A clinical and molecular genetic study of 112 Iranian families with primary microcephaly

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ABSTRACT

Background Primary microcephaly (MCPH) is a genetically heterogeneous disorder showing an autosomal recessive mode of inheritance. Affected individuals present with head circumferences more than three SDs below the age- and sex-matched population mean, associated with mild to severe mental retardation. Five genes (*MCPH1*, *CDK5RAP2*, *ASPM*, *CENPJ*, *STIL*) and two genomic loci, MCPH2 and MCPH4, have been identified so far.

Methods and results In this study, we investigated all seven MCPH loci in patients with primary microcephaly from 112 Consanguineous Iranian families. In addition to a thorough clinical characterisation, karyotype analyses were performed for all patients. For Homozygosity mapping, microsatellite markers were selected for each locus and used for genotyping. Our investigation enabled us to detect homozygosity at MCPH1 (*Microcephalin*) in eight families, at MCPH5 (*ASPM*) in thirteen families. Three families showed homozygosity at MCPH2 and five at MCPH6 (*CENPJ*), and two families were linked to MCPH7 (*STIL*). The remaining 81 families were not linked to any of the seven known loci. Subsequent sequencing revealed eight, 10 and one novel mutations in *Microcephalin*, *ASPM* and *CENPJ*, respectively. In some families, additional features such as short stature, seizures or congenital hearing loss were observed in the microcephalic patient, which widens the spectrum of clinical manifestations of mutations in known microcephaly genes.

Conclusion Our results show that the molecular basis of microcephaly is heterogeneous; thus, the Iranian population may provide a unique source for the identification of further genes underlying this disorder.

Microcephaly is a disorder characterised by an occipito-frontal head circumference (OFC) of at least three standard deviations (SD) below the age- and sex-matched population average.[1 2] This disorder is aetiologically heterogeneous, with environmental and genetic causes.[3] Among the environmental causes are congenital infections, maternal alcohol overconsumption and drugs taken during pregnancy. The majority of microcephalic cases are caused by a variety of genetic mechanisms, including cytogenetic abnormalities, single-gene disorders, etc.[4–6]

Microcephaly is divided into *primary microcephaly*, which is present at birth, and *secondary microcephaly*, which develops postnatally.[4] The birth prevalence of primary microcephaly varies from 1.3 to 150 per 100 000 live births depending on the population and the defined SD threshold,[7] with ~1 in 10 000 in consanguineous populations, less in non-consanguineous populations.[8] Primary nonsyndromal microcephaly has an birth prevalence of 1:30 000 to 1:250 000 live births.[3 7]

Autosomal recessive primary microcephaly (MCPH, for microcephaly primary hereditary) is a rare, genetically heterogeneous disorder reported in about 100 families worldwide.[5 9] The initial defining clinical features of MCPH are OFC ≤3 SD at birth with mild to severe mental retardation and no further neurological findings except for mild seizures. However, recently, studies have shown that the phenotype spectrum of patients with MCPH gene mutations is wider than reported in previous publications.[7 9 13 15 19]

There are at least seven MCPH loci, and the genes underlying six of these have been identified. These genes include *Microcephalin* at MCPH1, *CDK5RAP2* at MCPH3, CEP152 at MCPH4 *ASPM* at MCPH5, *CENPJ* at MCPH6 and *STIL/SIL* at MCPH7 (table 1).[3 5 7 8 20 35]

So far, different mutations have been detected for these loci in different countries, e.g. Pakistani Turkey, Yemen, Saudi Arabia, Jordan, The Netherlands, Brazil, India and Canada.[3 7 20–25 35] Furthermore, it is evident that mutations in additional genes are associated with MCPH, as 18 out of 56 northern Pakistani families,[23] five of nine Indian families[3] and 10 of 33 Pakistani families[20] showed no evidence of linkage to the known loci.

So far, no study has been carried out to evaluate the genetics of primary microcephaly in the Iranian population. Therefore, the aim of this study was to determine the molecular basis of primary microcephaly in the Iranian population.

MATERIALS AND METHODS

Clinical examination

Our study included a total of 642 families with mentally retarded patients in which the majority of them had two or more affected individuals. All had been referred to the Genetics Research Center, Tehran, Iran as part of a large collaborative study on the identification of causes of familial mental retardation. These families belonged to various ethnicities from different provinces of Iran. Informed consent was obtained from all family members who participated in the study.

Table 1 A review of the previous studies on loci for autosomal recessive primary microcephaly

Locus	Genomic region	Gene	Ethnicity	Reference
MCPH1	8p22-pter	Microcephalin	Northern Pakistani, Iranian	10–12
MCPH2	19q13.1–13.2	Unknown	Northern Pakistani, Indian, Pakistani	13
MCPH3	9q34	CDK5RAP2	Northern Pakistani	14 25
MCPH4	15q21.1	CEP152	Moroccan, Pakistani, Canada	15 35
MCPH5	1q31	ASPM	Northern Pakistani, Turkish, Jordanian, Dutch, Saudi Arabian, Yemeni, Indian	16–18
MCPH6	13q12.2	CENPJ	Northern Pakistani, Brazilian, Pakistani	21 25
MCPH7	1p32.3–p33	STIL	Indian	19

One hundred and twelve families out of 642 (17.5 %) showed primary microcephaly[2] and were divided into two groups: non-syndromic forms (92 families) and microcephaly associated with additional features (20 families). The number of affected individuals in the studied families ranged from one to eight. Consanguinity was observed in all families. Clinical histories indicated that microcephaly was present at birth in all of the affected individuals. On examination, head circumferences were 3 to 13 SD below the population age- and sex-related mean. All parents appeared to have normal intelligence and normal head circumferences.

DNA extraction and genotyping

DNA was extracted from peripheral blood samples following a standard protocol.[26] A panel of 70 (6 to 21 for the individual candidate regions) microsatellite markers (S1) was selected from the Genome Databases (http://www.gdb.org/ and http://genome.ucsc.edu/). Since population-specific allele frequencies were not available for the Iranian population, we verified the percentage of allelic heterozygosity for these markers in 10 unrelated individuals from diverse ethnic groups: Persians (Tehran and Esfahan), Azaris, Gilaki and Mazandarani, Kurds, Arabs (Bandar-e Abbas and Ahvaz), Baluchi and Lurs. A minimum of two microsatellite markers per locus was genotyped in the parents, at least two affected children and one healthy sibling in each family. Conditions for PCR amplification of the microsatellite markers are available in the supplementary information (S2). Polyacrylamide gel electrophoresis and a standard silver stain protocol were used to visualise the results. When the haplotype at a given MCPH locus was found to be homozygous in all affected individuals of a family, mutation screening was initiated. If heterozygous markers or different homozygous haplotypes were found in the patients, the respective locus was excluded.

Karyotyping

To exclude the possibility of chromosomal aberrations as the cause of microcephaly in these families, high-resolution G-banding chromosomal analysis was performed for one affected individual in each family.

Sequencing of *ASPM*

The probands of the 13 families linked to the MCPH5 locus on chromosome 1q31, all 28 exons and exon/intron splice junctions of the *ASPM* gene (National Center for Biotechnology Information GenBank Accession Number AF509326), were sequenced using a set of 33 PCR primers (S3). After PCR amplification, the amplicons (S4) were sequenced using an ABI 3730 genetic analyser (Applied Biosystems, Foster city, California, USA.). Sequences were compared with the reference genomic and cDNA sequence (NM_018136).

Sequencing of *microcephalin*

We amplified the 14 exons and exon/intron splice junctions of the *microcephalin* gene (GenBank accession number AX087870) using a set of 16 primers (S5 and S6), designed with the Primer3 software,[23] and sequenced the amplicons as described above.

Sequencing of *CENPJ*

Using a set of 17 primer pairs (S7), designed with the Primer3 software,[27] the 17 exons and exon/intron splice junctions of the *CENPJ* gene (GenBank accession number BC024209) were amplified and sequenced as described above.

Sequencing of *STIL*

We amplified and sequenced the whole coding region, untranslated regions and exon/intron junctions of *STIL* (GenBank accession number NM_003035.2) using 23 primer pairs (S8).

RESULTS

In total, we studied 112 families with 315 MCPH patients including 158 men and 157 women (ratio 1:1) The number of affected individuals in each family ranged from one to eight (table 2). The degree of microcephaly in the investigated patients ranged between 3 and 13 SD below the population age-related mean. There was no clinical correlation between sex and the degree of microcephaly. In most of the families with multiple affected individuals, except three families, differences in head circumferences did not vary by more than two SD (table 3). The ages of all patients were between 2 and 73 years, the mean age being 19. Intellectual quotient (IQ) scores measured in all patients revealed a degree of intellectual disability ranging from moderate to profound, with no correlation between sex and the

Table 2 Distribution of MCPH patients among the total families with known and unknown loci

Locus affected, number	MCPH1	MCPH2	MCPH3	MCPH4	MCPH5	MCPH6	MCPH7	Unlinked	Total families, number
1 affected	–	–	–	–	–	–	–	14	14
2 affected	3	2	–	–	4	3	–	23	35
3 affected	3	1	–	–	3	2	1	24	34
4≤ affected	2	–	–	–	6	–	1	20	29
Total families	8	3	–	–	13	5	2	81	112

Table 3 Results of Clinical manifestations and genotyping in patients with autosomal recessive primary microcephaly and novel mutations in Iran

Family	Affected number	Mental retardation severity	Head circumference	Locus	Gene	Mutation	Location	Predicted protein effect
M-019	6	Moderate	−3SD	MCPH1	Microcephalin	del exon 1−6*	Exon1-6	Truncated protein
M-282	2	Moderate	−10 to −11SD	MCPH1	Microcephalin	del exon 4	Exon4	Truncated protein
M-8600133	3	Moderate	−6 SD	MCPH1	Microcephalin	c.566_567insA	Exon6	p.Asn189fs
M-8700008	3	Mild to moderate	−6 to −8 SD	MCPH1	Microcephalin	del exon 2&3	Exon2 & 3	Truncated protein
M-8700012	2	Severe	−9 SD	MCPH1	Microcephalin	c.436+1G>T	Intron5	Truncated protein
M-8700070	4	Moderate	−7 to −9 SD	MCPH1	Microcephalin	c.147C>G	Exon3	p.His49>Gln
M-8700071	2	Mild to moderate	−6 to −10 SD	MCPH1	Microcephalin	del exon 3	Exon3	Truncated protein
M-8700173	3	Mild to moderate	−6 to −7 SD	MCPH1	Microcephalin	c.215C>T	Exon3	p.Ser72>Leu
M-064	4	Mild to moderate	−8 SD	MCPH5	ASPM	c.5584A>C	Exon18	p.Lys1862Gln
M-136	3	Mild to moderate	−9 to −11 SD	MCPH5	ASPM	c.9286C>T	Exon21	p.Arg3096X
M-273	8	Mild to moderate	−9 to −11 SD	MCPH5	ASPM	c.3055C>T	Exon11	p.Arg1019X
M-8500316	3	Mild to moderate	−6.5 to −8 SD	MCPH5	ASPM	c.9319C>T	Exon22	p.Arg3107X
M-8500319	4	Mild to moderate	−8 to −9 SD	MCPH5	ASPM	c.5188G>T	Exon18	p.Glu1730X
M-8600052	4	Moderate	−5.5 to −8 SD	MCPH5	ASPM	c.3229_3230delAA	Exon13	p.Lys1077fs
M-8600275	2	Mild to moderate	−7 to −8 SD	MCPH5	ASPM	c.3741+1G>A†	Intron15	Truncated protein
M-8600592	3	Mild to moderate	−8 to −10 SD	MCPH5	ASPM	c.3505_3506delGT	Exon14	p.Val1169fs
M-8700018	5	Moderate	−5.5 to −11 SD	MCPH5	ASPM	c.9091C>T	Exon21	p.Arg3031X
M-8700113	2	Moderate	−9 SD	MCPH5	ASPM	c.297+1G>C	Intron1	Truncated protein
M-8600570	6	Mild to moderate	−9 to −11 SD	MCPH5	ASPM	c.3506_3507delTG	Exon14	p.Val1169fs
M-238	2	Severe	−4 to −6 SD	MCPH6	CENPJ	c.2462C>T	Exon7	p.Thr821>Met

*This mutation detected in this study has been previously reported by Garshasbi *et al* 2006.[12]
†This mutation has been reported for the first time by Nicholas *et al* 2009.[28]
aa, amino acids; fs, frame shift; SD, standard deviations.

degree of mental retardation. In addition, there was no meaningful difference between the IQ levels and MCPH loci/ genes.

Clinical findings

In 20 of the 112 microcephalic families, other symptoms were observed such as short stature, ataxia, autism, facial dysmorphism and skeletal deformities. Facial dysmorphism included hypertelorism, long philtrum, micrognathia, lip thickness, downslanting palpebral fissure, teeth widening and strabismus. Skeletal deformities demonstrated in patients included toe shortening, joint stiffness, club foot, waddling gait, pectus incarnatum, large toes and sandal foot (see table 4). These features were observed in some of the families linked to different MCPH loci, and we can not conclude that the additional changes are the result of MCPH in these families.

Genotyping and sequencing results

In total, 112 families were screened for MCPH loci, and 31 families (27.7%) using microsatellite analyses revealed homozygosity at one of the seven known MCPH loci. Among the 92 nonsyndromic families, 29 (31.5 %) showed homozygosity at any of the known MCPH loci. In agreement with previous findings,[23] MCPH5 (*ASPM*) was the most prevalent locus with linkage in 13 families (14.1%). Eight families (8.7%) showed homozygosity at MCPH1, two (2.2%) at MCPH2, four (4.3%) at MCPH6, and two (2.2%) at MCPH7. Two out of 20 families with additional feature were linked to two loci, MCPH2 and MCPH6.

In total, 31 families with two or more affected individuals showed homozygosity at any of the MCPH loci, with all families having two or more affected individuals. Thus, the frequency of familial cases with linkage to either of the known MCPH loci was 31.6% (31 of 98 families).

Subsequent sequencing revealed eight novel mutations in *MCPH1* (one of which was previously reported by Garshasbi *et al*[12]), 10 novel mutations and one previously reported mutation in *ASPM* and one novel mutation in *CENPJ*. No mutations were found in the remaining families (11 of 31) with homozygosity at the MCPH loci. The results of mutation screening are summarised in table 3. All DNA changes and mutations not found in 160 German and 190 Iranian normal controls. Also, all mutations detected in affected individuals have been investigated in normal siblings and parents of these families.

We were unable to establish any linkage for the remaining 81 families (72.3%), of which 14 families had sporadic cases.

Karyotyping results

GTG banding, with the average resolution of 450–500 bands per haploid set, demonstrated a normal karyotype in all patients. However, in patients with *MCPH1* mutations, chromosomes appeared curly with a high level of breakage, and there was increased number of prophase looking cells (figure 1), representing about 80% of the total number of cells compared with 13% in normal controls. Both features are consistent with

Table 4 Microcephalic families with additional symptoms

Number	Family	Linkage results	Affected number	Additional feature
1	M-8600075	MCPH2	3	Facial dysmorphism†, IUGR*, wideness between the first/second toes
2	M-136	MCPH5	3	Short stature‡, congenital hearing loss
3	M-273	MCPH5	8	Short stature‡
4	M-8500316	MCPH5	3	Short stature‡
5	M-238¶	MCPH6	2	Facial dysmorphism§, developmental delay, joint stiffness (ankles), wheelchair boundaries, pectus incarnatum, finger deformities, seizure
6	M-8600278	MCPH7	4	Short stature‡, strabismus, ataxia, seizure
7	M-092	Unlinked	4	Malformed ear
8	M-108	Unlinked	2	Short stature‡
9	M-143	Unlinked	2	Short stature‡, joint deformity
10	M-160	Unlinked	3	Short stature‡, Cataract
11	M-177	Unlinked	3	Strabismus, club foot, imbalance walking
12	M-268	Unlinked	3	Short stature‡, strabismus, ataxia
13	M-279	Unlinked	2	Short stature‡
14	M-8600057	Unlinked	3	Wrist stiffness, lip thickness
15	M-8600083	Unlinked	2	Lip thickness, hand winging, widening teeth, down-slanting palpebral fissure
16	M-8700007	Unlinked	2	Neurodevelopmental delay, strabismus
17	M-8700069	Unlinked	3	Strabismus
18	M-8700073	Unlinked	3	Ataxia, autism
19	M-8700146	Unlinked	2	Ataxia
20	M-8700161	Unlinked	1	Micrognethia

*Intra-uterine growth retardation.
†Broad nasal bridge, long philtrum, micrognathia, thick lower lip.
‡With height <3 percentile.
§Small ears, hypertelorism, notched nasal tip, strabismus.
¶Some clinical features including seizure, wheelchair boundaries and joint stiffness were observed in both patients, but the others were only demonstrated in one of the siblings.

premature chromosome condensation, a previously reported characteristic in patients with *MCPH1* mutations.[24]

DISCUSSION

In this study, we investigated 112 Iranian families with nonsyndromic forms (92 of 112) of microcephaly and microcephaly associated with some additional features (20 of 112). Of the families with patients that had additional clinical features, one family showed linkage to MCPH2 and one to MCPH6 (*CENPJ*). In patients from the latter, we detected a novel mutation in the *CENPJ* gene. Interestingly, the patients showed seizures, a feature which was not observed in previous studies, where none of the reported patients with mutations in *CENPJ* had clinical features other than microcephaly.[21 22 25]

In two families with apparent homozygosity at the MCPH5 locus, no mutations in *ASPM* were detected, which might indicate that either the mutation is located in regulatory sequences of the gene (which had not been screened in this study) or that there might exist another MCPH gene in this region. Tentative evidence for a second causative gene in the region has been shown previously by Wallerman *et al*[29] in five families and Gul *et al*[20] in nine families linked to the MCPH5 locus.

The novel *ASPM* missense change (c.5584A>C) in exon 18 of the *ASPM* gene we found in family M-064 converts the conserved basic amino acid lysine to a neutral glutamine (K1862Q) in the IQ repeat motif 25 of the protein (amino acid 1854–1880). This novel mutation is the first missense mutation in *ASPM* gene. Even though our results lack a functional study for this missense mutation in the *ASPM* gene, the size of the family, lack of the homozygous mutation in normal siblings and lack of this mutation in the ethnically matched control subjects as well as absence of any other changes even in the regulatory region of the gene suggest that this missense change might disrupts the function of the protein. Mutations in IQ repeat motif 25 may disrupt the interaction of ASPM with calmodulin and calmodulin-related proteins,[30] and this may also be true for

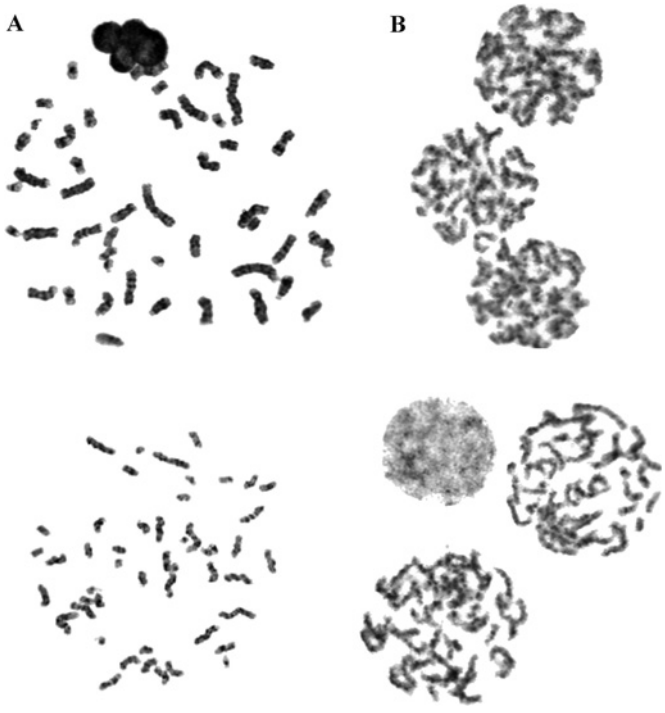

Figure 1 The metaphase spreads (A) and the prophase-like cells (B) in one of the patients with *MCPH1* mutation.

Table 5 The frequency of MCPH loci among the different population

Locus ethnic group	MCPH1	MCPH2	MCPH3	MCPH4	MCPH5	MCPH6	MCPH7	Linked	Unlinked	Reference
Pakistani	2/56 (3.5%)	10/56 (18%)	2/56 (3.5%)	0/56 (0%)	24/56 (43%)	0/56 (0%)	–	38/56 (68%)	18/56 (32%)	23
Pakistani	0/33 (0%)	2/33 (6%)	0/33 (0%)	2/33 (6%)	18/33 (55%)	1/33 (3%)	–	23/33 (70%)	10/33 (30%)	20
Pakistani	0/21 (0%)	0/21 (0%)	0/21 (0%)	0/21 (0%)	18/21 (86%)	0/21 (0%)	0/21 (0%)	18/21 (86%)	3/21 (14%)	34
Indian	0/9 (0%)	1/9 (11%)	0/9 (0%)	0/9 (0%)	3/9 (33.5%)	0/9 (0%)	–	4/9 (44.5%)	5/9 (55.5%)	3
Mixed population*					41/99 (41%)			†		28
Iranian	8/98 (8.2%)	3/98 (3.1%)	0/98 (0%)	0/98 (0%)	13/98 (13.3%)	5/98 (5.1%)	2/98 (2.1%)	31/98 (31.6%)	67/98 (68.4%)	This study

*Caucasian, Pakistani, Indian, Turkish, Arab, African, and Dutch.
†In this study only MCPH5 locus has been investigated.

the stop mutations observed in families M-8500319, M-8700018 and M-850036. The five novel nonsense mutations in the *ASPM* gene most probably induce non-sense-mediated mRNA decay and thus lead to a loss of ASPM function in homozygous mutation carriers. Three of the remaining novel *ASPM* mutations cause frame shifts in *ASPM*, and two affect splicing donor sites in intron15 (c.3741+1G>A) and in intron1 (c.297+1G>C), all of which alter the *ASPM* transcript, most likely leading to functional impairment of the gene product.[30] This is also the case in one of the mutations we found in intron5 (c.436+1G>T) of *MCPH1*.

In this gene, we also found four large deletions in families M-282 (del exon 4), M-8700071 (del exon 3), M-8700008 (del exon 2 and 3) and M-019 (del exon 1–6), which leads to truncation of microcephalin. Moreover, we found two missense mutations in two families M-8700173 (c.215C>T/S72L) and M-8700070 (c.147C>G/H49Q) that are located in the BRCT1 domain of the microcephalin protein. A frame shift mutation was found in family M-8600133 (c.566_567insA/N189fs). Microcephalin is predicted to contain three BRCA1 C-terminal (BRCT) domains. These domains interact to form homo/hetero BRCT multimers.[31]

We just found one novel missense mutation in *CENPJ* in family M-238 (c.2462C>T/T821M) which may be a disrupted TCP10 complex in CENPJ protein. The N-terminus of 4.1R-135 interacts with the C-terminal Tcp10 domain of CENPJ, and CENPJ is associated with the γ-tubulin ring complex. It has, therefore, been hypothesised that 4.1R-135 protein serves as an adaptor that anchors the CENPJ/γ-tubulin complex to the centrosome. In vitro evidence shows that CENPJ can inhibit microtubule nucleation from the γ-tubulin ring complex and is also able to depolymerise Taxol-stabilised microtubules. Therefore, this mutation can inhibit the normal function of CENPJ protein.[32]

With respect to the clinical manifestation of *ASPM* mutations, it is noteworthy that families with nonsense mutations in this gene (M-136, M-273 and M-8500316) also presented with short stature. Up to now, this was only observed in patients with mutations in *MCPH1*. In addition to that, we observed the first case of *ASPM* mutations in microcephalic patients with congenital hearing loss. We therefore excluded one of the most frequent causes of congenital hearing loss, mutations in the GJB2 gene encoding connexin 26, by sequencing. These observations show that the spectrum of clinical manifestations in affected individuals with mutations in *ASPM* is wider than previously reported.

In order to compare our data with the other previously reported study, we removed our 14 singleton families from our data. The remaining families all of which had two or more affected individuals were families with consanguineous marriages and autosomal recessive pattern of inheritance. We could establish homozygosity at one of the known MCPH loci for less than one-third of our families, and for two of three of our families, the genetic causes remained to be solved. Particularly *ASPM* defects among the 98 familial cases were 13.3% (13 of 98) much less prevalent than in the Pakistani's population, where frequencies between 43% and 55% were reported,[20 23] or in India where Kumar *et al*[3] found a frequency of 33%. We also observed much lower frequencies of MCPH1, MCPH7, MCPH6 and MCPH2 mutations in the Iranian compared with the other populations (table 5). Therefore, the Iranian population may provide a unique source for the identification of further genes with causative mutations in autosomal recessive primary microcephaly.

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