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Brief communication (Original)

Subtelomeric aberrations in Thai patients with idiopathic mental retardation and autism detected by multiplex ligation-dependent probe amplification

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Background: Chromosomal rearrangements involving telomeres account for approximately 1%–30% of causes of mental retardation (MR). It is therefore recommended that all cases of undiagnosed MR be screened for subtelomeric aberration. Nevertheless, resolution of a standard karyotyping using the G-banding technique is limited. Therefore, an additional technique with higher resolution should be performed to detect this type of anomaly.
Objectives: To screen for subtelomeric aberration in Thai patients with mental retardation and autism.
Methods: Multiplex ligation-dependent probe amplification (MLPA), was used to screen 114 Thai patients with idiopathic MR and 15 patients with autism. All positive results were confirmed by using a different set of MLPA probes or real-time PCR.
Results: We identified 5 patients with submicroscopic aberration in patients with MR. One patient had a submicroscopic deletion at the 1p36.33 region, which was confirmed by real-time PCR. There were 2 patients with subtelomeric duplication at the 15q11.2 and 11p15.5 regions sequentially. Two patients had the same duplication at the Xp22.33. region.
Conclusions: The present study shows that the incidence of a subtelomeric aberration in Thai patients with idiopathic MR is approximately the same reported previously (3.9%). Identifying these submicroscopic aberrations requires an advanced method with higher resolution than standard karyotyping. Although microarray techniques may be a more informative, they are costly and require an array facility, which are not widely available, especially in developing countries. Thus, MLPA in a routine cytogenetic test for MR patients with normal karyotypes in this setting can help to increase diagnostic yield.

Keywords: Autism, mental retardation, MLPA, subtelomere

Mental retardation (MR) is a common disorder found in children with a prevalence of approximately 1%–3% worldwide [1]. The cause of MR is heterogeneous, although majority are unknown. Various types of unbalanced chromosomal rearrangements are the cause of MR in 5%–30% of reported cases [1]. Rearrangements involving subtelomeric regions have been identified as contributing significantly to mental deficiencies, autism, and birth defects, at rates ranging from 1%–30% of cases depending on screening technique and sample selection method [2-10].

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Subtelomeres are gene-rich regions. Frequent rearrangement of 1 of these regions is thought to be caused by sequence similarity among nonhomologous chromosomes [11, 12]. Submicroscopic aberrations in 1 of these areas are generally too small to be detected by standard karyotyping using the G-banding technique. The introduction of the fluorescence in situ hybridization (FISH) method has helped to increase the resolution available to study such tiny details and thus increased our ability to detect such cryptic rearrangements; however, FISH is laborious and unsuitable for detecting sequential duplications or small deletions [13]. Whole-genome screening using a microarray technique for detecting any chromosomal rearrangements including subtelomeric aberrations is currently the best available first-tier diagnostic test for individuals with MR and dysmorphism [14, 15].

However, this technique is not available at many diagnostic laboratories because it requires a substantial investment in equipment and resources. Multiplex ligation-dependent probe amplification (MLPA) is another method for subtelomeric screening, and has the advantage of allowing rapid screening of multiple patients in a single assay. This method requires only capillary electrophoresis for fragment analysis. The MLPA technique is reliable and sensitive [16-19]. Additionally, the low cost of MLPA makes it a practical alternative to microarray analysis. Here we report a series of subtelomeric screenings by MLPA in pediatric Thai patients with MR and autism to determine whether implementing MLPA increases the diagnostic yield in our laboratory.

Materials and methods

Samples and DNA extraction

The study was approved by the Faculty of Medicine, Prince of Songkla University, Ethics Committee (approval No. EC 52-138-05-1-3). After written informed consent from their parents or legal guardians of all participants, we collected 129 peripheral blood samples (74 boys, 55 girls, average age = 3.7 years). We diagnosed 114 patients with idiopathic mental retardation (MR) with normal karyotypes detected by a standard G-banding technique. Fifteen patients (12 boys, 3 girls) fulfilled the diagnostic criteria of autistic disorder following DSMIV. All 15 patients with autistic features also had a below average IQ (<70, nonverbal form of the Standford–Binet Intelligence Scale, 5th edition). All had a normal karyotype and no CGG repeat expansion in the FMR1 gene. All autistic girls had no mutation in the coding regions of the MECP2 gene. Common mutation in the ARX genes was excluded in all autistic boys.

DNA was extracted from the peripheral blood samples using the Illustra blood genomic Prep Mini Spin Kit (GE Healthcare, Piscataway, NJ, USA) or standard phenol–chloroform method. The DNA in each sample was quantified and assessed for degradation.

Multiplex ligation-dependent probe amplification

A SALSA MLPA P070-B1 Human Telomere-5 kit (MRC-Holland, Amsterdam, The Netherlands) was used to screen the sample for subtelomeric aberrations. Subtelomeric MLPA probes from a SALSA MLPA P036-E1 Human Telomere-3 kit were used to validate any positive results. The screening was performed using 50 ng of genomic DNA according to the manufacturer's protocol. A positive control from either a trisomy 21 or a trisomy 13 patient and a negative control sample were included in each MLPA assay. Analysis of the MLPA data was performed as described previously [20].

Real-time PCR for result validation at 1p36.33

Exon 4b of *TNFRS18* was suspected of being deleted using probes from a MLPA P070-B1 screening kit. A real-time PCR was conducted to validate the result. Primers were designed to amplify *TNFRS4* and *TNFRS18* located in the 1p36.33 region. *TNFRS4* located approximately 7.9 kb from the targeted *TNFRS18* was used as a reference gene.

PCR reactions were conducted in triplicate using the CFX connect Real-time PCR Detection system (Bio-Rad, Hercules, CA, USA). Fifty nanograms of DNA was used in a 20 µl reaction containing 1× SsoFast EvaGreen Supermix (Bio-Rad), 0.2 µM of each primer for the *TNFRS4* and *TNFRS18* (*TNFRS4*_forward: ACCTTGGCTGGGAAGCAC AC, *TNFRS4*_reverse:TTCAGTGGGCTGGACAGT GATG, *TNFRS18*_forward: GTTCGGGTTTCTCA CTGTGTTC, *TNFRS18*_reverse: CCACATGCAC TGACTCCTCA). The PCR was conducted following an initial hot start at 95°C for 3 minutes, 40 cycles of 95°C for 10 s, 66°C for 10 s, and 72°C for 30 s. A comparative C_T method ($\Delta\Delta C_T$ method) as described in [21] was used to analyze real-time PCR results.

Results

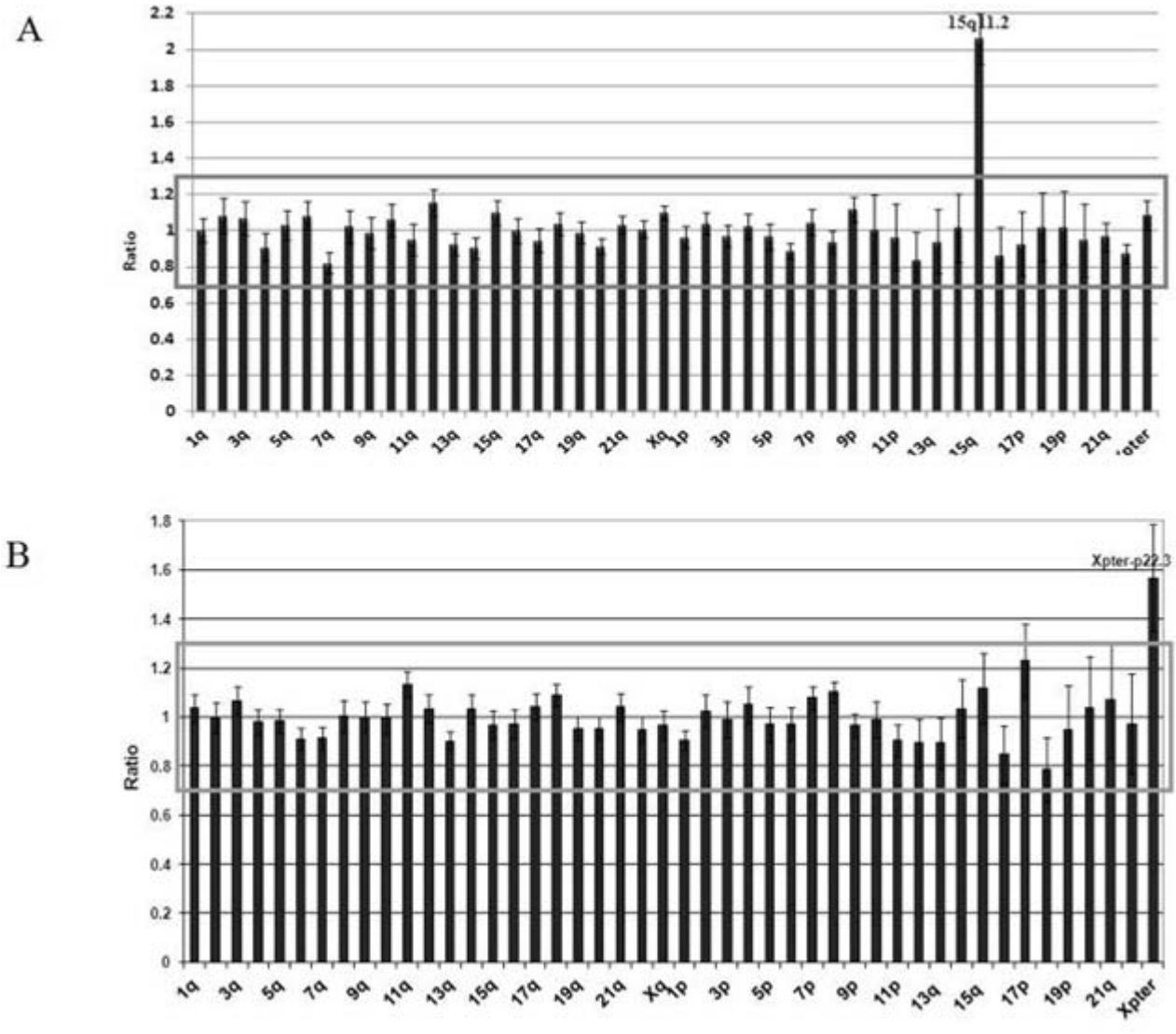
We detected 4 types of submicroscopic aberration in samples of 5 patients of 129 from the first screening. Three duplications at chromosome regions 15q11.2, Xp22.33, and 11p15.5, and 1 deletion at 1p36.33, were identified. All patients had been previously diagnosed with idiopathic MR. No abnormality was detected in any individuals diagnosed with autism. Two patients had the same duplication at Xp22.23. All initial results from the first screening were confirmed using different MLPA probes. The deletion of 1p36.33 was confirmed by real-time PCR. The results and the available clinical features of all affected patients are summarized in **Table 1**. Additionally, we also detected 3 samples with a 4q35.2 deletion in the initial MLPA screening, but these deletions were not confirmed by a different MLPA probe in the same region. Such false-positive results were noted in a previous study [9], which

suggested that they were possibly caused by a single nucleotide polymorphism (SNP) in the MLPA screening probe for the 4q region. The MLPA analysis from all positive samples is shown in **Figure 1**.

Table 1. Summary of MLPA results and clinical symptoms of all positive cases

Patient No.	Duplication	Verification Method	Sex	Age	Origin	Neurological symptoms	Other anomaly
1	15q11.2	MLPA	Male	7	de novo	MR	-
2	Xp22.3	MLPA	Male	6	N/A	MR	N/A
3	Xp22.3	MLPA	Male	6	N/A	MRseizure	- Bilateral cleft lips - Left cleft palate - Thalassemia hemoglobin E trait
4	11p15.5	MLPA	Female	3	N/A	MR	- Choanal atresia - Coloboma of right upper and lower eyelids - Right cleft lip
	Deletion						
5	1p36.33	Real-time PCR	Female	1	N/A	MR	Flat head with frontal bossing

MR = mental retardation, N/A = information is not available



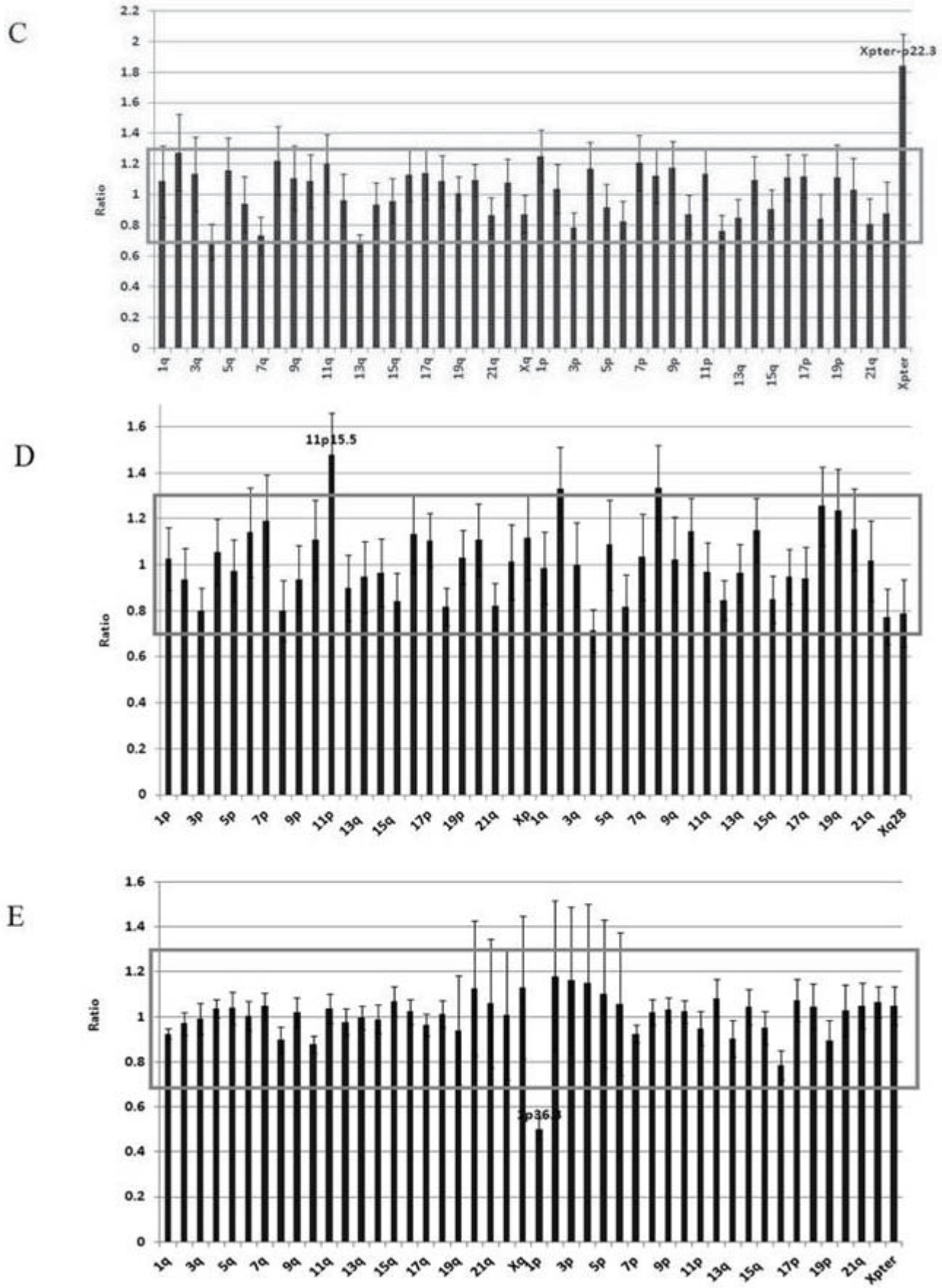


Figure 1. Multiplex ligation-dependent probe amplification (MLPA) analysis results of all 5 positive cases. (**A**) 15q11.2 duplication. (**B**) Xp22.3 duplication (patient No. 2). (**C**) Xp22.3 duplication (patient No. 3). (**D**) 11p15.5 duplication. (**E**) 1p36.33 deletion. Results in (**A–C, E**) were screened by a SALSA MLPA P070-B1 Human Telomere-5 kit and a result in (**D**) was from a SALSA MLPA P036-E1 Human Telomere-3 kit.

All 5 families among the affected individuals with submicroscopic aberrations were invited to participate in this research. Unfortunately, 4 of these families declined to participate because of personal beliefs or other reasons, and only 1 family whose child had a duplication of 15q11.2 agreed to further investigation. With consent, the pedigree of the 15q11.2 duplication family is shown in **Figure 2**.

The family with a submicroscopic duplication of the 15q11.2 region

Because the short arm of chromosome 15 is comprised only of repetitive sequences, the MLPA probe for 15p was relocated to the region of 15q11.2. Although this is not a subtelomeric region, this region is the closest to centromere and to 15p. Aberrations in this region are also known to cause abnormal phenotypes [22-24].

The proband of the family (IV-7) with submicroscopic duplication of 15q11.2 region was a 7-year-old Thai boy with moderate MR. His birth weight was 2700 g, and there was no remarkable history during pregnancy. The physical examination upon arrival found a 20 kg boy who was 117 cm tall. He had normal physical appearance and no dysmorphism was observed. His mother noticed that he had delayed development compared to his siblings since he was 4 months old, but the mother sought no medical opinion. At our first examination, he was able to understand words and short sentences and was now studying at a local kindergarten. His family history showed that he had 2 cousins also diagnosed with MR; the first (IV-9) a 15-year-old boy living in another province, who had been diagnosed with mild mental retardation (IQ = 65) at 8 years old, and the second (IV-10) a 16-year-old boy with MR and with repetitive hand movements; this second cousin had never been to a doctor and could not understand words or sentences. Neither of these cousins had any known dysmorphic features.

MLPA analysis of all available samples from this family (III-3, III-4, III-7, IV-7, IV-10) failed to detect the 15q11.2 duplication except in the proband. His cousin (IV-10) who also had MR did not have this duplication either. Considering the differences between the clinical symptoms of the 2 individuals, this finding was expected.

Discussion

The subtelomeric regions at both ends of the human chromosome arms are gene-rich. Any unbalanced rearrangement involving genes in these regions can cause mental retardation and an abnormal phenotype. Unfortunately, standard karyotyping, even with high resolution chromosome banding (550 bands), cannot detect such aberrations. In this study, we screened samples from 129 patients with idiopathic MR and autism for any rearrangements with MLPA and detected 5 patients with positive screening (4%), which is within the range reported previously [2-5, 10, 16, 19, 20, 25, 26].

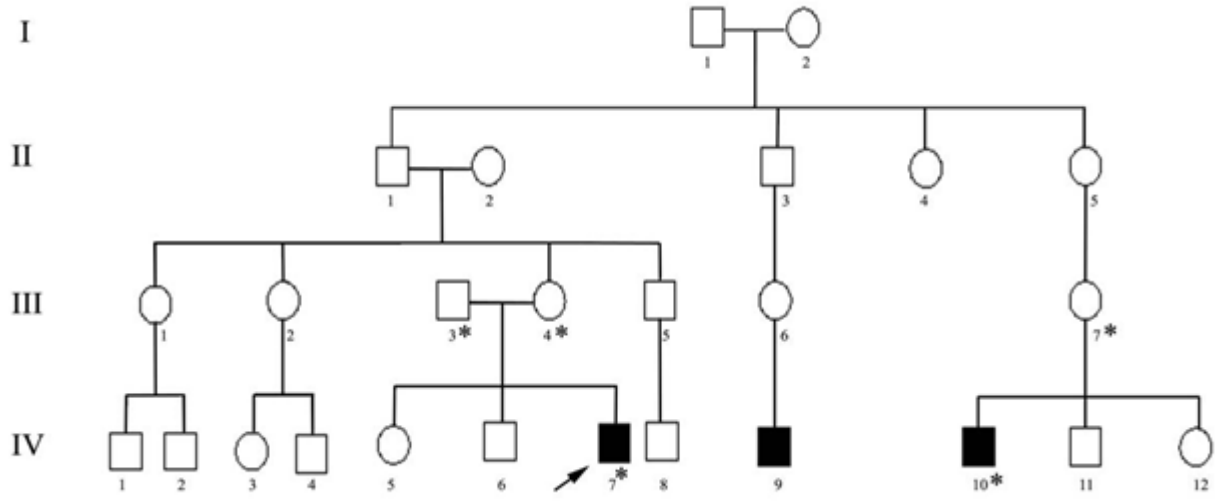


Figure 2. Pedigree of a family with a duplication of the 15q11.2 region. All individuals marked with (*) were screened for the duplication (with written informed consent for publication).

The duplication of 15q11.2 detected in our patient was a de novo event. The major anomaly manifested in our affected individual was developmental delay without any dysmorphism. Although there were 2 other affected individuals in the family, the clinical symptoms of each individual seemed to be distinctive, and it is likely that individuals IV-9 and IV-10 are differently affected. The proximal region of chromosome 15q is a region prone to genomic rearrangement. This region contains several low-copy repeats (LCRs) that mediate various duplications and deletions via nonhomologous recombinations [27]. The most well-known syndromes caused by deletions in this region are the Prader–Willi and Angelman syndromes. Mapping our MLPA probes to the region showed that they were centromeric to the *SNURF–SNRPN* imprinted region, but this is still an area where rearrangements are known to frequently occur [23, 27]. Duplication of 15q11.2 can cause a variety of phenotypes, primarily manifesting neurological symptoms. Developmental delay, especially speech delay, is the most frequent problem detected [22, 23], and was also detected in our patient. Because duplication of 15q11-13 accounts for approximately 0.5%–3% of autistic spectrum disorder (ASD), ASD is another feature frequently observed in patients with 15q duplication [24]. Even though dysmorphism has been noted in some cases with proximal 15q duplication, no consistent physical anomaly has been reported [23]. As in our patient, developmental delay without dysmorphism seems to be the main feature of 15q duplication. Because our finding was de novo, this duplication is likely to be pathologic.

We also had 1 patient with 11p15.5 duplication and 2 patients with Xp22.33 duplication in this study. The 11p15.5 region is known to contain a cluster of imprinted genes that together play an important role in the control of fetal growth. An abnormality in the imprinting mechanism of genes in this region or a uniparental disomy of chromosome 11 can cause either Beckwith–Wiedemann syndrome (BWS) or Silver–Russell syndrome (SRS) [28]. Paternal duplication of the 11p15.5 region has been reported in a few patients with BWS and maternal duplication of the same region can cause SRS [28, 29]. This duplication detected in BWS/SRS can arise as a de novo event or as a result of chromosomal rearrangement from inherited translocation or inversion. In addition to abnormal growth, psychomotor retardation is often detected in this group of patients [28-30]. From the medical records of our patient, in addition to MR, she also had several dysmorphic features (choanal atresia, coloboma of the right upper and lower eyelids, right cleft lip) (**Table 1**), but there was no record of postnatal overgrowth suggesting BWS. The patient also had no apparent evidence of intrauterine growth retardation or postnatal growth delay indicating SRS. Therefore, our patient is unlikely to fulfill the diagnostic criteria for either syndrome, although a thorough clinical examination should be performed to confirm these observations. However, considering the several dysmorphic features she clearly exhibits, this 11p15.5 duplication, if pathological, is more likely to be a complex submicroscopic chromosomal rearrangement than a simple duplication. Further investigation would be needed to elucidate the precise pathology.

We detected 2 patients with an Xp22.3 duplication in this study, both male with developmental delay (2/129 = 1.6%). Xp22.3 is a gene-rich region, and deletion of any of several genes in this region can cause a variety of abnormal phenotypes such as X-linked ichthyosis with developmental delay, attention deficit hyperactivity disorder, autism, and social communication deficit [31]. However, the clinical significance of the Xp22.3 duplication is still uncertain and has been classified as a normal variant [32, 33], a pathologic change [34-36] or an unclassified rearrangement [37]. The major gene thought to contribute to abnormal phenotypes in this region is the gene for steroid sulfatase (*STS*). A previous study of patients with developmental delay and Xp22.3 duplication suggested a possible association of the duplication with abnormal phenotypes, especially those involving *STS* [37]. The same study suggested the duplication might be a risk factor or a modifier of associated intellectual disability [37]. Mapping our MLPA probes to the Xp22.3 region showed that our probes laid approximately 7 Mb telomeric to the *STS* locus. Because of limitations of the MLPA method, the actual duplication size and *STS* involvement could not be determined in this study. Because parental DNA was not available, the significance of this duplication could not be examined, and thus remains uncertain. Therefore, there is still a possibility that this duplication may be a rare variant, and further screening of this aberration in a normal population should be performed.

Terminal deletion of the short arm of chromosome 1 (1p36) is generally difficult to identify by conventional cytogenetics due to the light-staining G-negative bands, but this deletion is thought to be a contiguous gene

syndrome [38]. This deletion can be derived from an unbalanced translocation, complex rearrangement or can be merely a simple deletion. A previous study found no correlation between the deletion size and abnormal phenotypes [38]. Common clinical phenotypes of 1p36 deletion include global developmental delay, hypotonia, hearing impairment, heart defects, and distinct dysmorphic features including microcephaly or brachycephaly, midface hypoplasia, deep-set eyes, flat nasal bridge, large anterior fontanelle, and a prominent forehead [38-40]. Because the family of the patient with the 1p36 deletion refused to be part of any further study, we could not perform additional tests that would indicate more conclusively that this deletion derived from any familial chromosomal rearrangement. However, the patient was noted to have frontal bossing with a flat head, which could have been brachycephaly, feature frequently found in patients with 1p36 deletion syndrome. Therefore, the 1p36.33 deletion detected in our study was likely to be pathologic.

Conclusion

Our study shows that implementing subtelomeric MLPA screening in Thai patients with idiopathic mental retardation and autism, who have a normal karyotype, can increase the diagnostic yield. Because some subtelomeric defects may be caused by unbalanced chromosomal rearrangements resulting in a complex submicroscopic aberration, MR and dysmorphism are frequently detected in this group of patients. Currently, a recommended test for MR patients with normal karyotype is microarray analysis, which, although being a useful tool for whole genome screening, is also costly and requires an array facility, which may not be widely available, especially in developing countries. MLPA is an effective alternative way to detect subtelomeric rearrangement of all chromosomes in a single reaction, and is also cheaper and less laborious than FISH. Therefore, we recommend MLPA screening in patients with idiopathic MR with dysmorphism and normal karyotype in diagnostic laboratories where a microarray facility is unavailable.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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