Clin Genet 2006: 69: 58–64
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CLINICAL GENETICS
doi: 10.1111/j.1399-0004.2005.00545.x

Short Report

Multiplex ligation-dependent probe amplification to detect subtelomeric rearrangements in routine diagnostics

Rooms L, Reyniers E, Wuyts W, Storm K, van Luijk R, Scheers S, Wauters J, van den Ende J, Biervliet M, Eyskens F, van Goethem G, Laridon A, Ceulemans B, Courtens W, Kooy RF. Multiplex ligation-dependent probe amplification to detect subtelomeric rearrangements in routine diagnostics. Clin Genet 2006: 69: 58–64. © Blackwell Munksgaard, 2005

Subtelomeric rearrangements are believed to be responsible for 5–7% of idiopathic mental retardation cases. Due to the relative complexity and high cost of the screening methods used till now, only preselected patient populations including mostly the more severely affected cases have been screened. Recently, multiplex ligation-dependent probe amplification (MLPA) has been adapted for use in subtelomeric screening, and we have incorporated this technique into routine diagnostics of our laboratory. Since the evaluation of MLPA as a screening method, we tested 275 unselected patients with idiopathic mental retardation and detected 12 possible subtelomeric aberrations: a der(11)t(11;20)(qter;qter), a 19pter duplication, a der(18)t(18;10)(qter; pter), a 15qter deletion, a 8pter deletion, a 6qter deletion, a der(X)t(X;1)(pter;qter), a der(X)t(X;3)(pter;pter), a 5qter duplication, a 3pter deletion, and two 3qter duplications. The patients can be subdivided into two groups: the first containing *de novo* rearrangements that are likely related to the clinical presentation of the patient and the second including aberrations also present in one of the parents that may or may not be causative of the mental retardation. In our patient cohort, five (1.8%) subtelomeric rearrangements were *de novo*, three (1.1%) rearrangements were familial and suggestively disease causing, and four (1.5%) were possible polymorphisms. This high frequency of subtelomeric abnormalities detected in an unselected population warrants further investigation about the feasibility of routine screening for subtelomeric aberrations in mentally retarded patients.

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Keywords: subtelomeric rearrangements – MLPA – routine diagnostics

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Received 22 July 2005, revised and accepted for publication 3 October 2005

Rearrangements involving the ends of chromosomes are commonly quoted to be responsible for approximately 5–7% of all mental retardation cases (1, 2). These estimates are based on the outcome of a series of screening studies that all are performed on selected populations of mentally retarded patients. While deletions of some chromosome ends cause a specific phenotype, including a deletion of the tip of chromosome 4p that causes Wolf–Hirschhorn syndrome and a deletion of the tip of chromosome 16p that causes alpha-thalassemia/mental retardation (ATR)-16 syndrome, deletions of most individual chromosome ends are not clinically recognizable. However, forced by the high number of mentally retarded patients in the population and the relatively laborious screening methods employed up to now, most studies tested only a selection of mentally retarded patients. Inclusion criteria aimed at increasing the diagnostic yield varied between studies but were mostly based on the severity of the mental retardation, the presence of facial dysmorphism, and familial history.

While most screening studies have used multiprobe fluorescence *in situ* hybridization (FISH), recently, a number of alternative techniques for

this reliable, but relatively labor-intensive method have been adapted for use in telomeric screening (3). Multiplex ligation-dependent probe amplification (MLPA) has been demonstrated to be a reliable and sensitive technique to detect subtelomeric rearrangements in two pilot studies (4, 5). Using MLPA, we were able to screen for subtelomeric aberrations in our patient population on a routine basis; for example, we have screened all patients referred to our genetic center who were suspected of mental retardation. In this study, we detected 12 subtelomeric rearrangements in 275 patients. Of these 12 rearrangements, eight (2.9%) are likely disease causing.

Methods

Patients

Patients studied were referred to the Department of Medical Genetics in Antwerp, Belgium, as mentally retarded. No further selection criteria were used.

MLPA probe kits

Telomere-specific MLPA kits P019, P020, P036, P036B, P069, and P070 were purchased from the manufacturer (MRC-Holland, Amsterdam, The Netherlands). The initial kits P019 and P020, in combination, cover all subtelomeric regions (5). These were jointly superseded by the P070 kit, containing 46 probes of which 22 were also present in the first generation P019/P020 set. The substituted probes are located closer to the telomere when compared with the corresponding probes in the P019/P020 set. Probe set P069 is identical to the P070 set but does not include the probes for the acrocentric chromosome arms 13p, 14p, 15p, 21p, and 22p. The P036 probe set was developed independently and contains probes close to each telomere different from the ones included in the P019, P020, P069, or P070 sets (4). More recently, it was ameliorated by replacing several probes that appeared polymorphic and renamed P036B (Table 1).

Patients were tested using one of these probe sets according to the protocol as described previously (5). If an aberrant MLPA result was observed, a second probe set containing a different probe for that specific telomere was used to verify the results.

FISH with a probe specific for the region of interest was performed to validate the results obtained with the MLPA probe sets as described (5).

X-inactivation

The methylation patterns of the maternally and paternally derived X-chromosomes were examined by a polymerase chain reaction (PCR)-based assay analyzing the polymorphic short tandem repeat in the human androgen receptor gene (*HUMARA*) as described previously (6).

Southern blotting

Southern blotting experiments were performed according to standard protocols. Following a 4-h restriction endonuclease digestion, genomic DNA was separated by 0.7% agarose gel electrophoresis. Subsequently, the restriction fragments were transferred to a nylon membrane (Hybond-N+, Amersham Biosciences, Buckinghamshine, England) and were cross-linked by alkali. Probes of PCR-amplified genomic DNA were labeled with ^{32}P deoxyadenosine triphosphate and ^{32}P deoxycytidine triphosphate by Klenow reaction with random hexamer primers. Probes were hybridized in hybridization solution [10% Denhardts, 15% ×20 Saline-sodium citrate (SSC), 4% sodium dodecyl sulfate (SDS), and 10% dextrane sulfate] with herring sperm DNA. Blots were washed with SSC/SDS wash solutions with elevating stringencies and exposed to a radiographic film. Primer pairs for amplification of the hybridization probes were based on the genomic sequence of contig NT_023133 and are as follows: GNB2L1 forward 5′-AGGTTCT-CATCTGCAATGTGG-3′ and GNB 2L1 reverse 5′-CTTCACCTCTTT CGCTTCTCG-3′.

Results

We tested 275 patients with idiopathic mental retardation. Twelve abnormalities were detected which can be subdivided into two groups. The first group contains five *de novo* rearrangements including a der(11)t(11;20)(qter;qter), a duplication of chromosome 19pter, a der(18)t(18;10)(q-ter;pter), a deletion of chromosome 15qter, and a deletion of chromosome 8pter. The second group contains aberrations, which are also present in one of the parents including a 6qter deletion in twin brothers, two independent patients with Xpter deletions, a 5q duplication, a 3pter deletion, and two independent patients with 3qter duplication (Table 2).

Patient 1: der(11)t(11;20)(qter;qter)

This girl presented with psychomotor retardation, growth retardation, cardiopathy, left-sided

Table 1. Overview of the different multiplex ligation-dependent probe amplification probe sets

Probe set	Description
P019/P020	First generation probe set Covers all subtelomeric regions in two reactions
P070	Contains 22 probes out of the P019/P020 set 24 new non-polymorphic probes
P069	Contains the same probes as the P070 set, excluding the probes for the acrocentric chromosome arms 13p, 14p, 15p, 21p, and 22p
P036	Second generation probe set Contains probes different from the ones included in the P019/P020 set
P036B	Improved version of the P036 set, with 10 polymorphic probes replaced

hydronephrosis, and dysmorphism. The aberration was detected by two different MLPA probe sets (P019/P020 and P036) and subsequently confirmed by FISH analysis using probe GS-26-N8 for the 11q subtelomeric region and GS-81-F12 for the 20q subtelomeric region. FISH analysis of both parents showed that the unbalanced translocation was *de novo*.

Patient 2: duplication of chromosome 19pter

This patient presented with psychomotor retardation, cardiopathy, growth retardation, hearing loss, and dysmorphic features. This duplication was detected by the P019/P020 MLPA probe set as well as by the P036 probe set and subsequently confirmed by FISH using clone GS-546-C11. The karyotype of the patient's mother showed no abnormalities. No material was available from the deceased father, but FISH analysis of the paternal grandparents showed no abnormalities.

Patient 3: der(18)t(18;10)(qter;pter)

This patient presented with psychomotor retardation, cardiopathy, and dysmorphism. This rearrangement was detected with the P036 probe set and subsequently confirmed with FISH using clone GS-23-B11 for the 18q subtelomeric region and clone GS-964-M9 for the 10p subtelomeric region. FISH analysis using the same clones showed no evidence for an abnormal karyotype in the parents suggesting that the rearrangement arose *de novo*.

Patient 4: deletion of chromosome 15qter

This boy presented with learning difficulties and a relatively short stature. He has small hands and feet and a mild clinodactyly of the left finger. A deletion of chromosome 15q was indicated by both probe sets P019/P020 and P036. FISH analysis using clone GS-154-P1 confirmed these results. This aberration appeared *de novo* as

Table 2. Overview of the detected aberrations

Patient	Aberration	Inheritance	Clinical manifestations
De novo			
1	11qter deletion 20qter duplication	*De novo*	MR, growth retardation, cardiopathy, dysmorphism
2	19pter duplication	*De novo*	MR, cardiopathy, growth retardation, dysmorphism
3	18qter deletion 10pter duplication	*De novo*	MR, cardiopathy, dysmorphism
4	15qter deletion	*De novo*	Learning difficulties
5	8pter deletion	–	MR, congenital heart anomalies, no dysmorphism
Inherited: presumably disease causing			
6	6qter deletion	Maternal	MR, microcephaly, seizures, dimpling, facial dysmorphism
7	Xpter deletion 1qter duplication	Maternal	Dysgenesis outer ear, epilepsy, facial dysmorphism
8	Xpter deletion 3pter duplication	Maternal	Psychomotor retardation, microcephaly, mild dysmorphism, behavioral problems
Inherited: probable polymorphisms			
9	5qter duplication	Paternal	MR, autistic behavior
10	3pter deletion	Maternal	MR, severe frontal epilepsy
11	3qter duplication	Paternal	MR, behavioral problems
12	3qter duplication	–	Mild MR, dysmorphic features

MR, mental retardation.

FISH analysis in both parents showed no aberrant results.

Patient 5: deletion of chromosome 8pter

This isolated patient was the third child of healthy non-consanguineous parents. Pregnancy and delivery were normal. His unaffected younger sister also has healthy children. He showed developmental delay already in early development. He had no dysmorphic features. He had a congenital endocardial cushion defect, characterized by ostium primum, atrial septum defect, and abnormally implanted mitral valve resulting in mitral insufficiency. A subtelomeric deletion of chromosome 8p was detected by both P019/P020 and P036 probe sets. There was no material available to perform FISH nor was there parental material.

Patient 6: deletion of the subtelomeric region of chromosome 6q

Two monozygotic twin brothers presented with moderate-severe mental retardation. Both have microcephaly, agenesis of the corpus callosum, and seizures. The patients have specific dysmorphic features including a high-arched palate and a tented upper lip and show dimpling on elbows and knees. We detected a subtelomeric deletion using the P036 probe set in both brothers. Subsequent FISH analysis using clone GS-57-H24 confirmed these findings. The same deletion was detected in the mother whereas no abnormalities were found in the karyotype of the father.

Patient 7: a der(X)t(X;1)(pter;qter)

The 4-year-old girl showed bilateral dysgenesis of the outer ear. She suffered from frontal nocturnal epilepsy. She had facial anomalies including a broad nasal bridge, epicanthus, and a long deep philtrum. Magnetic resonance imaging of the brain showed no aberrant results. She was included in the study because she was suspected of mental retardation before cognitive testing excluded it. MLPA using probe set P036 indicated a der(X)t(X;1)(pter;qter). The Xpter deletion was not observed using the P019 probe set probably as a consequence of the relatively long distance of the probe to the Xp telomere. The rearrangement was confirmed with FISH using clone GS-839-D20 for the Xp subtelomeric region and clone GS-167-K11 for the 1q subtelomeric region. FISH analysis of both parents using the same clones showed no evidence for abnormalities in the father's karyotype whereas the mother carried the same chromosomal rearrangements as the patient. Subsequently, we investigated the X-inactivation in the mother. The mother displayed a non-random X-inactivation pattern. Further investigations of the maternal grandfather revealed the same chromosomal aberration as detected in the patient and his mother.

Patient 8: a boy with a XXY karyotype carrying a deletion of Xpter and a duplication of 3pter

Karyotype analysis showed that this 6-year-old boy was mosaic 46XY/47XXY in 88% of his cells, which cannot be responsible for his clinical features. He presented with a severe psychomotor retardation: he could walk at the age of 20 months, and speech development was delayed as he could not talk at the age of 3 years. He had mild dysmorphic features such as a broad nasal bridge and a high-arched palate and had severe behavioral problems. He had microcephaly, brachydactyly, and ichthyosis. MLPA using probe set P036 gave evidence for a der(X)t(X,3)(pter,pter). FISH using clones GS-839-D20 for the Xp telomere and GS-1186-B18 for the 3p telomere confirmed these findings. The terminal parts of both copies of the X-chromosome were deleted. FISH analysis of the mother also revealed a der(X)t(X,3)(pter,pter), whereas no abnormalities were detected in the father.

Patient 9: a partial duplication of 5qter

A 10-year-old girl of African origin presented with mental retardation and autistic behavior. Dysmorphic features were absent. It was the second child of non-consanguineous parents. The firstborn daughter was normal. MLPA using two different probe sets, P036 with a probe in the *TRIM52* gene and P070 containing a probe for 5p in the *GNB2L1* gene, gave evidence for a 5pter duplication. FISH analysis using clone GS-240-G13 showed no aberrant results. This clone is located more distal to the telomere than the MLPA probes that were used. To determine whether we were dealing with an interstitial duplication, we performed Southern blotting experiments using a probe situated in the *GNB2L1* gene. We detected in the patient an additional *XmnI* fragment (Fig. 1) and a *HindIII* fragment with increased intensity when compared with the intensity of control fragments

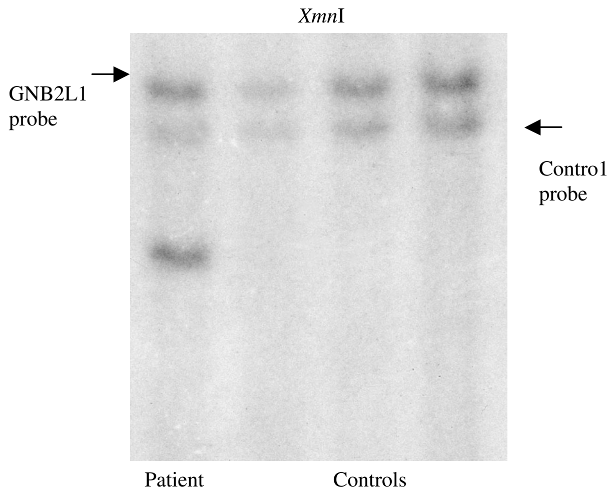

Fig. 1. Southern blotting experiments using probe GNB2L1. An additional *Xmn*I fragment is observed in the patient.

(data not shown). The same duplication was detected in the healthy father.

Patient 10: a polymorphism in the *CHL1* gene at 3pter

This boy is known for several years with a therapy-resistant frontal epilepsy caused by a ring chromosome 20, without detectable loss of chromosomal material. He has now, after 8 years of epilepsy, a moderate mental retardation and a somewhat peculiar but not really dysmorphic face. MLPA using probe set P019/P020 suggested a deletion of chromosome 3pter. Analysis of both parents showed the same aberration in the healthy mother. However, FISH analysis using probe GS-1186-B18 could not demonstrate a deletion. Therefore, we looked for abnormalities in the sequence of the MLPA primers. We detected a sequence change of an adenine to a guanine (Fig. 2), which inhibited the ligation in the MLPA probe and as a consequence its amplification in both patient and mother.

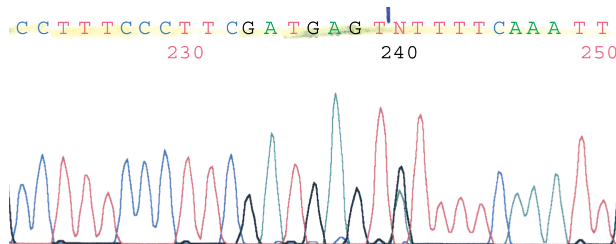

Fig. 2. Sequence at the site of the 3p probe. At nucleotide 240, there is an alteration visible of an adenine into a guanine.

Patient 11: a duplication of chromosome 3qter

This patient of healthy, non-consanguineous parents presented with moderate mental retardation and behavioral problems. Dysmorphic features were absent. She was somewhat obese. MLPA probe set P036 showed evidence for a duplication of chromosome 3qter. The probe for the 3q telomere in the P070 set is located in the *KIAA0226* gene telomeric to the BDH probe included in the P036 probe set and appeared not deleted. Subsequent FISH analysis using clone GS-196-F4 showed evidence for a regional duplication in the patient (Fig. 3). The same duplication was detected in the patient's healthy father.

Patient 12: a duplication of chromosome 3qter

This patient showed mild mental retardation, some dysmorphic features, and neurological signs. According to the results obtained with the P036 probe set, this patient has a duplication of chromosome 3q. This result was not confirmed using the P070 probe set that contains a probe for 3q that is located more distal. In contrast to the patient described above, FISH using probe GS-196-F4 did not show evidence for a duplication. Parental material was not available.

Discussion

We detected 12 rearrangements using different MLPA probe sets in 275 unselected patients referred to our laboratory for mental retardation. The rearrangements can be divided in two groups. The first group of five patients had *de novo* (or presumed *de novo*) large chromosomal deletions and/or duplications, including a der(11)t(11;20)(qter;qter), a duplication of the tip of chromosome 19p, a der(18)t(18;10)(qter;pter), a deletion of the tip of chromosome 15q, and a deletion of the tip of chromosome 8p. Such gross rearrangements are generally assumed to be disease causing. Deletions of chromosome ends 8p, 11q, and 18q have been described before. The deletion of chromosome end 15q has not been described before, but as it is a *de novo* deletion, we assume this rearrangement to be disease related. A duplication of the tip of chromosome 19p has also not been described before. However, as it concerns a *de novo* duplication of a relatively large chromosomal segment, we assume this to be related to the disorder in the patient.

The second group comprises aberrations inherited from one of the parents. In these cases, only

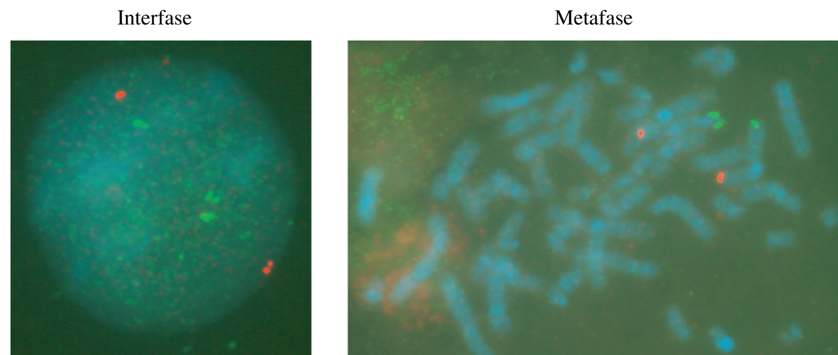

Fig. 3. Fluorescence *in situ* hybridization (FISH) detection of 3qter duplication. The 3q FISH probe is in green and the 3p FISH probe is in red. A double signal for the 3q region is detected.

a careful clinical examination of the patient and the parents can decide whether the aberration is related to the clinical manifestations in the patient or not. Both brothers with the 6q subtelomeric deletion show moderate-severe mental retardation, microcephaly, seizures, agenesis of the corpus callosum, and distinct facial characteristics. Clinical characteristics of the subtelomeric 6q-deletion include mental retardation, microcephaly, dysmorphic features, an abnormal corpus callosum, brain anomalies, hypotonia, and growth retardation (7). It seems therefore likely that the phenotype of both brothers is related to the chromosomal deletion. The mother of the twin brothers carries the same 6q subtelomeric deletion, seems to have a mild mental handicap, and clearly has a speech deficit. Her facial features are reminiscent of those of her sons. The mother may therefore represent a mild case of the disorder.

In contrast to the deletion of many chromosome ends that share more or less consistent phenotypes, a deletion of chromosome Xp appears to have a highly variable phenotype (1). The girl with the der(X)t(X;1)(pter;qter) presented with dysmorphic features, but, in contrast to initial suspicion, was not mentally impaired. She inherited this chromosomal aberration from her mother. It appeared that the mother showed a skewed X-inactivation in contrast to the patient that showed a random inactivation pattern. It is therefore possible that the clinical problems in the patient are due to the chromosomal deletion and that the mother is spared from the disease by non-random X-inactivation. However, the maternal grandfather shows the same Xpter deletion as both patient and patient's mother. This deletion is located in the terminal pseudoautosomal pairing region of Xp and Yp and contains at least four deleted genes including the *SHOX* gene. *SHOX* nullizygosity and even haploinsufficiency have been linked to skeletal anomalies and dysplasias. The findings in this family are therefore paradoxal. While the dysplasia of the outer ear in the child could be compatible with haploinsufficiency of the *SHOX* gene and the mother may be spared by skewed X-inactivation, it remains unclear how the grandfather can be without apparent phenotype despite the deletion of Xp. This family therefore adds further diversity to the Xp^{-} disorder.

In the case of the boy with the Klinefelter syndrome displaying a maternally inherited der(X)t(X;3)(pter;pter), the mother shows the same facial characteristics and appears mildly mentally impaired. Therefore she might represent a mild case of the disorder.

In addition to these aberrations that we consider disease causing, we detected three relatively small chromosomal rearrangements and a single base-pair change, each of which was also detected in a clinically unaffected parent: a duplication on chromosome 5q, two duplications on chromosome 3q, and a one base-pair change at the ligation site of the 3p probe of the P019 probe set. This MLPA probe is located in the *CHL1* gene (synonym *CALL* gene) a possible candidate gene for the symptoms of the 3p syndrome (8). The gene encodes a neural cell adhesion molecule with homology to L1CAM. The sequence alteration changes a tyrosine into a cytosine in the first exon of this gene (*Y52C*). The consequences of this amino acid substitution are difficult to predict. As both of these amino acids are hydrophobic, it seems unlikely that the structure of the protein is dramatically changed. Though the mutation itself is unlikely disease causing, it is intriguing that a single-nucleotide polymorphism located close to this missense mutation shows a highly significant association ($p < 0.00001$) with a brain disorder called schizophrenia in two separate populations (9). Thus, an association of this gene with brain dysfunctions might exist.

Both of the remaining inherited rearrangements are unique and have not been reported before. As the parents who carry the same alterations are clinically unaffected, it seems unlikely that these rearrangements *per se* are disease causing.

However, the duplications may represent risk factors that by itself are not disease causing but in combination with other factors might result in disease (10). After all, in each case, a series of genes has been duplicated.

In summary, we detected five (1.8%) *de novo* subtelomeric deletions/duplications that must be considered disease causing in a group of 275 unselected patients. An additional three (1.1%) familiar rearrangements that we consider disease causing were detected and we estimate that 2.9% of all patients referred to our center for mental retardation are caused by a subtelomeric rearrangement. This suggests that routine screening of mentally retarded patients is feasible. In addition, we found three (1.1%) small rearrangements using a primer pair from one MLPA probe set but not by other probe sets. Such polymorphisms are unlikely disease causing though these might be disease related. This emphasizes the importance to use two different MLPA probe sets in routine diagnostics to avoid false positives.

Acknowledgements

Work on the detection of subtelomeric rearrangements in Antwerp was supported by grants from the Marguerite-Marie Delacroix foundation and the Belgian National Fund for Scientific Research – Flanders (FWO). This research was performed in the framework of the Interuniversity Attraction Poles program P5/19 of the Belgian Federal Science Policy Office.

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