

Short Report

Genetic analysis of primary microcephaly in Indian families: novel *ASPM* mutations

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Patients with primary microcephaly, an autosomal recessive trait, have mild to severe mental retardation without any other neurological deficits. It is a genetically heterogeneous disorder with six known loci: MCPH1 to MCPH6. Only the genes for MCPH1 and MCPH5 have been identified so far. We have ascertained nine consanguineous families with primary microcephaly from India. To establish linkage of these nine families to known MCPH loci, microsatellite markers were selected from the candidate regions of each of the six known MCPH loci and used to genotype the families. The results were suggestive of linkage of three families to the MCPH5 locus and one family to the MCPH2 locus. The remaining five families were not linked to any of the known loci. DNA-sequence analysis identified one known (Arg117X) and two novel (Trp1326X and Gln3060X) mutations in the three MCPH5-linked families in a homozygous state. Three novel normal population variants (i.e., c.7605G > A, c.4449G > A, and c.5961 A > G) were also detected in the *ASPM* gene.

A Kumar^a, SH Blanton^b, M Babu^a, M Markandaya^a and SC Girimaji^c

^aDepartment of Molecular Reproduction, Development and Genetics, Indian Institute of Science, Bangalore, Karnataka, India; ^bDepartment of Pediatrics, University of Virginia Health Science Center, Charlottesville, VA, USA; ^cDepartment of Psychiatry, National Institute of Mental Health and Neurosciences, Bangalore, Karnataka, India

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Corresponding author: Dr Arun Kumar, MRDG, Indian Institute of Science, Bangalore 560012, Karnataka, India. Tel.: +91 80 2346 5523; fax: +91 80 2360 0999; e-mail: karun@mrdg.iisc.ernet.in

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Microcephaly (small head) is defined as a condition in which the head circumference of an affected individual is >3 SD below the population age-related mean (1). Microcephaly results from a smaller-than-normal cranial vault relative to the facial skeleton and the rest of the body (2). The small cranial capacity results from underlying hypoplasia of the cerebral cortex rather than abnormal development of the overlying skull (1), and there is no major abnormality in the cortical architecture (3).

Microcephaly is etiologically heterogeneous, with environmental and genetic causes (2). Among the environmental causes are intrauterine infections, drugs taken during pregnancy, prenatal radiation exposure, maternal phenylketonuria, and birth asphyxia (1, 4). The majority of microcephalic cases are caused by a variety of genetic mechanisms, including cytogenetic abnormalities, single-gene disorders, etc. (5).

Primary microcephaly (MCPH; OMIM 251200) is a distinct subtype that is defined by the absence of associated malformations and of

secondary or environmental causes (1). It is inherited as an autosomal recessive trait and has an incidence of 1/30,000 to 1/250,000 live births in western populations (2). The incidence of microcephaly is not known in India. However, it could be higher in certain parts of the country such as in the state of Karnataka (south India) where 33% of marriages are consanguineous. Mental retardation in primary microcephaly ranges from mild to severe, but other neurological deficits are absent. Primary microcephaly is diagnosed after exclusion of 1) craniosynostosis, 2) microcephaly occurring as a part of a malformation syndrome (e.g. Down syndrome), and 3) known causes of secondary microcephaly (e.g. birth asphyxia) (2). Prenatal diagnosis of microcephaly by serial ultrasonographic measurement of fetal head circumference has not been reliable until the third trimester (6). Therefore, the identification and characterization of the gene(s) responsible for microcephaly is important for both genetic counseling and prenatal diagnosis (2).

Mapping of primary microcephaly has been problematic due to unavailability of families with multiple affected individuals. However, some progress has been made recently with the mapping of six loci using relatively large families: MCPH1 on chromosome 8p22-pter mapped in two families from Mirpur region of Pakistan (2); MCPH2 on chromosome 19q13.1–13.2 mapped in two families from northern region of Pakistan (7); MCPH3 on chromosome 9q34 mapped in a single family from northern region of Pakistan (8); MCPH4 on chromosome 15q in a single family from Morocco (9); MCPH5 on chromosome 1q25–q32 mapped in two families from Turkey (10) and Multan, Pakistan (11); and MCPH6 on chromosome 13q12.2 in a single family from Brazil (12). Recently, the genes for the MCPH1 and MCPH5 loci have been isolated (13, 14). The *microcephalin* gene mutated in the MCPH1 families contains 14 exons and codes for an 835 amino-acid-long protein (13). The microcephalin protein contains three BRCT domains and is expressed in a wide variety of tissues including brain, kidneys, heart, lungs, etc. (13). The *ASPM* gene (MCPH5) contains 28 exons with a 10,434 bp long open reading frame (ORF) that codes for a 3477 amino-acid-long protein (14). The ASPM protein is predicted to contain an amino terminal microtubuline-binding domain, a calponin-homology domain, 74 isoleucine-glutamine (IQ) domains, and a C-terminal region (14). The *ASPM* gene is expressed in fetal brain (14). The ASPM protein is conserved in human, mouse, *Drosophila*, and *Caenorhabditis elegans* with a consistent correlation of brain complexity and protein length, principally involving an increase in the number of encoded IQ domains (14). Jackson et al. (13) have reported a common mutation, S25X in two MCPH1 families from northern Pakistan. A total of 22 protein truncating mutations have been reported in the *ASPM* gene so far in patients from Pakistan, Turkey, Yemen, Saudi Arabia, Jordan, and the Netherlands (14, 15). However, no study has been carried out to evaluate the genetics of primary microcephaly in the Indian population. We report here the results of genetic analysis of primary microcephaly in nine consanguineous families from the state of Karnataka, south India for the first time.

Materials and methods

Subjects

We have ascertained nine families with primary microcephaly from the state of Karnataka, south India. The number of affected individuals in these

families ranged from one to three. Consanguinity was observed in all families. According to clinical histories, microcephaly was present at birth, and there was no history of neurological deficits or other problems in the affected individuals. On examination, the head circumferences of the affected individuals were 8–13 SD below the population age-related mean. Except for the microcephaly, there were no dysmorphic features in affected individuals. Figure 1 shows photographs of microcephaly patients. All parents appeared to have normal intelligence and normal head circumferences. The Social Quotient (SQ) of affected individuals was measured by Indian adaptation of Vineland Social Maturity Scale by one of us (SCG) (16, 17). No maternal or environmental causes of microcephaly were identified in these families. Informed consent was obtained for research following the approval of the institute's ethical committee.

Chromosome analysis and genotyping

In order to exclude the possibility of chromosome aberrations as the cause of microcephaly in these families, high-resolution G-banding chromosomal analysis of one affected individual from each family was carried out as described in Kumar et al. (18). For genotyping, total genomic DNA samples were extracted from peripheral blood samples using a WizardTM Genomic DNA Purification kit (Promega Inc., Madison, WI). In order to determine whether these families were linked to one of the six known MCPH loci, we selected a minimum of three microsatellite markers from each of the candidate regions of these loci and genotyped all available individuals from these nine families (2, 7–12). Microsatellite markers used for genotyping were: D8S1798, D8S277, D8S1819, and D8S1825 for the MCPH1 locus; D19S226, D19S416, D19S245, D19S425, D19S224, D19S570, D19S881, D19S400, D19S420, and D19S418 for the



Fig. 1. Photographs of microcephaly patients. (a) Individual V-1 from family 4, (b) Individual V-2 from family 4, and (c) individual IV-2 from family 5 (for pedigrees see Fig. 2).

MCPH2 locus; D9S1872, D9S1682, D9S1881, and D9S1821 for the MCPH3 locus; D15S222, D15S659, D15S962, and D15S98 for the MCPH4 locus; D1S2757, D1S2816, D1S1660, D1S2622, D1S373, D1S1181, D1S1723, D1S2655, and D1S1678 for the MCPH5 locus; and D13S787, D13S1304, and D13S221 for the MCPH6 locus. Microsatellite markers were purchased from Research Genetics Inc. (Huntsville, AL) or were synthesized commercially according to data from the Genome Database (<http://www.gdb.org/>). Marker order was obtained from deCode genetic map (19). Genetic distances were obtained from the Marshfield Medical Research Foundation web site (<http://www.research.marshfieldclinic.org/genetics/>). For those markers whose order could not be resolved on the genetic linkage map, the order was established using the sequence map from the UCSC Genome Bioinformatics site (<http://www.genome.ucsc.edu/>). Physical distances between markers were obtained from the UCSC Genomics Bioinformatics Site. Amplification of

microsatellite markers was performed as reported by Kumar et al. (18). Radiolabeled polymerase chain reaction (PCR) products were separated on 6% denaturing polyacrylamide-sequencing gels and were either subjected to Phosphor Image analysis or exposed to X-ray films.

DNA-sequence analysis of the *ASPM* gene

In order to determine the mutations in the *ASPM* gene in MCPH5-linked families, a set of 41 PCR primers, which cover the entire 10,434-bp long ORF of this gene along with intron/exon junction, were made. These primers are available on request from the first author. Mutations in the *ASPM* gene were detected by sequencing the PCR products from one affected individual from each of MCPH5-linked families on an ABIprism A310-automated sequencer (PE Biosystems, Foster City, CA). Once a mutation was detected in an affected individual from a family, the rest of the family members were tested for the

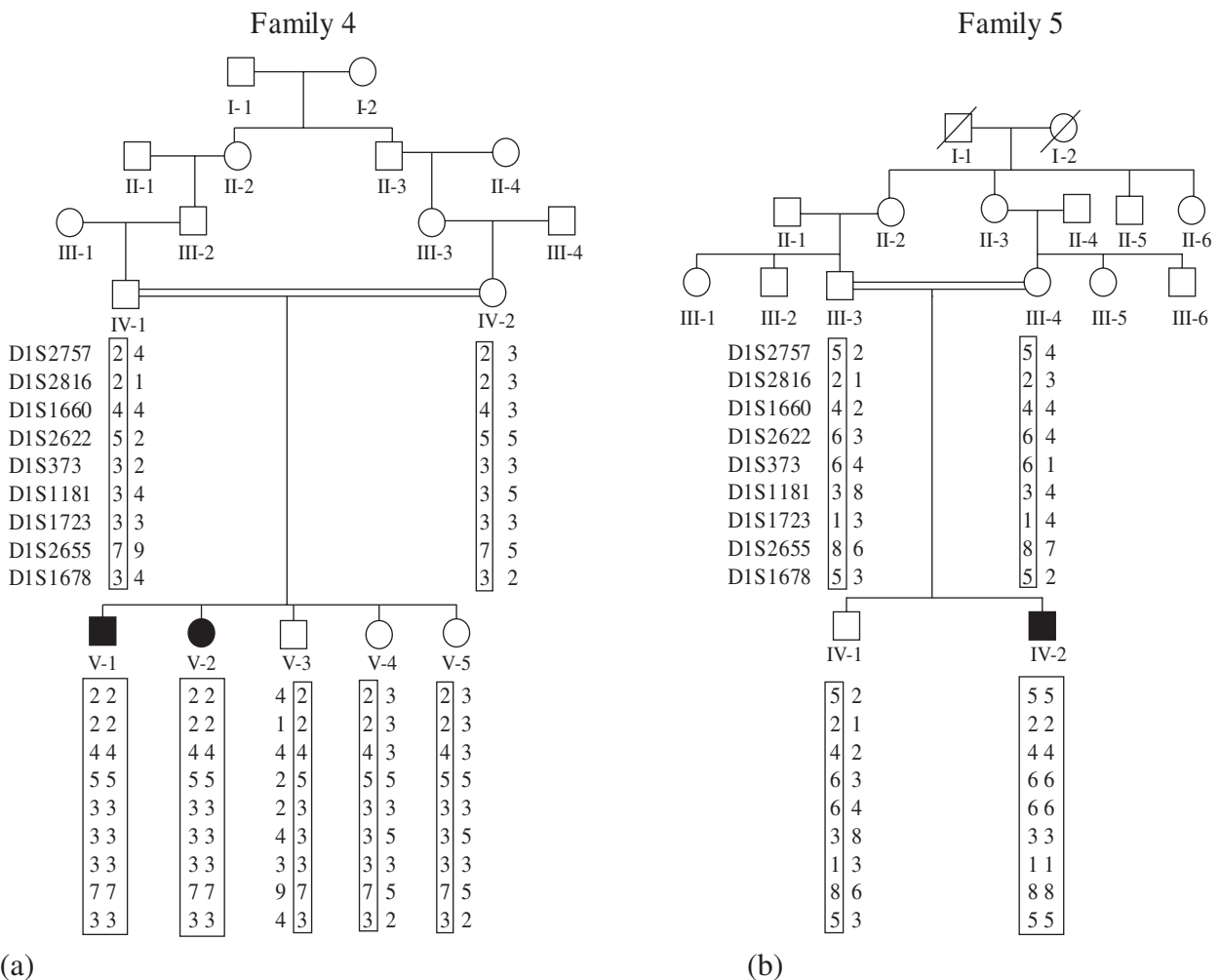


Fig. 2. Haplotype analysis of (a) family 4 and (b) family 5 with MCPH5 markers. Disease haplotype is boxed.

presence of the mutation by DNA-sequence analysis. Allele-specific oligonucleotide hybridization (ASOH) was used to determine if a mutation was present in 50 ethnically matched normal individuals as described by Cormand et al. (20).

Linkage analysis

Because all the nine families were consanguineous, identity by descent was sought and used to assess evidence of linkage to a particular locus (21, 22). Linkage of consanguineous families to a locus is based on the observation that if all affected individuals of a family had the same homozygous haplotype for a MCPH locus, the family was designated linked; if the affected individuals had different heterozygous marker results or different homozygous haplotypes, the family was considered as not linked (22). Two-point lod scores were calculated using the MLINK program from the LINKAGE Package version 5.1 (23), under the assumption of autosomal recessive mode of inheritance and a disease-gene frequency

of 1/300 (2). Population-specific allele frequencies are not available for the Indian population; therefore, equal marker allele frequencies were assumed for linkage analysis. Varying the allele frequencies did not substantially change the linkage results. Multipoint linkage analysis was conducted using Genehunter2 (24).

Results

High-resolution G-banding chromosome analysis revealed normal karyotypes in patients from all nine families (data not shown). Haplotype analysis using markers from six known MCPH loci suggested linkage of three families, family 4, family 5, and family 12 to the MCPH5 locus only (Figs 2 and 3a). Haplotype analysis of family 2 was suggestive of linkage of this family to the MCPH2 locus only (Fig. 3b). The remaining five families were not linked to any of the six known loci (data not shown). Affected individuals from family 4, family 5, and family 12 were

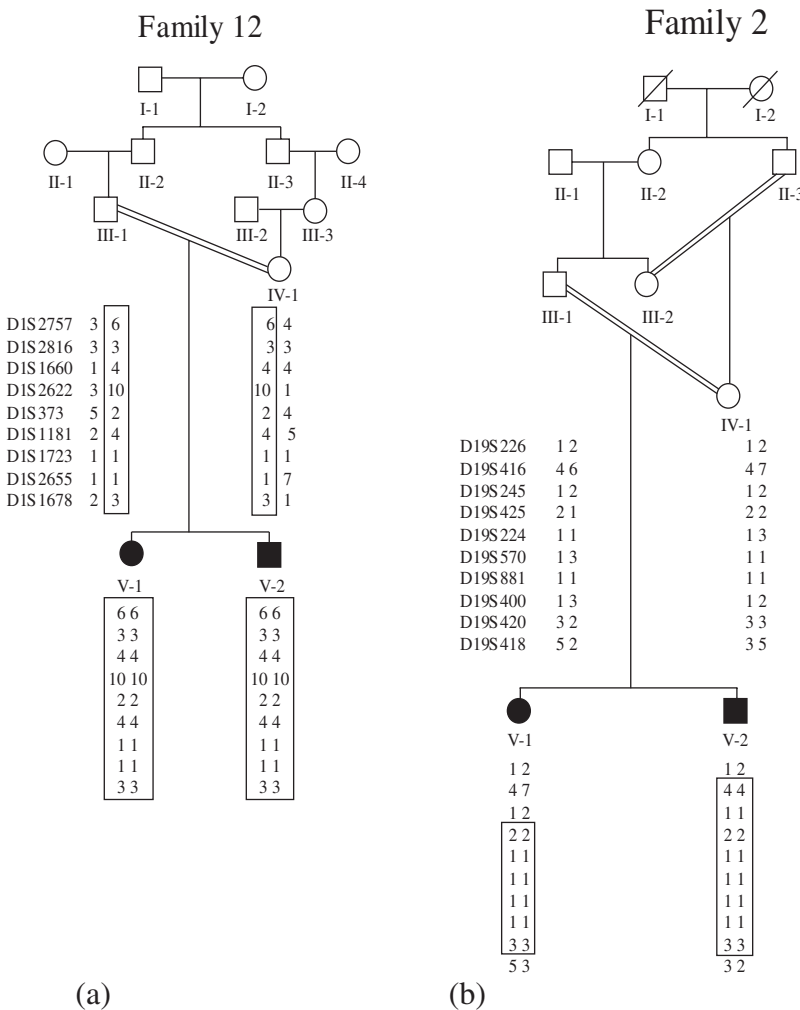


Fig.3. Haplotype analysis of (a) family 12 with MCPH5 markers and (b) family 2 with MCPH2 markers.

homozygous for the region flanked by D1S2757 and D1S1678, a distance of 9.3 cM (Figs 2 and 3a). A maximum two-point lod score of 1.92 at $\theta=0.0$ was observed at D1S2757, D1S2816, D1S373, and D1S2655 in family 4. A maximum two-point lod score of 1.52 at $\theta=0.0$ was observed at D1S2757, D1S2622, D1S373, D1S1181, and D1S1678 in family 12. None of the MCPH5-linked families (i.e., family 4, family 5, and family 12) shared a common disease haplotype (Figs 2 and 3a), suggesting that different mutations in the *ASPM* gene are responsible for the disease phenotype in these families.

A maximum two-point lod score of 1.31 at $\theta=0.0$ was obtained at D19S400 in family 2. Multipoint analysis yielded a maximum lod score of 1.43 in this family. A haplotype analysis in this family showed that the minimum region of homozygosity lies between D19S245 and D19S418 (Fig. 3b). Heterozygosities at D19S245 in affected individual V-1 and at D19S418 in affected individuals V-1 and V-2 placed the minimum critical region (MCR) for the MCPH2 candidate region between D19S245 and D19S418 (Fig. 3b).

DNA-sequence analysis of the affected individual V-1 from family 4 showed a C > T change at nucleotide position 9178 (c. 9178C > T) in exon 21 in a homozygous state, resulting in a nonsense mutation at codon 3060 (Gln3060X) (Fig. 4a). As expected from haplotype data, affected sibling V-2 was homozygous and both unaffected parents and three unaffected siblings were heterozygous for this change (Fig. 4b).

DNA-sequence analysis of the affected individual IV-2 from family 5 showed a G > A change at nucleotide position 3978 (c.3978G > A) in exon 17 in a homozygous state, resulting in a nonsense mutation at codon position 1326 (Trp1326X) (Fig. 4c). As expected from the haplotype data, both unaffected parents and the unaffected sibling, IV-1 were heterozygous for this change (Fig. 4d).

DNA-sequence analysis of the affected individual V-1 from family 12 showed a C > T change at nucleotide position 349 (c.349C > T) in a homozygous state, resulting in a truncating mutation at codon position 117 (Arg117X) (Fig. 4e). As expected from the haplotype data, the affected individual V-2 was homozygous for this change and both unaffected parents were heterozygous for this change (Fig. 4f).

The ASOH analysis showed that none of these three changes were observed in 100 ethnically matched normal alleles (data not shown). The presence of three different mutations in family 4, family 5, and family 12 was supported by the observations of different disease haplotypes at the MCPH5 locus in these families (discussed above). In addition

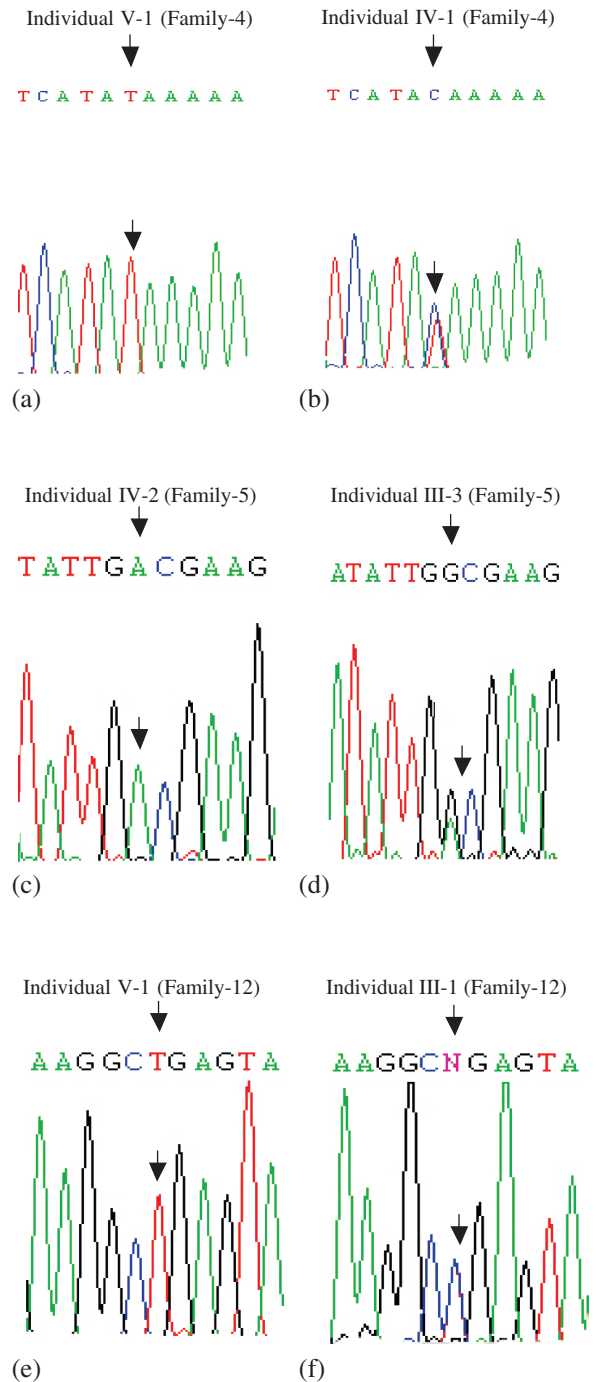


Fig. 4. DNA-sequence analysis of individuals from family 4, family 5, and family 12. (a) Sequencing chromatogram from affected individual V-1 from family 4, note homozygous change C > T marked by an arrow. (b) Sequencing chromatogram of the father IV-1 from family 4, note heterozygous change C > T (double peaks) marked by an arrow. (c) Sequencing chromatogram of affected individual IV-2 from family 5, note homozygous change G > A marked by an arrow. (d) Sequencing chromatogram of the father III-3 from family 5, note heterozygous change G > A (double peaks) marked by an arrow. (e) Sequencing chromatogram of affected individual V-1 from family 12, note homozygous change C > T marked by an arrow. (f) Sequencing chromatogram of the father III-1 from family 12, note heterozygous change C > T (double peaks) marked by an arrow.

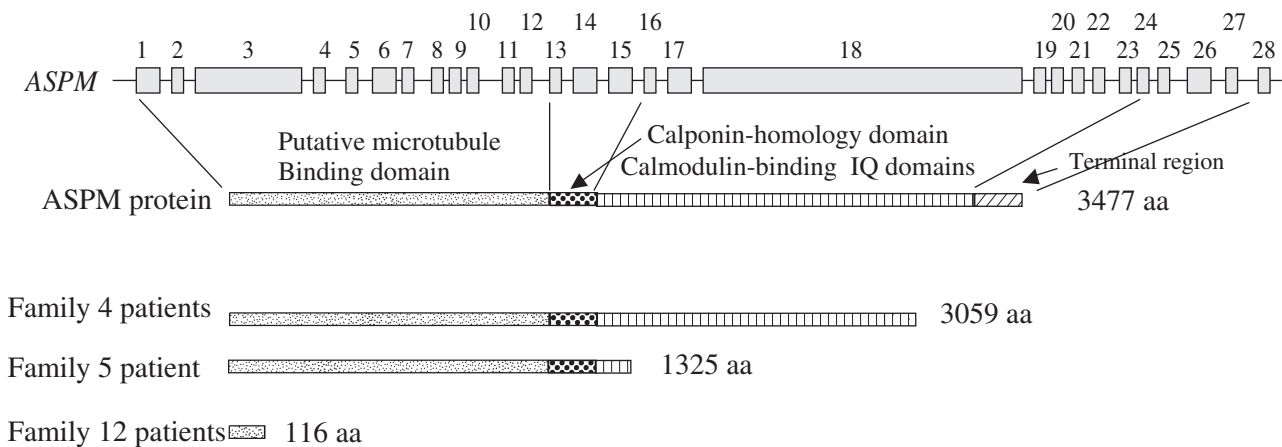


Fig. 5. Diagrammatic representation of the exon/intron structure of the *ASPM* gene according to Bond et al. (14). The sizes of the mutant *ASPM* proteins in patients from MCPH5-linked Indian families are shown.

to mutations, three normal population variants were also detected: c.7605G > A (V2535V) and c.4449G > A (K1483K) in family 4, c.7605G > A (V2535V) in family 5, and c.5961A > G (Q1987Q) in family 12 (data not shown).

Discussion

We have reported the results of a genetic analysis of primary microcephaly in nine consanguineous Indian families for the first time. Of nine families analyzed, 3/9 (33.33%) were linked to the MCPH5 locus and mutations were identified. One family (1/9, 11%), family 2, had genotype data which were consistent with linkage to the MCPH2 locus. While the maximum lod score for the markers surrounding this locus is below the accepted 2.0 to establish linkage to previously reported loci, this family was not linked to the remaining known loci. With both affected children homozygous for the region flanked by D19S245 and D19S418, MCPH2 as the cause of microcephaly in this family becomes even more likely. Haplotype analysis provides evidence for homozygosity for a 9.71-Mb region in affected individuals. The remaining five families were not linked to any of the six known loci. This suggests the involvement of an additional unknown locus (loci) for primary microcephaly and corroborates the observations of Roberts et al. (22). This study also suggests a major involvement of the *ASPM* gene in the etiology of primary microcephaly in Indian patients as has been observed in other populations (22). Interestingly, all three *ASPM* mutations reported in this study were nonsense mutations, resulting in premature truncation of *ASPM* proteins (Fig. 5).

In a study population of 56 consanguineous families originating from northern Pakistan, 24/

56 (42.85%) families were linked to the MCPH5 locus and 10/56 (17.85%) were linked to the MCPH2 locus (22). Roberts et al. (7) have previously reported that the MCR for the MCPH2 locus lies in a region of 9.74 Mb (7.61 cM) between markers D19S416 and 19S420 in two families from the northern region of Pakistan. Our analysis was unable to further narrow the region, as the MCR for this locus in our family lies between markers D19S245 and D19S418 in a region of 33.87 cM (Fig. 3b).

Jackson et al. (13) have recently identified the gene, *microcephalin*, responsible for the disease phenotype at the MCPH1 locus. A common protein truncating mutation, S25X was observed in two MCPH1-linked families from Pakistan. None of our families appear to be linked to the MCPH1 locus, suggesting that *microcephalin* mutations are not a major cause of primary microcephaly in India.

A total of 22 mutations in the *ASPM* gene have been reported so far in MCPH5-linked families from Pakistan, Turkey, the Netherlands, Jordan, Saudi Arabia, and Yemen (14, 15). Of these, five mutations, 1258delTCTCAAG, 9159delA, 9557C > G, 3663delG, and 3811C > T have been reported in more than one family (14, 15). The remaining 17 mutations have been reported in single families. One of the mutations, c.349C > T (Arg117X) reported in our family 12, has also been reported in one family from northern Pakistan (15). It is very unlikely that these two families have a common ancestral origin as they are geographically widely separated and are of different ethnic backgrounds. The northern Pakistani family is a Muslim family, whereas family 12 is a Hindu family from south India. The C residues in CpG dinucleotides are known to be mutational hotspots in many genes.

Table 1. Clinical details of affected individuals from family 4, family 5, and family 12 with mutations in the ASPM gene

Family/individual	Mutation	Age (years)	HC (SD) ^a	SQ	Behavioral and developmental characteristics	Other anomalies
Family 4/V-1	Gln3060X	8	40 (9 SD)	50	Sociable, low frustration tolerance, aggressive tantrums, hyperactive, occasional self-injurious behavior, walked at 19 months, says meaningful sentences and can relate experiences, can name few objects (food and water) and family members, no reading or writing skills, can partly take care of self	Sloping forehead, narrow bifrontal diameter
Family 4/V-2	Gln3060X	4	40 (8 SD)	62	Shy and withdrawn, walked at 12 months, talks in two-word phrases, can name familiar objects and family members, follows two- to three-step instructions, no reading or writing skills, can partly take care of self	Sloping forehead, narrow bifrontal diameter
Family 5/IV-2	Trp1326X	8	37.5 (11 SD)	48	Sociable, low frustration tolerance, hyperactive, occasional self-injurious behavior, walked at 18 months, talks in short meaningful sentences, can name family members and objects (food and water), partly take care of self, no reading or writing skills	Sloping forehead, narrow bifrontal diameter
Family 12/V-1	Arg117X	6	33.5 (13 SD)	31.5	Sociable, low frustration tolerance, aggressive tantrums, hyperactive, occasional self-injurious behavior, walked at 9 months, cannot talk, can follow simple instructions such as give or take, no reading or writing skills, can partly take care of self	Sloping forehead, narrow bifrontal diameter
Family 12/V-2	Arg117X	2	32 (12 SD)	44.5	Sociable, low frustration tolerance, aggressive tantrums, hyperactive, occasional self-injurious behavior, walked at 18 months, cannot talk, can follow simple instructions such as give or take, cannot take care of self	Sloping forehead, narrow bifrontal diameter

SQ, Social Quotient.

^aHead circumference in cm with SD below mean for age and sex in parentheses.

The C residue when methylated could demethylate and convert to a T residue. This could be the scenario with the mutation, c.349C > T, as the mutated C residue constitutes a CpG dinucleotide. The other two nonsense mutations, c.3978G > A (Trp1326X) in exon 17 in family 5 and c.9178C > T (Gln3060X) in exon 21 in family 4, are novel mutations. This raises the total number of reported mutations in the *ASPM* gene to 24. Interestingly, all of these mutations lead to premature truncation of the *ASPM* protein. Of the 24 truncating mutations, 12 are nonsense, nine are deletions, and three are splice-site mutations. These mutations are scattered throughout the *ASPM* gene, suggesting that mutation analysis will require screening of all the 28 exons of this gene. Due to the small size of our data, we are not able to study a correlation between the mutant protein length and head circumference (Fig 5; Table 1). No correlation was observed between the protein length and head circumference or the degree of mental retardation in 23 families studied by Bond et al. (15).

In summary, our genetic analysis of nine Indian families with primary microcephaly has shown that the most common cause for primary microcephaly in the Indian population is mutations in the *ASPM* gene as reported previously in other populations. Of three nonsense mutations detected in our MCPH5-linked families, two are novel and one is a known mutation reported earlier in a northern Pakistani family by Bond et al. (15). The observation of families unlinked to all loci in our family data set suggests the presence of one or more unknown loci for primary microcephaly.

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