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Original Article

Molecular analyses in Indonesian individuals with intellectual disability and microcephaly

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Abstract

Background Intellectual disability (ID) often coincides with an abnormal head circumference (HC). Since the HC is a reflection of brain size, abnormalities in HC may be a sign of a brain anomaly. Although microcephaly is often secondary to ID, hereditary (autosomal recessive) forms of primary microcephaly (MCPH) exist that result in ID.

Objective To investigate mutations in MCPH genes in patients with ID and microcephaly.

Methods From a population of 527 Indonesian individuals with ID, 48 patients with microcephaly (9.1%) were selected. These patients were previously found to be normal upon conventional karyotyping, fragile X mental retardation 1 (FMR1) gene analysis, subtelomeric deletion, and duplication multiplex ligation-dependent probe amplification (MLPA). Sanger sequencing for abnormal spindle-like microcephaly-associated (ASPM) and WD repeat domain 62 (WDR62) was performed in all 48 subjects, while sequencing for microcephalin (MCPH1), cyclin-dependent kinase 5 (CDK5) regulatory subunit-associated protein 2 (CD5KRAP2), centromere protein J (CENPJ), and SCL/TAL1 interrupting locus (STIL) was conducted in only the subjects with an orbitofrontal cortex (OFC) below -4 SD.

Results In all genes investigated, 66 single nucleotide polymorphisms (SNPs) and 15 unclassified variants which were predicted as unlikely to be pathogenic (UV2), were identified. Possible pathogenic variants (UV3) were identified in ASPM. However, since none of the patients harboured compound heterozygous likely pathogenic mutations, no molecular MCPH diagnosis could be established. Interestingly, one of the patients harboured the same variants as her unaffected monozygotic twin sister, indicating that our cohort included a discordant twin.

Conclusions This study is the first to investigate for possible genetic causes of MCPH in the Indonesian population. The absence of causative pathogenic mutations in the MCPH genes tested may originate from several factors. The identification of UV2 and UV3 variants as well as the absence of causative pathogenic mutations calls for further investigations. **[Paediatr Indones. 2013;53:83-8.]**

Keywords: *intellectual disability, microcephaly, MCPH genes*

The prevalence of intellectual disability (ID) has been estimated to be close to 3% worldwide due to a variety of genetic causes.[1] In patients with ID (intelligence quotient <70), an abnormal head circumference (HC) is often another main sign.[2,3] Since the HC or occipitofrontal circumference (OFC) is a reflection of brain size, abnormal HC may be a sign of a brain anomaly. Microcephaly is commonly classified as HC below -2 standard deviations (SD)

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or below the 2nd centile for the patient's age and gender.[4,5] Its incidence at birth was reportedly 1.3 to 150 per 100,000 live births.[2]

The etiologies of microcephaly can be divided into genetic causes and environmental insults to the brain during the prenatal, perinatal, or early postnatal periods.[6] Primary microcephaly / congenital microcephaly is described as a static developmental abnormality which presents at birth or as early as 32 weeks of gestation.[7] Secondary microcephaly is considered to be a progressive neurodegenerative condition, in which HC at birth is within normal range with microcephaly developing thereafter.[7,8] The presence of microcephaly at birth is one sign of genetic microcephaly.[3] In addition, eight loci associated with autosomal recessive primary hereditary microcephaly (MCPH1-MCPH8) have been found and causative mutations identified in these 8 MCPH genes.[9-25]

Several mutations have been identified in these loci in people from different countries.[24,26,27] However, as no genetic analysis has been performed in the Indonesian population, we aimed to investigate the presence of mutations in these genes. The understanding of possible genetic causes of microcephaly associated with ID in the Indonesian population is expected to assist appropriate etiological diagnosis and genetic counselling in the affected individuals and their families.

Methods

Subjects were selected from a cohort of 527 individuals with ID from special schools and institutions. They underwent physical examinations, dysmorphology assessments, blood collections, and several genetic screenings.[28] Duplicate peripheral blood samples in EDTA and heparin were collected from these individuals. Several genetic screenings including cytogenetic analysis, FMR1 gene, and subtelomeric re-arrangements were carried out previously.[28] Individuals whose cytogenetic analyses, *FMR1* gene, and subtelomeric rearrangement tests were normal and whose HC measurements were less than -2 SD (microcephaly) as measured by Nellhaus charts,[29] were included in this study. Informed consent was obtained from their parents or legal representatives. This study was approved by the Ethics Board of the University of Diponegoro Medical School/Kariadi Hospital Semarang, Indonesia.

Subjects' genomic DNA was isolated using a salting out method of DNA extraction[30] with slight modification. Sanger sequencing for ASPM and WDR62 was performed in all ID subjects who meet the inclusion criteria. While the remaining MCPH genes (MCPH1, CDK5RAP2, CENPJ, and STIL) was conducted only on the subjects who had the lowest OFC (below -4SD), considering the likely low prevalence of these genes. Centrosomal protein 152kDa (CEP152) and CEP135 were not sequenced in this study as they were only recently discovered. Polymerase chain reaction (PCR) products of all coding exons were generated using standard techniques. Sequences of the PCR primers are available upon request. In brief, 1μl DNA (~100 ng) from each specimen was diluted in a PCR mix solution, consisting of 7.6 μl of 360 PCR mix (*Life Technologies, Foster City, USA*), 0.5 μl of each primer (*Biolegio, Nijmegen, The Netherlands*) and 6 μl of purified water. The mixture was amplified in a *9700 Veriti PCR system* (*Life Technologies, Foster City, USA*) using the following protocol: initial denaturation at 95 ºC for 10 minutes, followed by 35 cycles at 94ºC for 30 sec, 60ºC for 60 sec and 72ºC for 60 sec, and ending with elongation at 72ºC for 1 minute. Subsequently, PCR products were purified using *Millipore columns* according to manufacturer's instructions (*Merck Millipore, New Jersey, USA*). One μl of purified PCR product was mixed with M13 primer (forward or reverse). Amplification and purification steps were performed according to manufacturer's instructions, followed by DNA sequencing analysis on an *ABI 3730 XL sequencer* (*Life Technologies, Foster City, USA*).

Sequence variations in the DNA of the patients were compared to the reference sequence in *Ensembl* assembly GRCh37 (GCA_000001405.6) from the Genome Reference Consortium,[31] using the following reference sequences: *ASPM* (NM_018136.4) *WDR62* (NM_001083961.1), *MCPH1* (NM_024596.3), *CDK5RAP2* (NM_018249.4), *CENPJ* (NM_018451), and *STIL* (NM_001048166). The DNA sequences were analyzed with *SEQPilot software version 3.2.1.0 (Softgenetics, Pennsylvania, USA)*. Variants were further analyzed using *Alamut 2.0 mutation interpretation software (Interactive Biosoftware, Rouen, France)*. The impact of protein alteration was calculated using Align-GVGD method,[32] SIFT,[33] and Polyphen.[34]

The variants found in this study were then classified according to the guidelines of the UK Molecular Genetics Society (CMGS) and the Dutch Society of Clinical Genetic Laboratory Specialists (*Vereniging Klinisch Genetische Laboratoriumspecialisten*). The classifications were as follows: Class 1, certainly non-pathogenic (UV1); Class 2, unlikely to be pathogenic (UV2); Class 3, likely to be pathogenic (UV3), and Class 4, certainly pathogenic (UV4).

Results

In this study, a total of 48 individuals were met the inclusion criteria. Clinical characteristics of the subjects are summarized in **Table 1**.

A total of 186 amplicons were sequenced. The Sanger sequencing of ASPM and WDR62 was performed in all 48 subjects who meet the inclusion criteria. Besides, the remaining MCPH genes (MCPH1, CDK5RAP2, CENPJ, and STIL), the sequencing of these 4 genes was conducted on 20 subjects.

For the sequencing of ASPM and WDR62, a total of 26 unclassified variants were identified, 15 of which were predicted to be UV2, 10 predicted to be likely to be UV3, and one which could not be classified. In addition, 15 and 13 SNPs (classified as UV1) were identified in the ASPM and WDR62 genes, respectively.

Table 1. Characteristics of children with intellectual disability and microcephaly (n=48)

Characteristics	n	%
Sex		
Female	23	47.9
Male	25	52.1
Level of intellectual		
Mild	22	45.8
Moderate	17	35.4
Severe	9	18.8
OFC		
-2SD<n<-3SD	6	12.5
-3SD<n<-4SD	22	45.8
<-4SD	20	41.7
Associated abnormalities		
Epilepsy	7	14.6
Age (years)		
< 14	24	50
> 14	24	50

Note: OFC: occipitofrontal circumference; ID: intelectual disability

Only one subject harboured two ASPM variants that were classified as UV3. For the patient and her family, an identifiler test was conducted in a follow up study using *AmpFlSTR Identifiler Direct PCR Amplification Kit (Life Technologies, Foster City, USA)* using the manufacturer's protocol. The follow up study was performed to investigate if these variants resided on different chromosomes. Sequence analysis of the variants in both parents and a twin sister revealed that both variants were present in the unaffected father and twin sister, indicating that they were located on the same chromosome. As such, these variants did not explain the subject's phenotype. To exclude the possibility of an unidentified mutation in the second (maternal) allele, an identifiler test was performed to examine whether the twins were dizygotic or monozygotic. Interestingly, the result showed that the twin sisters were monozygotic, leading us to conclude that autosomal recessive inheritance of the condition in this family was highly unlikely.

In the remaining genes, only known SNPs were identified: 15 MCPH1 variants, 14 CD5KRAP2 variants, 6 CENPJ variants, and 3 STIL variants. Since MCPH is an autosomal recessive disease, only the presence of homozygous or two compound heterozygous mutations would explain the phenotype.

Discussion

This study is the first to investigate some of the possible genetic causes of microcephaly-associated ID in the Indonesian population. Albeit pathogenic mutations were not found, the identification of numerous SNPs may be useful in future association studies in our population. In addition, we have not excluded the possibility that some of the UV3 variants may be pathogenic.

An interesting finding in our study was the identification of a female monozygotic twin, whose twin sister was affected. Underlying mechanisms may contribute to this so called twin discordance phenomenon including alterations in gene structure and expression during meiosis and/or mitosis, epigenetic changes or phenocopy.[35] As the variants were identified in the unaffected father and the unaffected monozygotic twin sister, autosomal recessive primary microcephaly was a highly unlikely

diagnosis in the affected sister. Therefore, we conclude that the disease was more likely caused by a *de novo* mutation with a low recurrence risk for future children of these parents. This knowledge is not only useful for counselling the family, but paves the way for future identification of the genetic defect in this family, e.g., by exome sequence comparison of the genotypes of the two twin sisters.

The absence of causative pathogenic mutations in the MCPH genes investigated in this study might be due to several reasons. First, the microcephaly and ID in these individuals might not be genetic. Several non-genetic factors, such as under nutrition, infections, ischemia, metabolic disturbances, exposure to toxic substances, and trauma are known to play a role in microcephaly.[6,36] As there was limited information on the onset of microcephaly in these individuals (birth HC measurement was rarely available), non-genetic factors could not be ruled out. Second, microcephaly in these individuals may have been due to secondary microcephaly. Third, there was a possibility that causative mutations were present in the tested genes, but were missed or not recognized by the methods utilized. For example, the presence of exonic or whole gene deletions was not investigated, since only direct sequencing was performed.[25,37] Fourth, the microcephaly may have resulted from mutations in genes not yet discovered, since more than half of reported families with MCPH have not been linked to any of the eight MCPH loci (see **Table 1**). Fifth, the genetic defects may have occurred in the CEP152 and CEP135 genes that we did not investigate in this study.

The overall frequency of microcephaly with ID in this study was 9.1%, comparable to studies performed in other Asian ID populations, such as in India (9.8%)[36] and Israel (15.4%).[38] However, the frequency of microcephaly in this study might have been overestimated, since the mean height and weight of Indonesian children in a nationwide survey was found to be smaller compared to the US Centers for Disease Control and Prevention growth charts.[39] Considering the correlation between height, weight and HC, Indonesian individuals would be expected to have smaller HCs. In addition, in a pilot study conducted in Semarang, Central Java, the mean HC of normal Indonesian school children aged 7-12 years was smaller than the normal range in the Nellhaus charts. Boys' mean HCs (n = 128) were between the 3rd and 50th percentiles, while those of the girls (n = 146) were between the 10th and 50th percentiles. For a better estimation of microcephaly incidence, future studies are required to establish a normal HC chart for the Indonesian population.

To conclude, this is the first large-scale study to investigate possible genetic causes of microcephaly with ID in Indonesian subjects. We find the frequency of microcephaly and ID to be similar to previously reported studies in Asian populations. The absence of causative pathogenic mutations in the MCPH genes tested may originate from several factors such as non-genetic influences, secondary microcephaly or mutations in other genes not investigated in our study. The identification of UV2 and UV3 variants, as well as the absence of causative pathogenic mutations, calls for further investigations, such as sequencing of other MCPH genes (CEP152 and CEP135) and a whole exome sequencing study, preferably in a larger and better defined sample.

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