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High rate of detection of subtelomeric aberration by using combined MLPA and subtelomeric FISH approach in patients with moderate to severe mental retardation

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Abstract

Objectives: (1) To evaluate the prevalence of subtelomeric deletion in moderate to severe mental retardation population, (2) to assess the feasibility and cost-effectiveness of combined methodology in routine workup of this sub-population.

Method: Twenty unrelated patients using strict selection criteria were recruited for the study from the Clinical Genetic Service. Patients were initially screened by Multiplex Ligation-dependent Probe Amplification (MLPA) for subtelomeric imbalance followed by FISH analysis for anatomical integrity. This is then followed by parental subtelomeric FISH analysis.

Results: Three subtelomeric deletions were identified. They were Deletion 1p36, Deletion 1q44 and Deletion 10q26; these were previously unidentified by conventional technique.

Conclusions: The prevalence of subtelomeric deletion in our cohort of moderate to severe mental retardation patients is consistent with published findings of around 10%. The figure is on the higher side if more stringent criteria is used. The combination of strict clinical criteria, MLPA and selective subtelomeric FISH was shown to be feasible and cost-effective.

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Keywords: Moderate to severe mental retardation; Subtelomeric deletions; MLPA; Subtelomeric FISH; MR, mental retardation; FISH, fluorescence in situ hybridization

Introduction

Mental retardation is common and occurs in approximately 2% of the population. 5–30% of moderate to severe mental retardation can be accounted for by chromosomal disorders; and the majority have associated malformations, growth retardation, dysmorphism and family history of similar occurrence.

Many studies have shown that subtelomeric aberrations are a significant cause of idiopathic moderate to severe mental retardation with figures varying from 5 to 10% [1,8,12,15,17] depending on sample selection criteria, as compared to less than 1% in mild mental retardation.

Subtelomeric regions are gene-rich. A small deletion in these regions would involve many genes, thus deleterious conse-

quences result. Many of these subtelomeric deletions are now recognized as clinically recognizable phenotypes [3]. These terminal regions stain lightly on karyotyping and deletion sizes are variable, which are properties that often are difficult to detect on routine conventional examination. Although in retrospect, with awareness of the deletion, repeat culture with higher banding can often detect such deletions.

Multiplex Ligation-dependent Probe Amplification (MLPA) is a new, sensitive, economical and simple method for relative quantification of multiple nucleic acid sequences in a single reaction. Introduced by MRC-Holland in January 2002, the principle is a relatively simple one, in which denatured genomic DNA after standard extraction is hybridized with a mixture of standardized probes. Each MLPA probe consists of two oligonucleotides, the two parts of each probe hybridized to adjacent target sequences and are ligated by a thermostable ligase. All probe ligation products are amplified by PCR using

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Table 1
Subtelomeric patient selection criteria

Major criteria

- 1) Normal karyotype at 550 band level (46XY, 46XX)
- 2) Idiopathic moderate to severe MR (IQ < 50)

Plus minor criteria 3 out of 6 of the following

- a) Facial dysmorphism (at least 2 areas from eyes, nose and ears that markedly differ from parents)
- b) Non-facial dysmorphism or congenital anomalies (hands, brain, internal organ anomaly or abnormal genitalia)
- c) Abnormal growth (pre or postnatal onset of growth retardation/or overgrowth)
- d) Behavioural disorder (autistic symptom, hyperactivity, sleep disturbance, aggression, self-mutilations, etc.)
- e) Family history of mental retardation
- f) Family history of miscarriages or perinatal death



Fig. 2. Case 1 patient.

only one primer pair. Since the amplification product of each probe has a unique length, they can therefore be separated by electrophoresis. The relative amounts of probe amplification products reflect the relative quantity of target sequences.

Subtelomeric FISH probe is the most commonly used technique in detecting subtelomeric rearrangement [7]. Subtelomeric FISH probe sets are commercially available and are fully tested by their manufacturer.

Due to the laborious, time-consuming and costly procedures of using a complete set of subtelomeric FISH to analyze these selected patients, we developed a strategy of selecting the high risk group, screening with MLPA and then confirming with selective subtelomeric FISH probe(s), hoping to be of cost-effectiveness for future routine clinical use.

Methods

Subtelomeric testing selection criteria

The Clinical Genetic Service is a tertiary referral center and is also the only genetic center in Hong Kong. Twenty patients were selected consecutively from the genetic clinic from our service in year 2003 who satisfied major criteria of having a normal karyotype at 550 band level (46XY, 46XX) and with a diagnosis of idiopathic moderate to severe mental retardation (IQ < 50). They were all examined by a clinical geneticist. In

addition, they all had 3 out of 6 of the followings: (a) facial dysmorphism (at least 2 areas from eyes, nose and ears, etc., that markedly differ from their parents), (b) non-facial dysmorphism or congenital anomalies (hands, brain, internal organ anomaly or abnormal genitalia, etc.), (c) abnormal growth (pre- or postnatal onset of growth retardation/or overgrowth), (d) behavioral disorder (autistic symptom, hyperactivity, sleep disturbance, aggression, self-injurious, etc.), (e) family history of mental retardation, (f) family history of miscarriages or perinatal death. When positive cases were detected in probands, karyotype (minimum 550 bands), MLPA and subtelomeric FISH analyses were performed in proband's parents and their mentally retarded relatives, where indicated (Table 1).

Subtelomeric FISH probes

Subtelomeric FISH studies were performed using Vysis ToTel Vysion selective probe(s) according to the manufacturer's procedure. It is comprised of a different combination of probes with different colors. The probe size ranged from 70 kb to 191 kb, and each contained a locus estimated to be within 300 kb of the end of the chromosome. We followed the standard hybridization method which consisted of 2× SSC aging of

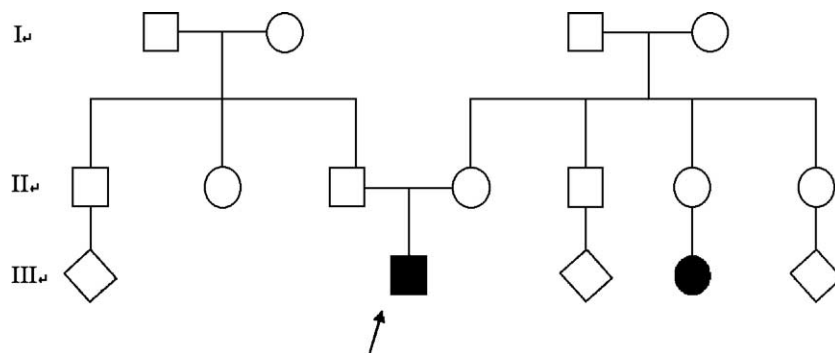


Fig. 1. Case 1 family pedigree.

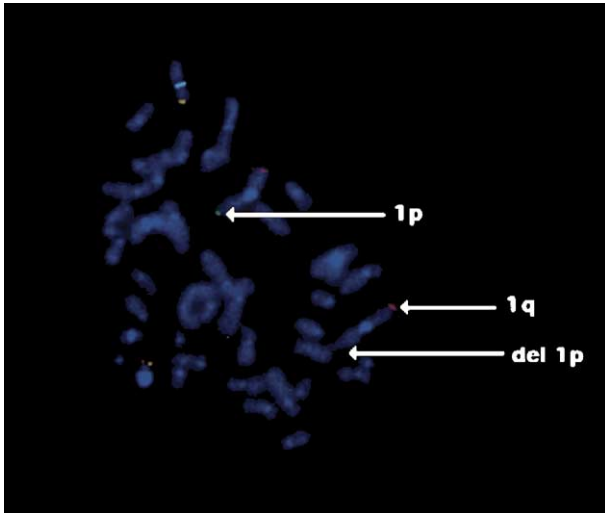


Fig. 3. Case 1 subtelomeric FISH showing deletion 1pter.

slide, 70% formamide denaturation of slide, denaturing the probe mixture followed by hybridization and post-hybridization washing. This is then followed by visualization.

Multiplex Ligation-dependent Probe Amplification (MLPA)

Multiplex Ligation-dependent Probe Amplification (MLPA) was developed from a standardized kit (2003, first generation MLPA) to compare the amount of PCR products generated from 46 subtelomeric regions of the human chromosomes, with the amount of products generated from reference regions.

MLPA was performed according to the manufacturer's protocol. 50–500 ng of genomic DNA in 5 μ L Tris–EDTA was denatured for 5 min at 98°C, cooled and then mixed with the probe set and a high salt buffer (MLPA buffer). The mixture was heated for 1 min at 95°C and incubated overnight at 60°C for hybridization. Ligation was then performed with the Ligase-65 enzyme for 15 min at 54°C. The ligase was then inactivated by incubation at 98°C for 5

min. Ten microliters of this ligation product was premixed with 30 μ L of PCR buffer and put in a thermocycler at 60°C. Subsequently, a 10 μ L reaction mix was added, which contained dNTPs, Taq polymerase and one unlabeled and one labeled PCR primers which are complementary to the universal primer sequences on the MLPA probes. PCR was carried out for 33 cycles (30 s at 95°C, 30 s at 60°C, 1 min at 72°C). The fragments were analyzed on an ABI model 3100 capillary sequencer (Applied Biosystems) with the Genescan software using Genescan 500 size standards (Applied Biosystems). Individual peaks corresponding to each exon were identified based on the difference in migration relative to the size standards. The peak area of each fragment was compared to that of a control sample.

The manufacturer's protocol recommends that one determines the relative probe signals of each probe by dividing each measured peak area by the sum of all peaks area of that sample. This relative peak area is then divided by the relative peak area of the corresponding probe obtained from a control DNA sample. Comparison of results should preferably be performed within the same experimental environment. To automate the interpretation of fragment analysis, the relative peak areas of the amplified probes were analyzed using a Microsoft Excel (Microsoft, <http://www.microsoft.com>) template. Deletions will give a 35–55% reduced relative peak area, while duplications will give a 30–55% increase in relative peak area of the amplification product of that probe.

A consistent, reproducible one half reduction (or 1.5 \times increase) in peak area (fluorescent signal) of a subtelomeric fragment, compared with the internal controls, was taken as indicative of a deletion or duplication of 1 copy of subtelomeric region in a given individual.

Results

Among 20 patients tested, 3 subtelomeric deletions were identified in this study. They were Deletion 1p36, Deletion 1q44 and Deletion 10q26; these were previously unidentified

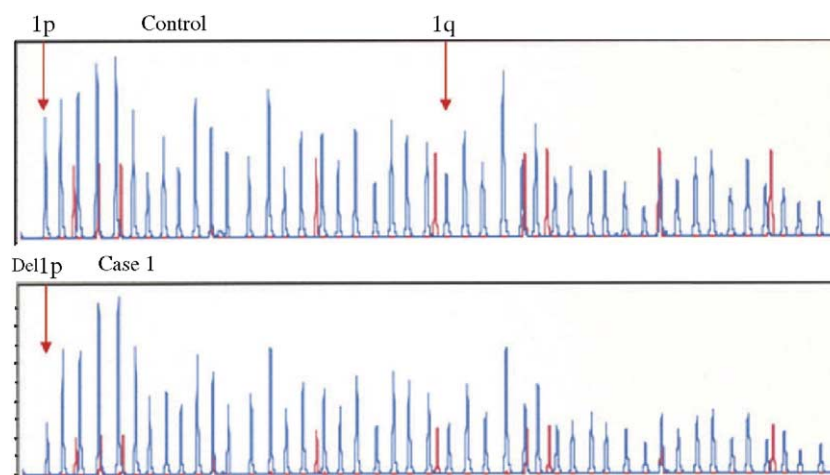


Fig. 4. Case 1 MLPA for subtelomeric probes showing heterozygous 1p deletion.

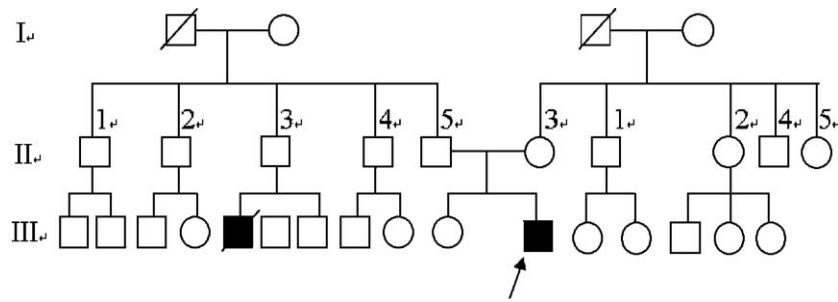


Fig. 5. Case 2 family pedigree.

by conventional technique. The pick up rate was 3/20 (15%). All parental investigations (MLPA and subtelomeric FISH) of all three positive cases were normal.

Case 1 identified

Case 1: Male born in 1993 was referred for global delay, myoclonic epilepsy, hydrocephalus and cleft lips. He was born at 38 weeks gestation after normal delivery with birth weight of 2.1 kg to non-consanguineous parents. His maternal aunt's daughter was mentally retarded. On examination, all growth parameters were less than third percentiles. The patient could not speak since birth and could not sit or walk at 12 years of age. He had a hairy forehead, synophry, long eyelashes, epicanthic folds, deep set eyes, repaired cleft lip, small hands and feet, hypotonia and fourth and fifth fingers clinodactyly. Brain imaging showed hydrocephalus, hypoplasia of corpus callosum and absent septum pellucidum (Figs. 1–4).

Case 2 identified

Case 2: Male born in 1997 was referred for occipital meningocele, dandy-walker malformation, absent corpus callosum, hypotonia, global delay, seizure, micropenis and

cryptorchidism. Antenatally, it was noted that there was oligohydramnios and intrauterine growth retardation. Fetal ultrasound showed meningocele. He was born at 39 weeks gestation after caesarean section for fetal anomaly with birth weight of 2.56 kg to non-consanguineous parents. His paternal uncle's son had global delay and hydrocephalus. All growth parameters were less than third percentiles at 6 years. There was no speech. He was able to sit and stand with support. He was microcephalic with metopic ridging, with a narrow forehead, deep set eyes, high myopia and astigmatism, short upturned nose, big ears, long philtrum and thin upper lips. He also had moderate hearing loss and required hearing aids, micropenis and cryptorchidism (Figs. 5–9).

Case 3 identified

Case 3: Female born in 1996 was referred for dysmorphism and developmental delay, failure to thrive and congenital heart disease (PDA). She was born at 41 weeks plus 3 days requiring vacuum extraction with birth weight of 2.76 kg to non-consanguineous parents. The birth was complicated by birth asphyxia requiring assisted ventilation for 2 weeks. Her paternal aunt's son was mentally retarded. All her growth parameters were less than third percentiles. She sat at 1 year, walked at 3.5 years, said Ba–Ba and Ma–Ma at 2 years. She had an asymmetric



Fig. 6. Case 2 patient.

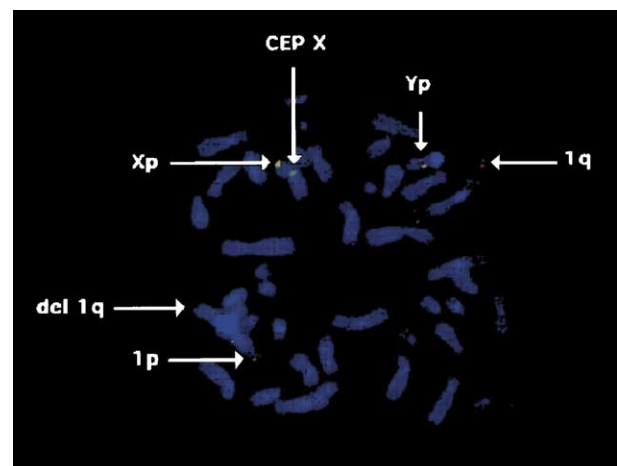


Fig. 7. Case 2 subtelomeric FISH showing deletion 1qter.

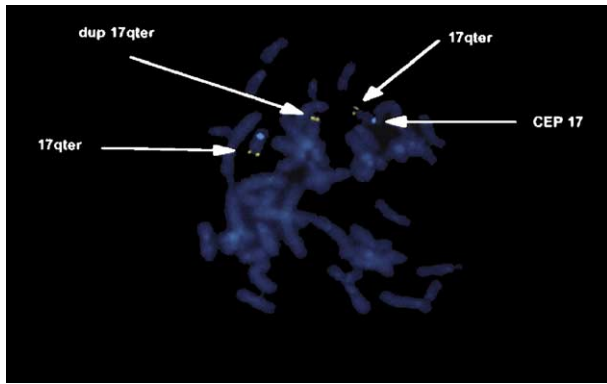


Fig. 8. Case 2 subtelomeric FISH showing duplication 17qter.

face with left side bigger than right, plagiocephaly, bilateral abnormal ear configuration and bilateral fifth finger clinodactyly (Figs. 10–13).

Discussion

Subtelomeric regions of chromosomes are gene-rich. Unbalanced chromosomal rearrangement in these regions leads to severe consequences; e.g. multiple congenital anomalies, moderate to severe mental retardation, dysmorphism and/or abnormal growth or behaviors. Therefore, investigating patients with moderate to severe mental retardation for subtelomeric rearrangement should have a higher yield of results.

Commercial subtelomeric FISH is universally costly and labor-intensive. Many centers tried different alternative approaches with variable success [8,13,14,16,19]. We therefore adopted a cost-effective approach in applying a strict selection criteria prior to using MLPA to screen for chromosomal imbalances and then confirmed by subtelomeric FISH using the indicated probe(s) only. Once confirmed, parental subtelomeric FISH using the same probe was performed for structural rearrangements.

The MLPA HUMAN TELOMER test kits (SALSA P036 and P019/020) were obtained from MRC-Holland, Amsterdam, The Netherlands. According to the manufacturer's recommen-

dation, SALSA P036 is used for screening purposes while SALSA P019/020 is used for confirmation. For SALSA P036, one probe was made for each of the subtelomeric region. For SALSA P019/020, in addition to one probe for each of the subtelomeric region, another probe was directed to a sequence in the middle of each chromosome. These 72 probes were divided into two probe mixes, P019 and P020. Details on probe sequences and gene loci can be found at the company's website (<http://www.mrc-holland.com>). All probes in P036 are different from the ones that are present in these P019/020 probe mixes.

The advantages of MLPA over other techniques such as FISH or microarray are relative simplicity, speed and low cost. The turn-around time for an MLPA reaction is within 24 h. The current price for an MLPA reaction is only \$14 (USD). The equipment necessary for MLPA is only a thermocycler and a sequencing electrophoresis equipment. The high cost of full sets of subtelomeric FISH probes and microarray is not available or affordable to all countries for daily routine use. The present study emphasized the affordable novel strategy of combining strict clinical selection criteria, use of less expensive commercially available MLPA technique and the selective use of expensive subtelomeric FISH probe. It is an alternative that can be considered by less affordable centers so that no family would be disadvantaged by economic situations of their region.

There is no general consensus on which technique is markedly superior to any other currently used techniques for detecting subtelomeric rearrangements that include deletions, duplications and translocations. Further studies are needed to compare various methodologies in order to calculate the sensitivity and specificity of individual technique. Multi-probe FISH has been the most commonly used technique to detect subtelomeric rearrangements. The major drawback of multi-probe FISH is the low throughput (8 days per case), high cost (\$250 (USD) per probe set) and that it is very labor-intensive. In addition, multi-probe FISH may not be sensitive enough to detect the subtelomeric duplication. The aim of the present study is only to introduce a novel strategy that combines the advantages of different methodologies for screening of subtelomeric rearrangements. Future studies are encouraged to test the efficacy of this novel approach.

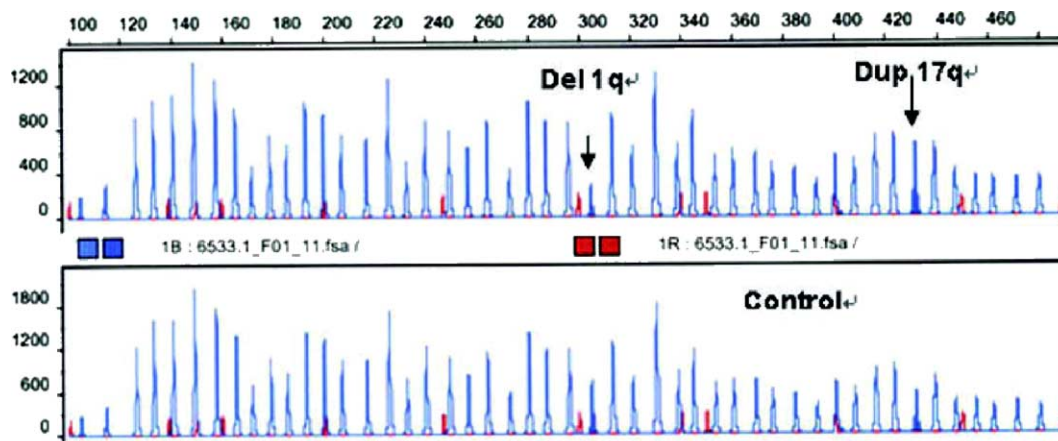


Fig. 9. Case 2 MLPA for subtelomeric probes showing heterozygous 1q deletion and 17q duplication.

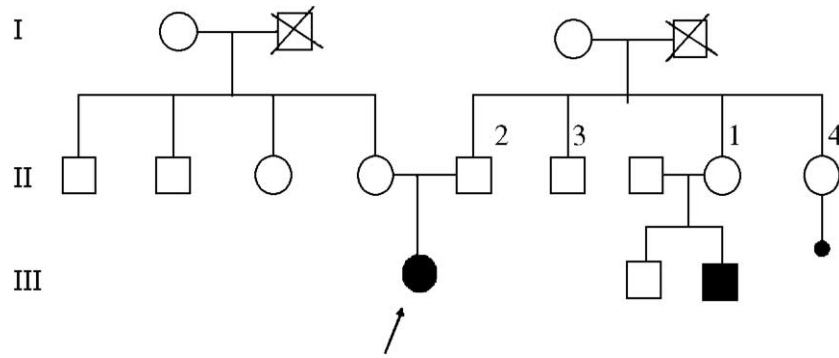


Fig. 10. Case 3 family pedigree.

The three cases of Deletion 1p36, 1q44 and 10q26 were common findings in most subtelomeric series [2,8]. Since our sample size is small, effectiveness of this approach awaits a future larger size study. No attempt was made to compare the efficacy of either MLPA or subtelomeric FISH alone due to limitation of our clinical service settings.

Case 1 showed typical features of 1p36 deletion, which include severe mental deficiency, deep set eyes, cleft lips and hypoplasia of corpus callosum. This is said to be the most common of all subtelomeric deletion syndromes, and detailed studies of phenotype–genotype correlation of published cases allowed narrowing of critical region for the major features of the syndrome and their respective candidate genes [11].

Case 2 had combination of 1qter deletion and 17q duplication. Clinically, his features were more suggestive of 1qter deletion with midline neurological defects [18]; absent corpus callosum, occipital meningocele, dandy-walker malformation and seizures; deafness, micropenis and cryptorchidism. Typical craniofacial features include microcephaly, prominent metopic suture, long philtrum and thin upper lips.

Case 3 had typical 10qter deletion characteristics [5,9] with marked hypotonia and respiratory distress at birth requiring ventilatory support. Other typical features include

microcephaly, broad nasal bridge, malformed ears, triangular face with facial asymmetry and patent ductus arteriosus.

Couples who are carriers of balanced subtelomeric chromosomal rearrangement may present with recurrent miscarriage [20] or neonatal death; or child with multiple anomalies [4]. Therefore, family history of recurrent miscarriage was included in one of our patient selection criteria. Correct cytogenetic diagnosis of proband would lead to examination of parental carrier status. The results of which would allow proper genetic counseling, as most families would be too worried to have further pregnancy after the birth of an affected child, and the discussion of possible option of prenatal diagnosis. Prenatal diagnosis has been shown to be feasible by using subtelomeric FISH [6,10].

Parental testing of all 3 cases was normal. It was counseled that the recurrence risk for all 3 families was low. Parents of case 3 indicated after genetic counseling that they wanted further children and asked for prenatal diagnosis “just to be sure”. Prenatal diagnosis with MLPA on amniotic fluid cells was performed and showed normal result; and the couple subsequently delivered a normal baby. Parents of case 2 expressed interest in planning for further pregnancy at the time of writing this paper.



Fig. 11. Case 3 patient.

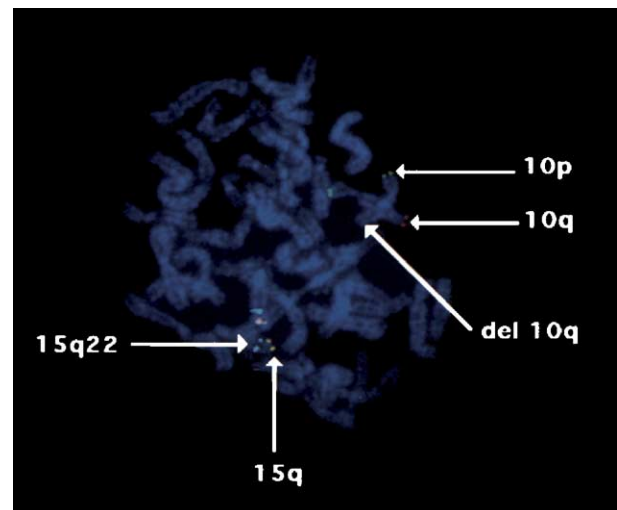


Fig. 12. Case 3 subtelomeric FISH showing deletion 10qter.

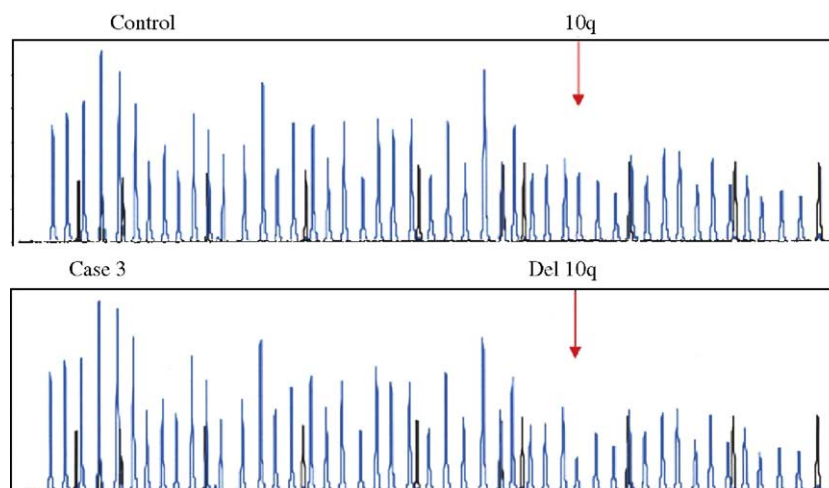


Fig. 13. Case 3 MLPA for subtelomeric probes showing heterozygous 10q deletion.

The prevalence of subtelomeric deletion in our cohort of moderate to severe mental retardation patients is consistent with published findings of around 10%. The figure is on the higher side when more stringent criteria is used. The combination of strict clinical criteria, MLPA and subtelomeric FISH was shown to be feasible and cost-effective. This marked an important and novel approach to the management of these patients in terms of genetic counseling and family planning of affected families.

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