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ORIGINAL ARTICLE

Prevalence of fragile X syndrome in males and females in Indonesia

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Abstract

AIM: To investigate the prevalence of fragile X syndrome (FXS) in intellectually disabled male and female Indonesians.

METHODS: This research is an extension of a previously reported study on the identification of chromosomal aberrations in a large cohort of 527 Indonesians with intellectual disability (ID). In this previous study, 87 patients had a chromosomal abnormality, five of whom expressed fragile sites on Xq27.3. Since FXS cannot always be identified by cytogenetic analysis, molecular testing of the fragile X mental retardation 1 CGG repeat was performed in 440 samples. The testing was also conducted in the five previously identified samples to confirm the abnormality. In total, a molecular study was conducted in 445 samples (162 females and 283 males).

RESULTS: In the cohort of Indonesian ID population, the prevalence of FXS is 9/527 (1.7%). The prevalence in males and females is 1.5% (5/329) and 2% (4/198), respectively. Segregation analysis in the families and X-inactivation studies were performed. We performed the first comprehensive genetic survey of a representative sample of male and female ID individuals from institutions and special schools in Indonesia. Our findings show that a comprehensive study of FXS can be performed in a developing country like Indonesia where diagnostic facilities are limited.

CONCLUSION: The prevalence of FXS is equal in females and males in our study, which suggests that the prevalence of FXS in females could be underestimated.

Key words: Fragile X syndrome; Intellectual disability; Fragile X mental retardation 1; CGG repeat; Indonesia

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INTRODUCTION

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability (ID), with an estimated prevalence of 1 in 4000-6000 males and 1 in 7000-10 000 females[1]. Expansion of a CGG repeat in the 5' untranslated region of fragile X mental retardation 1 (*FMR1*) is the most frequent cause of FXS[2,3]. When the expansion exceeds the number of 200 repeats (full mutation), the promoter region becomes hypermethylated and the *FMR1* gene is silenced. This leads to deficiency of the FMR1 protein[4]. FXS is inherited as an X-linked dominant disease with variable expressivity and reduced penetrance in females. The level of ID in FXS males ranges from mild to profound, whilst females are usually less affected[5,6].

Several behavioral characteristics associated with FXS include autism spectrum disorders, poor eye contact, short attention span, hyperactivity, several stereotypic behaviors (hand flapping, hand biting, preservative speech, echolalia), tactile defensiveness and anxiety related to social contact[7-10]. The classical facial phenotype of FXS includes a prominent forehead, a long, narrow face, a prominent jaw and prominent ears. The palate is often highly arched. Macro-orchidism is reported in more than 80% of post-pubertal and adult males. Connective tissue abnormalities such as soft velvet-like skin, joint hypermobility, pes planus, congenital hip dislocation, scoliosis and clubfoot are also commonly observed[5,11].

Diagnostic analysis of FXS is mainly based on direct amplification of the CGG-repeat using flanking primers and Southern blot analysis[5,12-16]. Standard PCR testing allows amplification of alleles up to 120-150 CGGs. Although this method cannot reveal full mutations, it allows precise sizing of premutation alleles. On the contrary, Southern blot analysis allows sizing of full mutations but is unable to discriminate between large normal and small premutation alleles[16]. To overcome these problems, several diagnostic laboratories recently changed their procedure to PCR-based tests that can amplify repeat alleles up to full mutations and are able to distinguish between female samples homozygous for a normal allele or heterozygous for a normal and an expanded allele (e.g., tests by Abbott, IL, United States and Asuragen Inc., Austin, United States). While these procedures are routinely performed in the Western world, they are not being used as standard diagnostic tools in Indonesia, mainly due to costs and lack of adequate health insurance coverage.

In a previous study, the prevalence of FXS in the male Indonesian population was determined to be 1.9% (5/262)[17]. However, diagnostic testing for FXS is not routinely performed and widely available in Indonesia. Therefore, we aimed to identify unrecognized FXS individuals and to determine the prevalence in both male and female individuals with ID. In view of the fact that genetic testing is still uncommon practice in Indonesia, the detection of new FXS cases gives insight in to the prevalence of FXS in Indonesia and should promote awareness of this disease among medical doctors and health professionals in Indonesia. For the families involved, establishing a diagnosis will be beneficial since genetic counseling and carrier testing can be provided.

MATERIALS AND METHODS

Selection and setting

This research is an extension of a previously reported study on the identification of chromosomal aberrations in a large cohort of 527 Indonesian ID patients from several special schools and institutions in Java Island, Indonesia. In this previous study, 87 patients had a chromosomal abnormality, five of whom expressed fragile sites on Xq27.3[18]. Since FXS cannot always be identified by cytogenetic analysis, molecular testing of the *FMR1* CGG repeat was performed in 440 samples. The testing was also conducted in five previously identified samples to confirm the abnormality. In total, a molecular study was conducted in 445 samples (162 females and 283 males).

Genomic DNA of each patient was isolated using the salting out method as described elsewhere[19], with slight modification. The CGG repeat in the *FMR1* promoter was amplified as described by Fu *et al*[12]. Fragment length analysis was carried out on an ABI Prism 3730 DNA Analyzer (Life Technologies, Foster City, United States) and the Genemapper software (Version 4.0, Apache) was used to determine the exact length of the CGG repeat. Southern blot analysis of the *FMR1* CGG(n) repeat was performed as described previously[20]. In families 5, 6 and 7 (Figure 1), a more detailed analysis of the repeat length was performed using a three-primer CGG repeat primed *FMR1* PCR method (Asuragen Inc., Austin, United States), according to the manufacturer's protocol. The difference between the distribution of the full mutation allele in males and females was calculated using a χ^2 test.

A clinical reinvestigation was done in the positive cases and family members at risk of being a carrier were molecularly tested. X chromosome inactivation (XCI) analyses were performed in all full mutation females in order to explain their phenotypes. Family members from all affected individuals were counseled and extended pedigrees were drawn. Thirty nine family members were available for molecular testing and clinical examination was only performed in family members with obvious signs of ID. The XCI pattern was studied in female samples with a full mutation (either clinically affected or unaffected) as described before[20].

RESULTS

In a total of 445 (162 females and 283 males) molecularly tested individuals (607 alleles), 593 alleles were within the normal range (15-44 CGG repeats), 3 alleles in the intermediate range (45-55 CGG repeats), 2 alleles in the premutation range (between 55 and 200 CGG repeats) and 9 alleles in the full mutation range (> 200 CGG repeats) (classification according to the American College

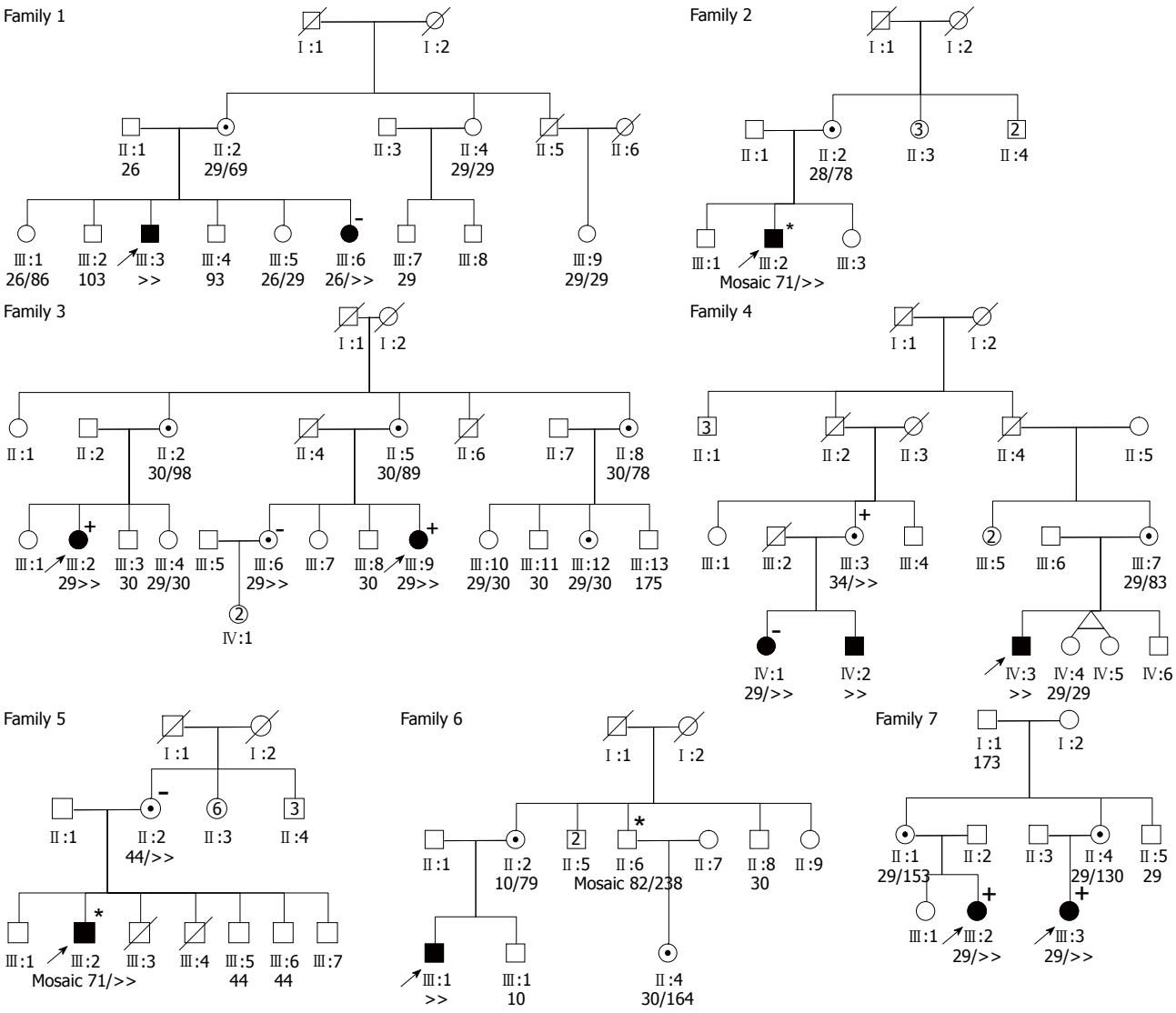

Figure 1 Pedigrees of fragile X syndrome families. Besides the nine index cases (indicated by an arrow), six additional family members with a full mutation (five females, full black circle and one male, indicated by full black square) and 17 individuals [11 males; 5 females (indicated by dotted circle)] with a premutation have been identified. The length of the CGG repeats is depicted below the pedigree number of each tested individual. The X-inactivation (XCI) of full expansion females is shown at the upper right of the pedigree symbol (+ for non-random XCI and - for random XCI). Asterisk at the upper right indicated a mosaic permutation to full mutation.

of Obstetricians and Gynecologists Committee Opinion, No. 469)[21]. The 29 allele (n = 245) was the most frequent allele in this population, followed by 28 CGG repeats (n = 127) and 30 CGG repeats (n = 93).

The five samples (4 males and 1 female) in which fragile sites were shown in previous chromosome analyses indeed showed a full mutation with Southern blot analysis, therefore confirming the diagnosis of FXS. Another four samples (1 male and 3 female) were newly identified to have a full mutation. Two of the positive male samples showed a mosaic pattern of premutation to full mutation (Family 2/Ⅲ:2 and Family 5/ Ⅲ:2, Table 1). A χ^2 test revealed no statistically significant differences in the distribution of full mutation alleles between males and females (χ^2 = 0,184; df = 1; P = 0.67).

Pedigree analysis of the nine FXS cases showed that two individuals were related to two other index cases in other families (first cousins in two different families) (Family 3/Ⅲ:2 and Ⅲ:9, Family 7/Ⅲ:2 and Ⅲ:3, Table 1). Therefore, only seven families were identified in this study (Figure 1). Molecular testing of potential carriers in those families resulted in the identification of 17 samples with a premutation (11 females and 6 males). In one of the 11 females (Family 6/Ⅲ:4), the Southern blot result could not clearly distinguish between a large premutation or full mutation. More detailed analysis using repeat-primed PCR (Asuragen Inc., Austin, United States) revealed a premutation. Furthermore, another six full mutation cases were identified: five females (two mildly affected and three clinically unaffected) and one clinically affected male.

X-inactivation studies were performed in all nine females with a full mutation. Four females (two mildly affected and two unaffected) showed random patterns

Table 1 Fragile X mental retardation 1 gene analysis of index fragile X syndrome subjects

Patient	Sex	*FMR1* gene analysis
Family1/Ⅲ:3	Male	Full mutation
Family 2/Ⅲ:2	Male	Mosaic premutation/full mutation
Family 3/Ⅲ:2	Female	Full mutation
Family 3/Ⅲ:9	Female	Full mutation
Family 4/Ⅳ:3	Male	Full mutation
Family 5/Ⅲ:2	Male	Mosaic premutation/ full mutation
Family 6/Ⅲ:1	Male	Full mutation
Family 7/Ⅲ:2	Female	Full mutation
Family 7/Ⅲ:3	Female	Full mutation

FMR1: Fragile X mental retardation 1.

Table 2 X chromosome inactivation pattern in all full mutation females

Pedigree	XCI	Clinical features
Family 3/Ⅲ:2	87/13 (non random)[1]	Affected
Family 3/Ⅲ:9	96/4 (non random)[1]	Affected
Family 7/Ⅲ:2	93/7 (non random)[1]	Affected
Family 7/Ⅲ:3	82/18 (non random)[1]	Affected
Family 1/Ⅲ:6	75/25 (random)	Affected (mild)
Family 4/Ⅳ:1	74/26 (random)	Affected (mild)
Family 3/Ⅲ:6	60/40 (random)	Not affected
Family 4/Ⅲ:3	84/16 (non random)[2]	Not affected
Family 5/Ⅱ:2	67/33 (random)	Not affected

[1]The normal allele is inactive by X chromosome inactivation (XCI) and the expanded allele is active but methylated because of the expansion; [2]the normal allele is active and the expanded allele is not active.

of inactivation (Table 2). Four out of five samples with non random patterns of inactivation (> 80:20, defined by Amos-Landgraf *et al*[22]) are from clinically affected females, while the 5th one is not affected. Southern blot analysis showed that in the unaffected female the normal allele was active and the expanded allele was inactive, whereas in the affected females the normal allele was inactive. A summary of the most common features of the index patients and affected family members is shown in Table 3, for male and female individuals, respectively. Clinical pictures of index patients and affected family members are depicted in Figure 2. In male individuals, shy behavior (shy and timid behavior with a tendency towards social withdrawal) and social anxiety are the most frequent features (detected in all six males = 100%), followed by large cupped ears, elongated face and joint laxity (detected in 83%). Four of post pubertal individuals had large testicular size (67%). Highly arched palate, scoliosis and flat feet were found in three patients (50%), whereas neurological problems (seizure, spasticity of the extremities and strabismus) were found in one patient. In females, shy behavior and social anxiety are also the most frequent features (100%), whereas joint laxity and flat feet were found in five (83%). Four females showed large cupped ears, elongated face and high arched palate (67%). Scoliosis and strabismus were found in three and two individuals, respectively (50% and 33%).

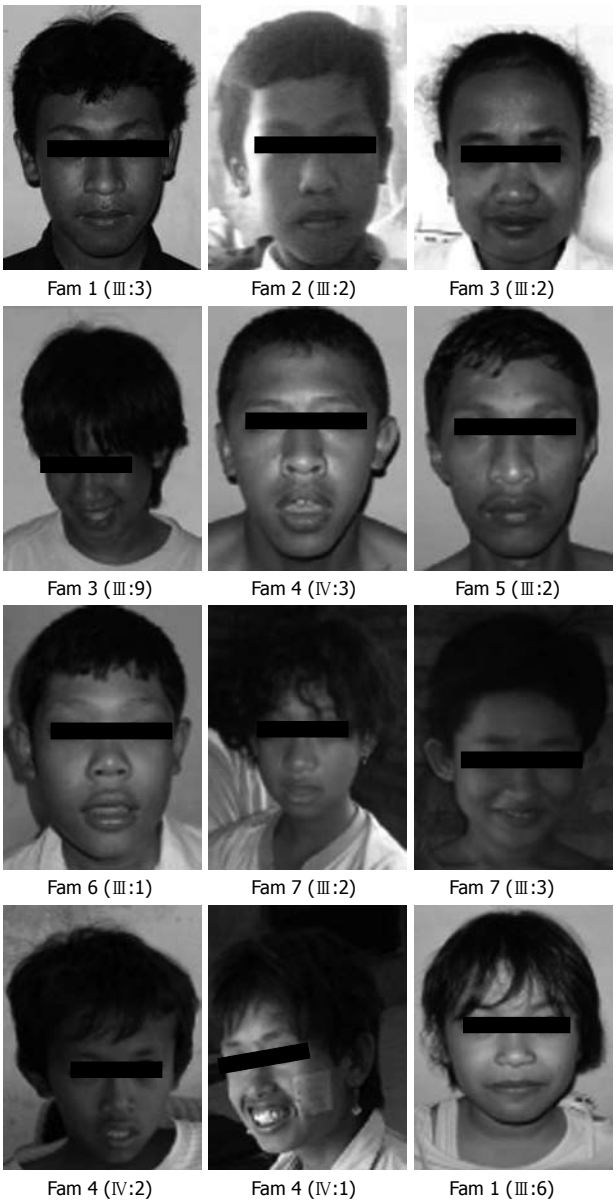

Figure 2 Clinical pictures of patients with fragile X syndrome. Fam: Family.

DISCUSSION

Few studies have been carried out to determine the contribution of FXS as a cause of ID in the Indonesian population[17,23]. In this study, we performed a comprehensive genetic survey of a representative sample of male and female ID individuals from institutions and special schools. The prevalence of FXS found in this study was 1.7% (9/527); 1.5% (5/329) in the male population and 2% (4/198) in the female population. This prevalence of FXS is similar to that in other Asian populations (approximately 1%-3%)[24-26] and is about the same as found in a previous study from Indonesia with a prevalence of 1.9% (5/262) in the male population[17].

The prevalence of FXS among females was estimated to be about half of that of males[1]. The actual distribution of full mutation alleles in the general population, however, is considered to be equal in both males

Table 3 Clinical features of male and female patients with fragile X syndrome

	Male							Female						
	Fam 1/ Ⅲ:3	Fam 2/ Ⅲ:2	Fam 4/ Ⅳ:3	Fam 5/ Ⅲ:2	Fam 6/ Ⅲ:1	Fam 4/ Ⅳ:2	%	Fam 3/ Ⅲ:2	Fam 3/ Ⅲ:9	Fam 7/ Ⅲ:2	Fam 7/ Ⅲ:3	Fam 4/ Ⅳ:1	Fam 1/ Ⅲ:6	%
Intellectual disability level	2	1	2	2	1	2		2	2	3	2	1	1	
Shy behavior and social anxiety	+	+	+	+	+	+	100	+	+	+	+	+	+	100
Large cupped ears	+	+	+	-	+	+	83	-	+	+	+	+	-	67
Elongated face	+	+	+	+	-	+	83	-	+	+	+	+	-	67
High arched palate	+	-	+	-	+	-	50	+	+	+	-	+	-	67
Scoliosis	-	-	+	+	-	+	50	-	+	+	-	+	-	50
Joint laxity	+	+	+	+	-	+	83	-	+	+	+	+	+	83
Neurological problems	-	-	+	-	-	-	17	-	+	+	-	-	-	33
Macroorchidism	+	+	+	+	-[1]	-[1]	67							
Flat feet	+	+	+	-	-	-	50	+	+	+	+	+	-	83

[1]Prepubertal. 1: Mild; 2: Moderate; 3: Severe. Fam: Family; %: Percentage.

and females[27], but due to the X-inactivation in females, they are usually less severely affected. With regards to the allele distribution of full mutation alone, this study yielded no statistically significant differences in males 1.5% (5/329) and females 2% (4/198). This finding is in line with the results of previous studies[26,27]. The equal distribution of clinically affected females and males in the present study, however, was unexpected. In order to explain why most of the females with a full mutation in this study are clinically affected, the X inactivation status was determined. All female index patients (n = 4) demonstrated a non random XCI, in which the normal unexpanded allele was preferentially inactivated. Although the XCI pattern in blood does not necessarily represent the pattern in the brain, the results in this family provide evidence for the fact that XCI patterns play a role in the development of cognitive disturbances in females with a full mutation. The results of the XCI assay in the five female family members with a full mutation are also in concordance with their clinical status: the two mildly affected females and two of the unaffected females showed random X-inactivation. The difference in intellectual abilities between these females might possibly be explained by a difference in the X-inactivation pattern in brain. One of the unaffected females showed non-random X-inactivation (Family 4/Ⅲ:3), but since the normal allele was preferentially active, this explains why she has a normal phenotype. This study clearly demonstrates why females with full mutation alleles can be affected or unaffected, depending on their XCI pattern, a feature that has been recognized before[28-31]. The percentage of clinically affected females (due to non random X-inactivation) in the present study is considerably higher than what has been reported among the full mutation female population: 44% (4/9) in this study *vs* 24.1% (7/29) reported by Reiss *et al*[31]. Further studies on larger numbers of full mutation females have to be performed in order to confirm this high percentage of non-random X-inactivation in our female population.

In family 5, the affected index male (Ⅲ:2) showed a mosaic premutation to full mutation (71/>>). Segregation analysis using Southern blot in the family demonstrated that the mother was a carrier of a full mutation. In order to exclude the possible presence of a low amount of premutation alleles in the mother, an additional test using a repeat-primed PCR was performed. The analysis confirmed that the mother was a carrier of a normal allele (44 CGG repeats) and a full mutation allele (294 CGG repeats) without evidence of mosaic premutation allele. This indicates that the full mutation allele of the mother was transmitted to her son in reduced size. Although the molecular mechanisms responsible for the reduction of the CGG repeat in the *FMR1* gene are largely unclear, several other cases where full mutation carrier females had affected sons with a mosaic pattern, have been described[32,33]. One of the mechanisms explaining repeat contraction (but also expansion) is slipped strand mispairing[34,35]. Another explanation is that the contraction could be a postzygotic event due to somatic instability of the CGG repeat[36-38].

Individuals who have a mosaic premutation to full mutation may have a milder phenotype compared to those with a full mutation[39]. Besides patient Ⅲ:2 from family 5, patient Ⅲ:2 from family 3 also showed a mosaic pattern on the Southern blot. Notably, one of the male family members with a normal intelligence was also identified to have a mosaicism of a premutation (81 CGG repeats) and a full mutation allele (Family 6/Ⅱ:6). However, the full mutation allele was not visible on the Southern blot and was only detected after the repeat-primed PCR which was performed in order to better characterize the repeat number in his daughter. This may indicate that the fully expanded allele was present only in a small percentage of cells, explaining the normal phenotype.

The most frequent clinical features found in both sexes in our population were shy behavior and social anxiety, large cupped ears, elongated face and joint laxity. These features were consistent with those described for FXS[5,8,11].

Cytogenetic testing to detect FXS is no longer considered to be sufficiently accurate because of its high false negative and false positive rates[11], the main difficulty being the detection of females with a full muta-

tion[40,41]. Indeed, in our study, cytogenetic analysis only picked up five out of nine samples, most of which were males. Although cytogenetic diagnosis is still useful and affordable to establish a FXS diagnosis in developing countries, this study emphasizes the significance of molecular screening. Moreover, despite the fact that the PCR-based test is available at the Center for Biomedical Research (CEBIOR) at Diponegoro University, testing for FXS in the ID population in Indonesia is not routinely performed and CEBIOR is the only laboratory to perform FXS diagnosis in Indonesia. It is recognized that FXS is an inherited disease; however, establishing a diagnosis and providing possibilities for genetic counseling and carrier testing is not seen as useful in Indonesia. Due to its high costs and limited accessibility, prenatal diagnosis is only available to a minority of the population. Even though termination of pregnancy is legal when based on a medical emergency, e.g., genetic diseases (Republic Indonesia Laws No. 36/2009) [42], in practice it still is a very complex procedure. Also, other options such as preimplantation genetic diagnosis are financially and culturally complex. Still, as common infectious diseases and nutritional problems are becoming less prevalent in Indonesia, diagnostic facilities for inherited diseases such as FXS need a higher priority. In addition, medical personnel and stake holders at the Ministry of Health should be continuously informed about the problem of genetic diseases and its management.

FXS testing is a common diagnostic procedure performed in all non-microcephalic males with ID of unknown origin in Western countries[43]. However, routine FXS testing in females with ID of unknown origin is said not to be warranted unless there are other indicators (e.g., a positive family history)[44]. On the other hand, the American College of Medical Genetics strongly recommends fragile X testing to be considered in both genders with unexplained ID, especially in the presence of any physical or behavioral characteristics of FXS, a positive family history and relatives with undiagnosed ID[45]. Our findings support the notion to broaden FXS testing to include females, in view of the fact that the prevalence of FXS in females could be higher than thought up to now.

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COMMENTS

Background

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability (ID). Expansion of a CGG repeat in the 5' untranslated region of fragile X mental retardation 1 (*FMR1*) gene is the most frequent cause of FXS.

Research frontiers

Diagnostic analysis of FXS is mainly based on direct amplification of the CGG-repeat using flanking primers and Southern blot analysis. While these procedures are routinely performed in the Western world, they are not being used as standard diagnostic tools in Indonesia, mainly due to costs and the lack of adequate health insurance coverage.

Innovations and breakthroughs

In the previous study, the prevalence of FXS in the male Indonesian population was determined; however, diagnostic testing for FXS is not routinely performed and not widely available in Indonesia. Therefore, the authors aimed at identifying unrecognized FXS individuals and determining the prevalence in both male and female individuals with ID. They performed the first comprehensive genetic survey of a representative sample of male and female ID individuals from institutions and special schools in Indonesia.

Applications

Their findings show that a comprehensive study of FXS can be performed in a developing country like Indonesia where diagnostic facilities are limited. Moreover, their findings support the notion to broaden FXS testing to include females, in view of the fact that the prevalence of FXS in females could be higher than thought up to now.

Terminology

FXS is the most common inherited cause of ID. The spectrum of ID ranges from mild to severe, while physical features can include an elongated face, large and prominent ears, larger testes/macroorchidism (in males), behavioral characteristics such as stereotypic movements, and social anxiety. FXS is caused by mutations in the *FMR1*-gene. *FMR1* is a gene in humans which encodes a protein called fragile X mental retardation protein. This protein is important for normal cognitive development.

Peer review

This is a good descriptive study in which the authors investigate the prevalence of FXS in intellectually disabled male and female Indonesians. The results are interesting and suggest that the prevalence of FXS in females could be underestimated.

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