bittles, 1998). Autosomal recessive primary microcephaly show considerable locus heterogeneity and may emerge worldwide in a population as the prevalence of a deleterious gene or when degree of consanguinity increases. Molecular genetic analysis of MCPH in consanguineous families has been instrumental for mapping disease loci and for identification of causative genes and mutations.

In this study, we reported a consanguineous family and a non-consanguineous family from Karak district of NWFP in Pakistan with autosomal recessive microcephaly. Khattak tribe lives in Karak district, and the traditional system of marriage within family/tribe results in high rate of consanguineous marriages. Analysis of pedigree is strongly suggestive of autosomal recessive mode of inheritance and marriage within family/tribe could account for all affected individuals being homozygous for the abnormal allele. A common homozygous missense mutation in the both Pakistani families is also indicative of an autosomal recessive inheritance of MCPH either due to deleterious gene or consanguinity.

The identification of common mutation in ASPM gene in families with primary microcephaly analyzed in this study and several other families of same ethnic group, will not only help to educate people about the anticipated genetic consequences and genetic counseling but will also help the patients in prenatal diagnosis, postnatal diagnosis

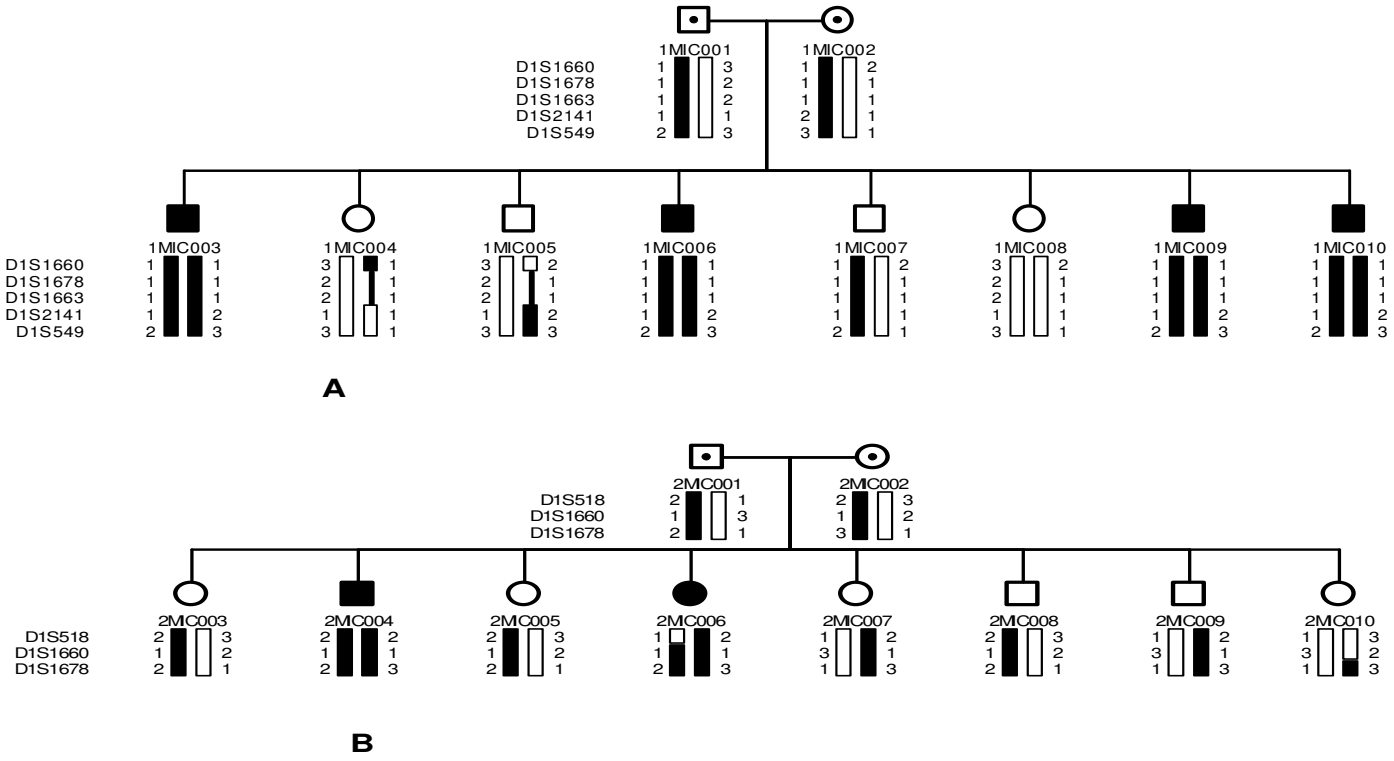

Figure 2. Pedigrees of 1MIC and 2MIC Pakistani families with STR genotyping data for MCPH5 locus on chromosome 1q31.3. Filled circles and squares represent affected females and males, respectively. Double lines between symbols are representatives of consanguineous marriages.

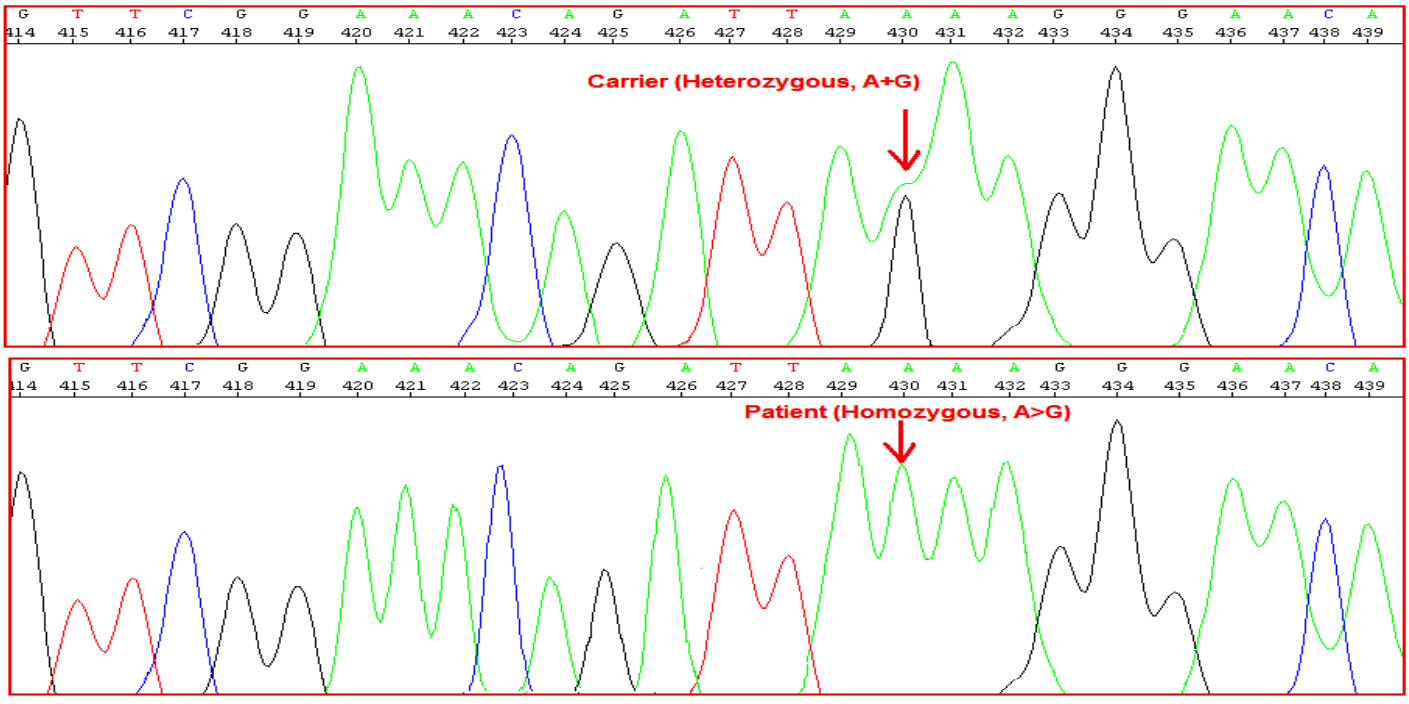

Figure 3. DNA sequence analysis of the ASPM gene in 2 families (1 and 2MIC) with microcephaly. DNA sequencing analysis revealed a homozygous G>A pathogenic mutation (c.1326G>A) in exon 17 of the ASPM gene in all the affected individuals of the both families. The upper electropherogram represents the sequence in the carrier individual, while the lower electropherogram represents the sequence in the affected individual. Arrows indicate the site of mutation.

and carrier testing to reduce the prevalence of MCPH in a particular ethnic group of Pakistan.

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