Research Paper

Linkage Study of Primary Microcephaly in Pakistani Kindred

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Abstract

Microcephaly is heterogeneous, autosomal recessive trait with reduced head circumference of at least 4 SD below age and sex means due to reduction in neuron production. The brain of microcephalic patient is architecturally normal but severe to mild mental retardation. It is rare disease affecting 2-2.5% of total population specifically in Asia and Arab where the incidence of cousin marriages is relatively high. From seven known currently mapped loci ASPM is found to be the frequent causative agent. In the current investigations exclusion mapping of a microcephalic family was done. DNA from all blood samples was extracted using standard procedure and after gene specific PCR amplifications, 8% non-denaturing PAGE was done. Linkage was observed at MCPH5 locus where ASPM is a candidate gene on chromosome 1q31. The results of DNA sequencing showed G to A transition and Leucine (CTG) to Leucine (CTA) was noted. There are six triplet codons which differ by single nucleotide encoding for Leucine. Hence, no overall change in the effect of protein expression was observed due to the degeneracy of codons. Therefore, the sequencing of the entire ASPM gene with intervening sequences was suggested in order to find the actual cause of microcephaly.

Keywords: Exclusion, Mapping, Microcephaly, ASPM

1. Introduction

Microcephaly (MCPH) is an autosomal recessive neurodevelopmental disorder which is described by various terms that explain the same phenotype in literature such as “True microcephaly” “microcephaly vera” and “autosomal recessive primary microcephaly”. The cerebral cortex is severely affected due to decreased production of neurons leading to overall reduction in brain size (Wood et al, 2005).

The early clinical features which included; congenital microcephaly at least 4 SD below age and sex means, mental retardation but no other neurological finding such as spasticity, seizures, or progressive cognitive decline and normal height and weight, appearance (Jackson et al, 2002).

Genetic heterogeneity is well established in MCPH with seven loci which have been currently mapped. MCPH1/Microcephalin localized at 8p23.1 in Northern Pakistani families (Jackson et al, 2002). MCPH2/WDR62 localized at 19q13.1-13.2 in Northern Pakistani families (Nicholas et al, 2010). MCPH3/CDKRAP2 localized at 9q33.2 in Northern Pakistani families (Bond et al, 2005). MCPH4/CEP152 localized at 15q15-21 in Canadian families (Guerney et al, 2010). MCPH5/ASPM localized at 1q31.1 in Turkish/Pakistani families (Bond et al, 2002). MCPH6/CENPJ localized at 13q12.12 in Brazilian families (Bond et al, 2005). MCPH7/STIL localized at 1p33 in Indian families (Kumar et al, 2009).

MCPH5 is localized on chromosome 1q31 and is mapped to an 8-cM region (Pattison et al, 2000). The ASPM gene comprises 10,434 bp long coding sequence with 28 exons and traverse 65 kb of genomic DNA. ASPM is upregulated...
in malignant cells and is widely expressed in fetal and adult tissues. MCPIH is the consequence of impairment in mitotic spindle regulation in cortical progenitors due to mutations in \textit{ASPM} (Gul et al, 2006). \textit{ASPM} mutations have been strongly isolated (Kumar et al, 2004).

Until now 57 \textit{ASPM} mutations have been reported. Out Of which 17 mutations are frequently occurring. One of the mutations involved premature termination codon in the \textit{ASPM} gene. 23 nonsense mutations, 28 small deletions or insertions mutation which lead to an alteration in the reading frame, and five were splice site mutations, once more lead to the use of a premature stop codon (Nicholas et al, 2009).

The aim of the study is linkage analysis of primary microcephaly in Pakistani population.

2. Materials and Methodology

2.1. Sample Collection

The data of the family of autosomal recessive primary microcephaly was collected to generate an individual’s medical and developmental history considering the inheritance and chance occurrence of disease in offspring. Informed consent was obtained from all family members who participated in the study. MCP Family having six individuals was collected from Shirkpur, out of which two sons were affected with primary microcephaly (as shown in figure 1), while father was unapproachable.

![Figure 1: Photographs of Microcephalic individuals of MCP family from Shirkpur. (a) II: 2; (b) II: 4.](image)

The clinical findings of the individuals were evaluated. Individual II: 2 (6yrs 10months) had slopping forehead, prominent ears, mild to moderate mental retardation and the Head circumference was measured to be 36.5 cm. Individual II: 4 (8 1/2 months) had slopping forehead, small head size and prominent ears. The Head circumference was found to be 36 cm.

2.2. DNA Extraction and Genotyping with MCPH5

\subsection*{2.2.1. Microsatellite Markers}

The patients were clinically evaluated and the information was collected individually. The extraction of Genomic DNA from peripheral blood was performed by using standard phenol-chloroform extraction procedure (Sambrook et al, 2001). Microsatellite markers used for genotyping were; D1S2757, D1S2840, D1S1660, D1S2622, D1S373, D1S1723, D1S2655, D1S2686 and D1S1724. The master mixture for PCR (Thermocycler I Biorad) was prepared for five reactions. 65 µl autoclaved distilled water was added first and then 12.5 µl of 10X PCR buffer (NH\textsubscript{4}SO\textsubscript{4}, 12.5 µl of (25 mM) MgCl\textsubscript{2}, 10.0 µl of (2.5 mM) dNTPs , 5.0 µl of (50 pM) forward and 5.0 µl (50 pM) reverse primer solutions, 10.0 µl of Taq DNA polymerase were added to the master mixture. Master mixture was mixed well and was equally distributed in PCR tubes (24 µl each). In the last 1 µl of DNA template was added and final volume was made up to 25 µl in each PCR tubes. This method of setting reactions saves times an also minimizes the pipetting errors. Amplification was performed with an initial denaturation for 4 min at 95°C, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 57°C for 45 sec, elongation at 72°C for 45 sec, final extension at 72°C for 10 mins and hold at 4°C for ∞. The PCR products were separated on 8% non-denaturing polyacrylamide gels. The staining of gel was done with ethidium bromide and photographed under UV illumination Gel doc system (Syngene).

\subsection*{2.3. DNA sequencing}

Polymerase chain reaction (PCR) amplification of exon 18 of \textit{ASPM} gene was done by using gene-specific primer D1S1660. The amplification was done following the previously described thermocycler profile. The PCR products were analyzed by 2% agarose gel. PCR product were then purified using PCR Purification Kit (Fermentas GeneJET\textsuperscript{TM} Gel Extraction Kit # K0691, # K0692). 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 BioEdit viewer software.

3. Results

After pedigree construction and DNA extraction, genotyping with MCPH5 microsatellite markers was done and linkage was observed on the basis that all the affected individuals had the same homozygous banding pattern whereas the normal individuals showed heterozygous banding pattern with the MCPH5 locus. MCP family showed complete linkage on chromosome 1q31 which harbours \textit{ASPM} gene, with the microsatellite marker D1S1660 and D1S2840 as shown in the figure 2 and 3.
Haplotype was constructed and the region was defined with the microsatellite marker D1S2757 and D1S1678 as shown in the figure 4. DNA sequence analysis revealed a G to A transition [(CTG) to (CTA)] in affected individual (II: 4) as shown in the figure 5 and 6 for exon 18 of ASPM gene.

Figure 2. Electropherogram of ethidium bromide stained 8% non-denaturing PAGE gel of MCP for primer D1S1660 (212.44 cM) from Left to right, on chromosome 1q31 showing homozygosity among all affected (A) and normal (N) individuals.

Figure 3. Electropherogram of ethidium bromide stained 8% non-denaturing PAGE gel of MCP for primer D1S2840 (212.04 cM) from Left to right, on chromosome 1q31 showing homozygosity among all affected (A) and normal (N) individuals.

Figure 4. Pedigree of family MCP showing primary microcephaly associated haplotype. The genetic map distance is according to Marshfield genetic map in centimorgans (cM) is shown next to the marker name. Where circles shows females and squares for males, filled for circles and squares are for affected individuals.
Figure 5. Chromatogram showing the sequence of exon 18 of phenotypically normal/carryer (II: 2) individual. Arrows indicate the site of mutation.

Figure 6. Chromatogram showing the sequence of exon 18 of affected (II: 4) individual. Arrows indicate the site of mutation.

4. Discussion

The viability of using a simple and defined PCR followed by 8% native PAGE and sequencing is supportive in screening possible mutations in ASPM and all other MCPH genes. Family MCP was selected for screening for all possible known loci MCPH1-MCPH7. The selection of the locus was clearly on the basis of chance for the disease to occur. So first of all MCPH5 was selected as it is the most frequent causative agent. More than 50% of the MCPH families were mapped to MCPH5 locus in Pakistan (Saleha et al, 2011). In Pakistan the incidence of MCPH is ~ 1 in 10,000 individuals (Woods et al, 2005). In our study, MCPH5 markers were selected which include D1S2757 (209.15 cM) to D1S1678 (218.05 cM) in centromeric to telomeric direction. Screening was preceded with the marker D1S2840, D1S1660, D1S2622, D1S373, D1S1723, D1S2686, D1S2655, D1S1724 and D1S1678 which indicated the homozygous banding pattern of the affected individuals where as phenotypically normal or carrier individuals showed heterozygous banding pattern thus, linkage was observed in the region starting from 209.15cM to 218.05 cM. Some microsatellite markers which had been tested were non informative i.e., could not differentiate between affected and normal individual and same banding pattern was observed. Allelic frequency of all five individuals was observed in MCP family by difference in heterozygous and homozygous banding pattern and hence showed complete linkage on chromosome 1q31.

In present study, G to A transition [(CTG) to (CTA)] was
noted in affected individual (II: 4) as shown in the figure 5 and 6. The observed two triplet codons were from six triplet codons code for leucine and represented the degeneracy of codons. Hence, no change in the overall protein structure was observed. The observed G to A mutation was not present in all previously reported mutations of ASPM gene (Nicholas et al, 2009).

Thus, the sequencing of entire ASPM gene including intervening sequences is suggested to determine the real cause of MCPH phenotype. Further investigations are recommended for more molecular elucidations to prevent the risk of disease. Hence, mutation screening will help in genetic counselling and prenatal diagnosis for microcephaly in Pakistani population, which may in turn help reduce the incidence of microcephaly in consanguineous population.

Acknowledgments

This study is funded by the University of the Punjab, Pakistan. We wish to thank Dr. Muhammad Yaqoob, Head of the Genetics department at the Children’s Hospital, Lahore for his essential assistance in blood sample collection and reviewing the patient’s clinical history and pedigree for this study.

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