HUMAN MUTATION 26(5), 477–486, 2005

METHODS

Detection of Cryptic Subtelomeric Chromosome Abnormalities and Identification of Anonymous Chromatin Using a Quantitative Multiplex Ligation-Dependent Probe Amplification (MLPA) Assay

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Communicated by John McVey

The need to detect clinically significant segmental aneuploidies beyond the range of light microscopy demands the development of new cost-efficient, sensitive, and robust analytical techniques. Multiplex ligation-dependent probe amplification (MLPA) has already been shown to be particularly effective and flexible for measuring copy numbers in a multiplex format. Previous attempts to develop a reliable MLPA to assay all chromosome subtelomeric regions have been confounded by unforeseen copy number variation in some genes that are very close to the telomeres in healthy individuals. We addressed this shortcoming by substituting all known polymorphic probes and using two complementary multiplex assays to minimize the likelihood of false results. We developed this new quantitative MLPA strategy for two important diagnostic applications. First, in a group of cases with high clinical suspicion of a chromosome abnormality but normal, high-resolution karyotypes, MLPA detected subtelomeric abnormalities in three patients. Two were de novo terminal deletions (del(4p) and del(1p)), and one was a derivative chromosome 1 from a maternal t(1p;17p). The range of these segmental aneuploidies was 1.8–6.6 Mb, and none were visible on retrospective microscopy. Second, in a group of six patients with apparently de novo single-chromosome abnormalities containing anonymous chromatin, MLPA identified two cases with simple intrachromosomal duplications: dup(6p) and dup(8q). Three cases showed derivative chromosomes from translocations involving the distal regions of 9q and 4q, 5p and 11q, and 6q and 3p. One case showed a nonreciprocal, interchromosomal translocation of the distal region of 10p–7p. All abnormalities in both groups were confirmed by fluorescence in situ hybridization (FISH) using bacterial artificial chromosomes (BACs). This quantitative MLPA technique for subtelomeric assays is compared with previously described alternative techniques. Hum Mutat 26(5), 477–486, 2005. **© 2005 Wiley-Liss, Inc.**

KEY WORDS: chromatin; MLPA; aneuploidy; karyotype

INTRODUCTION

The development of techniques for the detection and characterization of cryptic chromosome abnormalities is currently of great interest due to the growing realization that clinically significant chromosome segmental aneuploidy exists throughout the genome and extends well beyond the range of light microscopy. Multiplex ligation-dependent probe amplification (MLPA) is a technique that is ideally suited to the dosage measurement of genomic sequences, including those in the subtelomeric regions of all human chromosomes [Schouten et al., 2002]. The ideal probes are those that target the most distal genes; however, our own experience and previous reports describing the use of MLPA for subtelomeric testing have revealed polymorphic copy number variation in some of the genes targeted by the probe sets used [Koolen et al., 2004; Rooms et al., 2004]. However, the selection of more proximal, nonpolymorphic gene targets increases the risk of false-negative results. To overcome this problem, we substituted known polymorphic probes with suitable nonpolymorphic ones within 1 Mb of the telomere, and used two complementary multiplex assays that target two separate intragenic, subtelomeric sequences per chromosome end. We applied this technique

The Supplementary Material referred to in this article can be accessed at www.interscience.wiley.com/jpages/1059-7794/suppmat.

Received 20 January 2005; accepted revised manuscript 11 July 2005.

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Grant sponsor: Murdoch Children's Research Institute; Grant sponsor: ANZ Trustees; Grant sponsor: National Health and Medical Research Council of Australia.

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DOI 10.1002/humu.20243
Published online 16 September 2005 in Wiley InterScience (www.interscience.wiley.com).

successfully to address two important diagnostic challenges in current clinical cytogenetic practice, as described below.

First, we studied a group of patients (n = 51) with idiopathic mental retardation (MR) with multiple congenital abnormalities, who previously showed a normal high-resolution G-banded karyotype. It is now widely recognized that unbalanced chromosome abnormalities involving the subtelomeric regions are relatively common in mentally retarded individuals, especially those who are moderately to severely affected. There has been particular interest in chromosome abnormalities that are submicroscopic and therefore not detectable using high-resolution G-banded metaphase preparations. Many studies have used molecular techniques with probes mapping to the subtelomeric regions one by one, in pooled sets, or in microarrays [reviewed in De Vries et al., 2003; Flint and Knight, 2003]. A smaller number of studies used polymorphic markers [Borgione et al., 2001; Colleaux et al., 2001; Flint et al., 1995; Rio et al., 2002; Rosenberg et al., 2001; Slavotinek et al., 1999] or high-resolution comparative genomic hybridization [Harada et al., 2004; Kirchhoff et al., 2004; Ness et al., 2002]. These studies showed that 1–8% of mentally retarded individuals have subtelomeric imbalances of $\geq$150 kb, and therefore represent a hitherto undetected category of mutations.

Second, we applied subtelomeric MLPA to identify incompletely characterized, unbalanced chromosome abnormalities in a group of patients with a variety of developmental disabilities. The identification of anonymous chromatin in de novo derivative or marker chromosomes is problematic, and has been approached with the use of chromosome dissection/reverse painting, multiplex fluorescence in situ hybridization (FISH), and metaphase or microarray comparative genomic hybridization (CGH). These techniques have not been widely adopted, for a variety of reasons. In this work we demonstrate the effectiveness of MLPA for this application and compare it with the currently available alternative techniques.

MATERIALS AND METHODS

Study Design

The patients selected for analysis were divided into two main groups. Group A (n = 51) consisted of patients who showed a normal karyotype on a previous referral yet were still deemed by clinical geneticists to have a high likelihood of carrying a chromosome aberration. Typically these patients show MR varying from mild to severe and/or developmental delay with dysmorphic features. Group B (n = 6) consisted of patients with the same clinical spectrum but with known de novo, unbalanced chromosome abnormalities. Additional chromatin was apparent from inspection of the G-banding pattern, but its identity and the donor chromosome identity were not discernible. Furthermore, whether the recipient chromosome was intact or partially deleted was also unknown.

Sample Preparation

Genomic DNA was extracted from blood anticoagulated with EDTA or lithium heparin using the Progenome^{TM} II Extraction Kit (Progen; www.progen.com.au). DNA was diluted in distilled water to a concentration of 50 ng/μl and stored at −20°C until use.

MLPA Reaction and Fragment Analysis

Two sets of subtelomeric probes were used. The probe target sequences were all different, although they were selected from the same gene for eight subtelomeric regions (2p, 3p. 8p, 13p, 17p, 7q, 21q, and X/Yq). For instance, each set uses a different probe target sequence within the ACP1 gene in the subtelomeric region of chromosome 2p. SALSA P036 consists of one unique probe per subtelomeric region for all chromosomes except the acrocentric and sex chromosomes. The acrocentric chromosomes (13, 14, 15, 21, and 22) contain predominantly repeated sequences in their short arms, and both probe sets contain probes that are actually located in the pericentromeric long-arm regions (hereafter referred to as 13*, 14*, 15*, 21*, and 22*). The X and Y chromosomes have short- and long-arm homologous pseudoautosomal regions of 2,500 kb (PAR1) and 800 kb (PAR2), respectively. The P036 set has one probe in each of the PAR regions and in two Y-chromosome specific, nonsubtelomeric regions. The second probe set (SALSA P070) does not contain the Y-chromosome specific probes. The two probe sets have no subtelomeric probes in common.

The probe sets were developed by MRC-Holland (Amsterdam, The Netherlands), and details regarding the gene targets can be found at www.mrc-holland.com. Since the first-generation (SALSA P019/P020) and second-generation (SALSA P036) subtelomeric sets were first released for assessment, several probes have been replaced in the SALSA P036 and P070 probe sets. This was due to the realization that some genes close to the telomere are polymorphic in copy number (i.e., duplicated or deleted in healthy individuals), and the availability of improved maps of the human genome. New genome targets were therefore selected on the basis of being within the most telomeric yet nonpolymorphic gene sequences. The median distance of target sequences from the telomere is 0.30 Mb for both SALSA P036 and P070.

The principle of MLPA is described in detail elsewhere [Schouten et al., 2002]. Samples were processed using capillary electrophoresis (MegaBace, General Electric; www.ge.com/en). Five normal controls (46,XX and 46,XY) and one abnormal control (48,XX,+8,+21) were included in each run.

Data Analysis

For the data analysis we used the relative peak height (RPH) method recommended by MRC-Holland, as previously described [Slater et al., 2003, 2004]. Briefly, each individual subtelomeric region's peak height is normalized by dividing it by the total of all peak heights. Each normalized peak height is then divided by the corresponding, average, normalized peak height from five control samples to obtain the RPH. Theoretically, heterozygous deletions and duplications show an RPH of 0.5 and 1.5, respectively. Normal variation was determined from the total sample data to determine thresholds. Intrasample subtelomeric variation was determined using replicates of normal samples and intersample variation from within-run total normal data (see Results section).

All copy number changes demonstrated with both probe sets were verified using FISH with bacterial artificial chromosomes (BACs) or painting probes. Any result that showed an apparent copy number change with one set but not the other was repeated. If a consistent result was not obtained on the repeat, a negative result was concluded and a polymorphism was recorded for the relevant probe.

FISH Analysis

All abnormal findings in the MLPA analysis were validated using FISH performed on metaphase chromosome preparations using standard techniques [Lichter and Cremer, 1992]. The analysis was performed using a Zeiss Axioskop microscope

equipped with a Cytovision Image Analysis System (Applied Imaging Ltd., Newcastle, UK; www.aicorp.com).

All of the FISH probes used were derived either from a commercial supplier (Vysis; www.vysis.com) and used in accordance with the manufacturer's instructions, or from BACs sourced from the RPCI-11 library and selected using the UCSC Genome Browser (http://genome.cse.ucsc.edu, v16). All cultures used for DNA isolation were grown from single colonies after plating out. DNA was obtained from 2.5 ml of L-broth cultures using a standard alkali lysis method [Sambrook et al., 1989]. DNA was labeled with digoxigenin-11-dUTP by nick translation using a commercial kit (Roche Diagnostics GMBH; www.rochediagnostics.com) following the manufacturer's instructions.

RESULTS

Normal Variation

Normal variation was determined for both probe sets using 50 P036 samples and 40 P070 samples from normal individuals. This is shown as the mean and standard deviation (SD) of RPH for all probes (Fig. 1a and b). Although considerable effort was made to select nonpolymorphic probe targets located within 1 Mb of each telomere, several probes still showed considerable normal variation. In particular, those for 2p, 20p, 22p*, 4q, 21q, and 22q (P036) and 6p, 8p, 10p, 11p, 15p*, 16p, 17p, 18p, 19p, 22p*, and 4q (P070) (see Materials and Methods for an explanation regarding the asterisks), all show coefficients of variation (CV) of 20% or more. The intrasample probe variation, determined using five replicates of five normal samples, showed an average CV of 7.3%. The CV for the subtelomeric region RPH in the P036 and P070 probe sets showed averages of 13.9% and 14.8%, respectively. Identifications of clinically significant copy number changes must take this polymorphic variation into consideration. By taking an empirical approach based on collating all the data for deletions (n = 33; 21 and 12 for P036 and P070, respectively) and duplications (n = 30; 20 and 10 for P036 and P070, respectively) detected in this study, including the abnormal controls, we set conservative thresholds to minimize the chance of false-negative results. These are the mean (P036 and P070) RPH values of <0.70 and >1.33 for deletions and duplications, respectively (Fig. 2), and will be refined as the data sets grow. Knowledge of the normal distributions, means, and SDs for each probe in each set (Fig. 1) assists the interpretation of data points outlying the above thresholds. Since the results are interpreted as normal when either both values are normal or only one of the pair is abnormal after repeat testing, the false-negative rate is unknown.

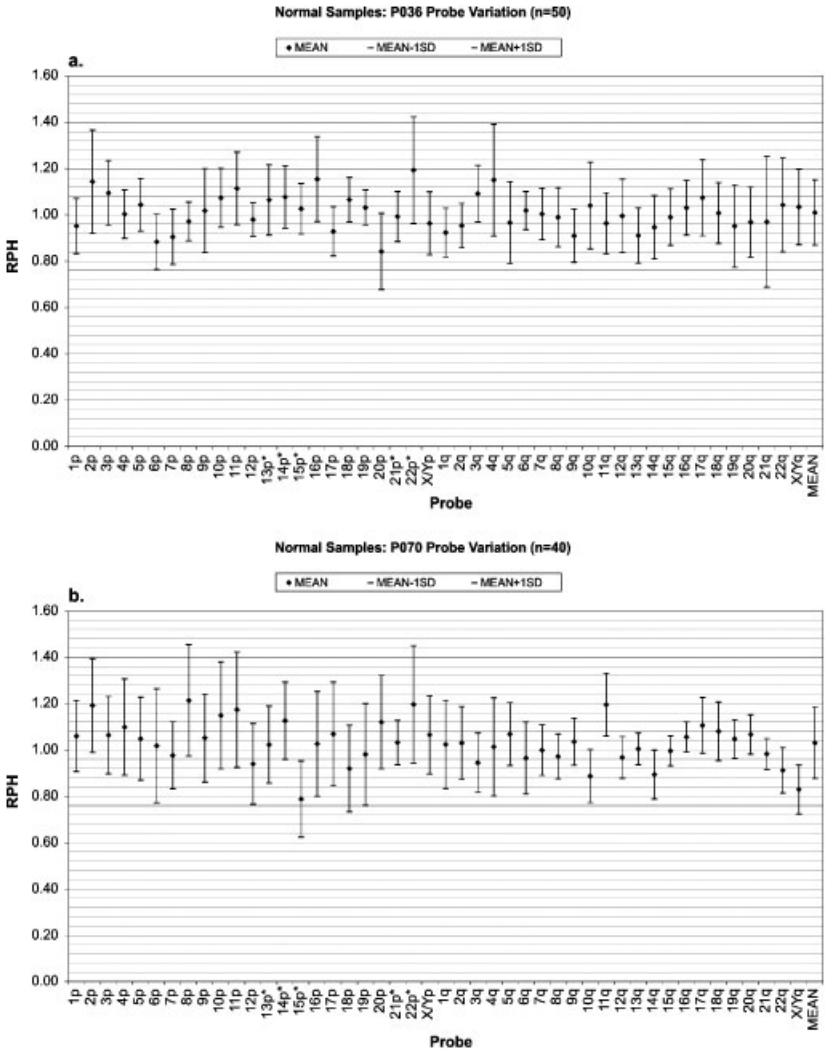

FIGURE 1. **Variation in normal individuals of RPH for each subtelomeric probe. The mean ±1 SD is shown for the (a) P036 set (n = 50) and (b) P070 set (n = 40).**

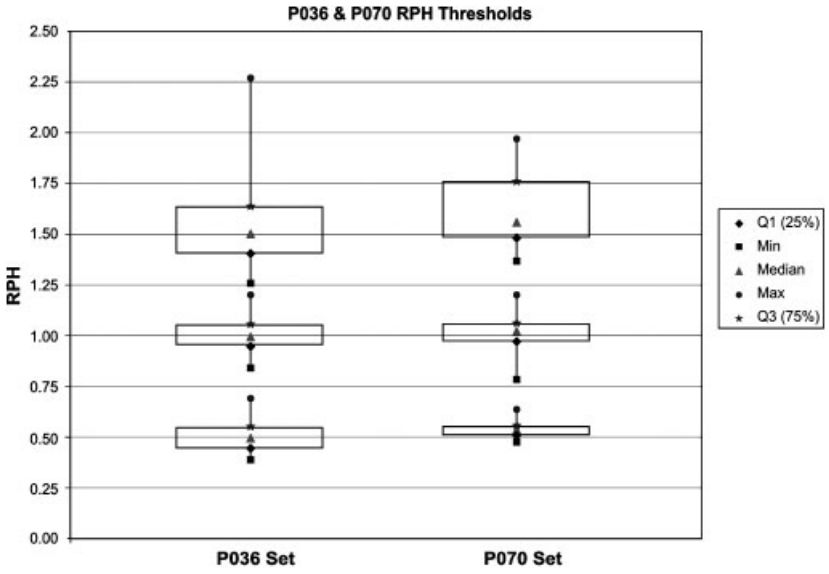

FIGURE 2. **Distributions of RPH for normal, deleted, and duplicated samples using the P036 and P070 probe sets.**

TABLE 1. **Summary of Abnormal Results**

Case	MLPA Result	RPH			Origin
"Normal" karyotype		P036	P070	Mean	
A9	Del (1p subtel)	0.46	0.57	0.52	Maternal/interchromosomal
	Dup (17p subtel)	1.53	1.97	1.75	
A40	Del (4p subtel)	0.44	0.55	0.50	De novo/intrachromosomal
A45	Del (1p subtel)	0.47	0.57	0.52	De novo/intrachromosomal
Anonymous chromatin					
B1	Dup (6p subtel)	2.02	1.73	1.88	De novo/intrachromosomal
B2	Dup (8q subtel)	1.47	1.46	1.47	De novo/intrachromosomal
B3	Del (11q subtel)	0.55	0.54	0.55	De novo/interchromosomal
	Dup (5p subtel)	1.48	1.61	1.55	
B4	Del (4q subtel)	0.54	0.38	0.46	De novo/interchromosomal
	Dup (9q subtel)	1.36	1.50	1.43	
B5	Del (3p subtel)	0.45	0.46	0.46	De novo/interchromosomal
	Dup (6q subtel)	1.43	1.43	1.43	
B6	Dup (10p subtel)	1.39	1.76	1.58	De novo/interchromosomal

The nature and origins of the abnormalities found are summarized in Table 1.

Group A Patients

MLPA analysis of Group A (n = 51; patients with strong clinical indications of a chromosome abnormality but a normal karyotype analyzed at a resolution of 600–850 bands) demonstrated that three patients had subtelomeric abnormalities. Patient A9 was referred for investigation of moderate MR with bilateral brain polymicrogyria; short, palpable fissures; and dysmorphic toes and fingers at the age of 14 months. MLPA showed a decreased RPH of 0.52 (average RPH for P036 and P070 assays) for the subtelomeric chromosome 1p probe and an increased RPH for chromosome 17p of 1.75 (Fig. 3a). These abnormalities were verified as a deletion and duplication, respectively, using FISH with BAC probes RP11-581I24 (Fig. 3b, part i) and RP11-629C19 (Fig. 3b, part ii). Using the location of the MLPA 1p probe as a starting point, further mapping (data not shown) indicated that the deletion on chromosome 1 was 5–6.6 Mb in size with a breakpoint between RP11-58I24 and RP11-262K21. The breakpoint on chromosome 17 was approximately 1.8 Mb in size between LIS1 (MIM# 601545) and RP11-806J5. A balanced reciprocal translocation involving these subtelomeric regions was subsequently demonstrated using FISH on the patient's mother, indicating that A9 inherited only the derivative chromosome 1 in an unbalanced karyotype.

MLPA showed abnormalities for A40 and A45 involving a single subtelomeric region. Patient A40, who was referred for investigation at the age of 3 years for microcephaly, developmental delay, and failure to thrive, showed a decreased RPH of 0.50 for the subtelomeric chromosome 4p probe (Fig. 4). This was verified as a deletion using the BAC probe RP11-784E7 (Supplementary Fig. S1; available online at www.interscience.wiley.com/jpages/1059-7794/suppmat) and a Wolf-Hirschhorn Syndrome Critical Region probe (LSI WHS, Vysis; data not shown). This abnormality is not present in either of A40's parents and is therefore de novo.

Patient A45, who was referred at the age of 16 years for investigation of cardiomegaly, osteopenia, and diabetes showed a decreased RPH of 0.52 for the subtelomeric chromosome 1p probe (Fig. 5). This was confirmed as a deletion by FISH using the subtelomeric BAC probe RP11-421C4, which is located

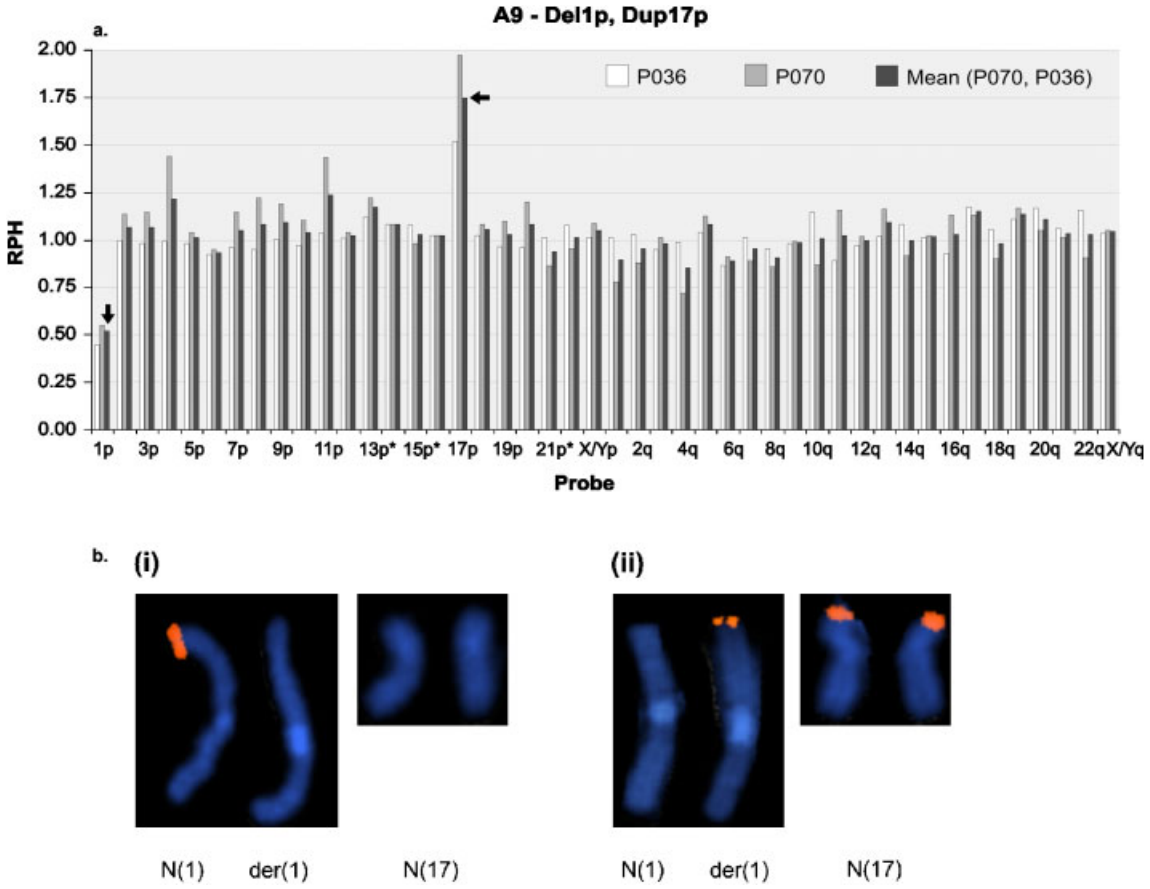

FIGURE 3. **a: Histogram of RPH for each subtelomeric probe measured in Case A9 showing deletion of 1 psubtel and duplication of 17psubtel (arrows). b: (part i) FISH showing deletion of 1psubtel using BAC probe RP11-581I24; (part ii) FISH showing duplication of 17psubtel using BAC probe RP11-629C19. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

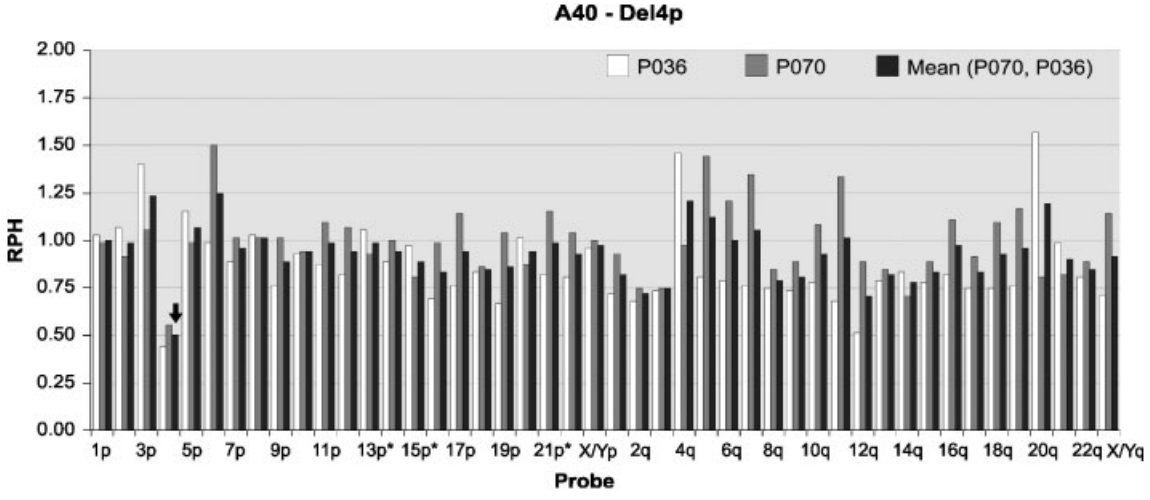

FIGURE 4. **Histogram of RPH for each subtelomeric probe measured in Case A40 showing deletion of 4psubtel (arrow).**

1.3–1.5 Mb from the short-arm telomere (Supplementary Fig. S2). This abnormality is not present in either of A45's parents and is therefore de novo. Interestingly, a commercial chromosome1p subtelomeric probe (TelVysion 1p; Vysis), which is located 3 Mb from the short-arm telomere, did not detect this deletion. Starting from the MLPA probe locus, further mapping showed the deletion to be 2.5 Mb in length with a breakpoint at BAC RP11-70N12.

A retrospective high-resolution microscopic analysis did not detect any of the abnormalities detected by MLPA in probands A9, A40, and A45.

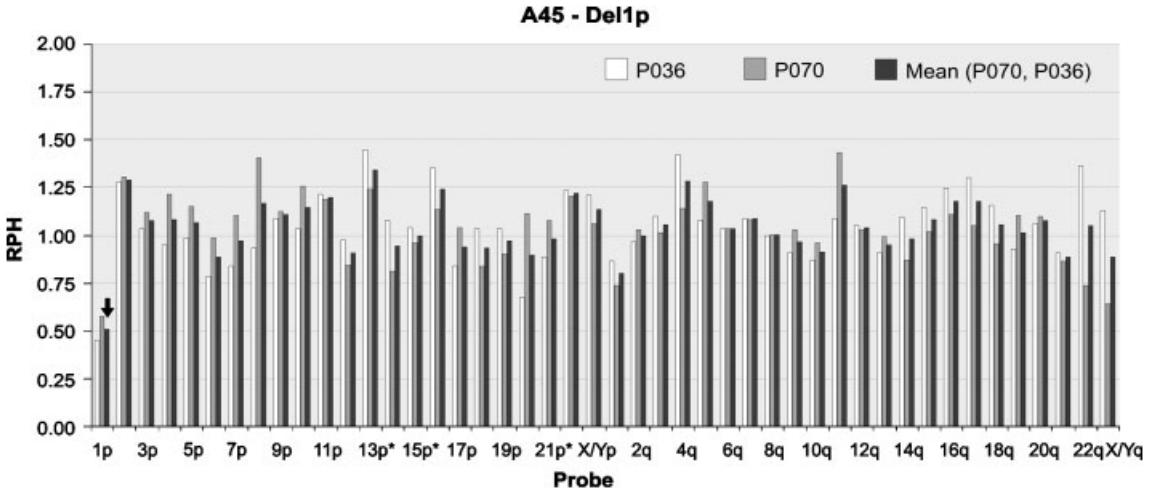

FIGURE 5. **Histogram of RPH for each subtelomeric probe measured in Case A45 showing deletion of 1psubtel (arrow).**

Group B Patients

Six Group B patients (B1–6) who showed anonymous chromatin in de novo and unbalanced karyotypes were selected for subtelomeric MLPA with the aim of identifying the segmental aneuploidies present. In each case, G-banding analysis did not identify the donor chromosome. The abnormalities revealed were of three types: intrachromosomal duplication (cases B1 and B2), interchromosomal reciprocal translocation (cases B3–5), and interchromosomal, nonreciprocal translocation (case B6). Data are presented for one of each.

Case B1, a 16-year-old male who was referred for intellectual disability, showed a karyotype with a single abnormal chromosome (Fig. 6a). One chromosome 6 showed additional unidentified chromatin at band p25. MLPA showed an RPH of 1.88 for the subtelomeric region of 6p (Fig. 6b). Chromosome painting covered the entire abnormal chromosome 6 (Supplementary Fig. S3). This is consistent with the interpretation that the unidentified chromatin originated from the terminal region of 6p (i.e., this region was duplicated). Case B2 was a 29-year-old female with intellectual disability with atrial-septal and renal defects, who showed a similar abnormality with a mean RPH of 1.47, verified as an intrachromosomal duplication of a chromosome region including 8qsubtel (Table 1).

The karyotypes in cases B3, B4, and B5 also contained a single chromosome abnormality with unidentified additional chromatin. The regions involved were 11q (Supplementary Fig. S4a), 4q, and 3p, respectively. In each of these cases however, MLPA profiles contained one peak with a decreased RPH and one peak with an increased RPH. Case B3, a 9-year-old boy with developmental delay, epilepsy, and mild dysmorphic features showed a decreased subtelomeric copy number of 0.55 for 11q and an increased copy number of 1.55 for 5p (Fig. 7). FISH using BAC clone probes (RP11-27H17 for 11qsubtel (Supplementary Fig. S4a) and D5S23, D5S721(Vysis) for 5psubtel (Supplementary Fig. S4b) confirmed these copy number changes as a deletion and duplication, respectively, resulting from interchromosomal translocation. Cases B4 (a dysmorphic neonate referred for suspicion of trisomy 18) and B5 (a dysmorphic neonate with signs of intrauterine growth retardation at birth, short neck, hypospadias, and camptodactyly) showed similar interchromosomal abnormalities with decreased subtelomeric copy number for chromosomes 4q and 3p, respectively, and increased copy number for chromosomes 9q and 6q, respectively (Table 1). FISH using a probe for ABL (Vysis; data not shown) confirmed the translocation between chromosome region 9qsubtel and 4qsubtel in case B4. FISH using BAC clones RP11-465F15 and RP11 confirmed the translocation between 3psubtel and 6qsubtel-125M18 in case B5 (data not shown). The recipient chromosome (i.e., the one identified as abnormal in the karyotype) corresponded with the deleted chromosome in all cases.

Case B6 was a 2-year-old female with mild developmental delay and mild facial dysmorphism whose karyotype (Fig. 8a) contained an abnormal chromosome 7 with unidentified additional chromatin located at the end of the short arm. MLPA showed an increased copy number of 1.58 for subtelomeric region 10p (Fig. 8b). This was confirmed as a "duplication through translocation" using the subtelomeric BAC RP11-709J19 (Supplementary Fig. S5a). In contrast to cases B3–B5, the recipient chromosome, identified as an abnormal chromosome 7p in the karyotype, showed no decreased copy number, which suggests that there was no reciprocal exchange of chromatin in the original translocation. FISH using the subtelomeric BAC RP11-23D23 for chromosome 7p (Supplementary Fig. S5b) confirmed this finding. The borderline low-mean RPH value for 4qsubtel was not confirmed as an abnormality.

DISCUSSION

The alignment of cytogenetic and physical maps in the human genome database has prompted a revolution in clinical cytogenetics. It is now possible to describe a chromosome duplication/deletion or rearrangement in genomic terms to indicate the location of breakpoints, the sequences involved, and the identity of deleted, duplicated, or disrupted genes. It is hoped that as we learn more about the roles specific genes play in pathology, significant clinical benefits in diagnosis, prognosis, and treatment will ensue. This goal is driving the development, selection, and assessment of molecular techniques that will be suitable for use in routine diagnostic settings. One particularly powerful and flexible technique is MLPA, which can detect copy number changes in any unique sequence of greater than approximately 50 nucleotides with high sensitivity. More than 40 exons or loci can be assayed in a single multiplex assay, and these can be located within single or multiple genes [Gille et al., 2002; Slater et al., 2004], dispersed

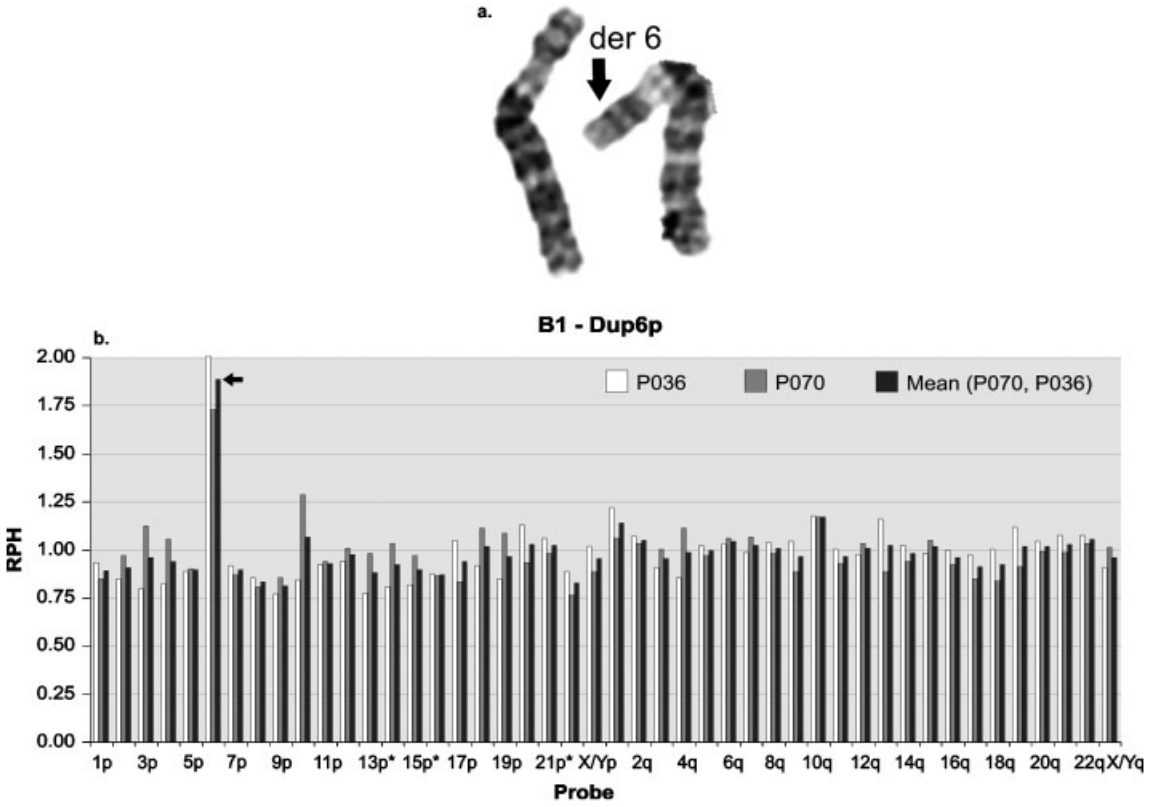

FIGURE 6. **a:** G-banded normal and derivative chromosome 6 homologs. **b:** Histogram of RPH for each subtelomeric probe measured in Case B1 showing duplication of 6psubtel (arrow).

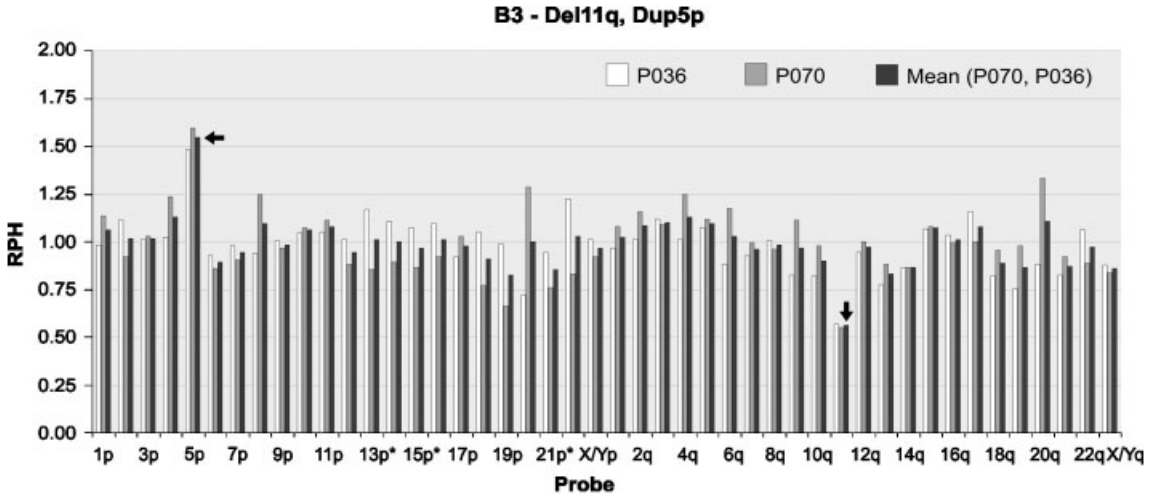

FIGURE 7. **Histogram of RPH for each subtelomeric probe measured in Case B3 showing deletion of 11qsubtel and duplication of 5psubtel (arrows).**

across the genome in subtelomeric regions [Koolen et al., 2004; Rooms et al., 2004], or across groups of chromosomes [Slater et al., 2003].

Two previous studies of patients with MR [Koolen et al., 2004; Rooms et al., 2004] have demonstrated the potential of subtelomeric MLPA. The first of these detected five subtelomeric aberrations in 75 patients using the SALSA P019/P020 probe set. One deletion was not confirmed due to inadequate sample and another was a false-positive due to a 3-basepair (bp) deletion at the relevant probe binding site. The second study detected 14 subtelomeric aberrations in 210 patients using the SALSA P036 set. Four of these were also found in a phenotypically normal parent, and one duplication was not confirmed on retesting. Both of these studies used early-generation probe sets, which were subsequently found to contain too many polymorphic probe targets. Neither study used quantitative data analysis.

We assessed a quantitative subtelomeric MLPA technique that uses complementary SALSA P036 and P070 sets of probes to target exon sequences in genes located near the ends of each

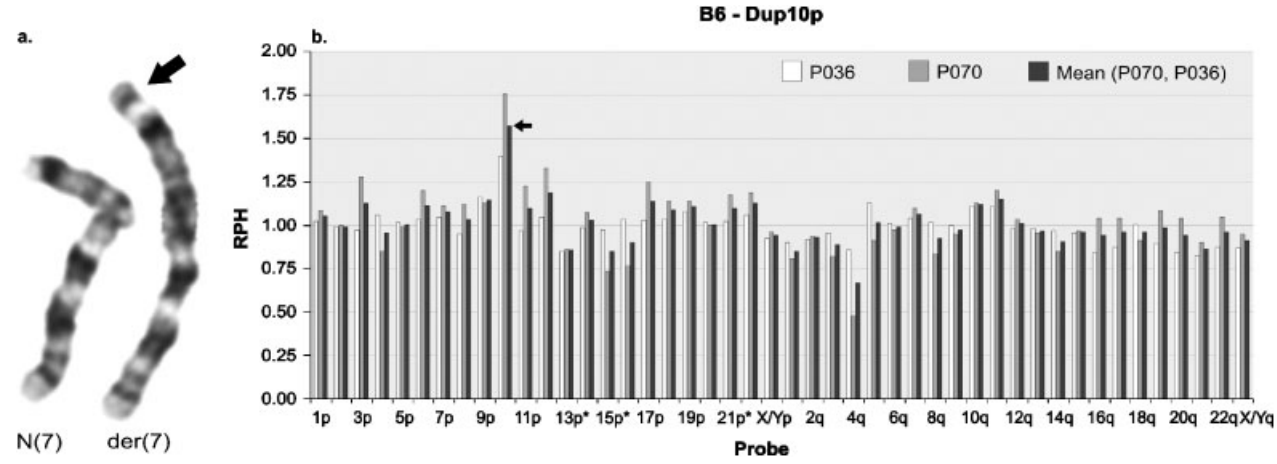

FIGURE 8. **a: G-banded normal and derivative chromosome 7 homologs. b: Histogram of RPH for each subtelomeric probe measured in patient B6 showing duplication of 10psubtel (arrow).**

human chromosome. We used the assay in two new applications. First, we requested clinical geneticists who refer patients to our laboratory to recall any patient who was considered very likely to have a chromosome abnormality but previously showed a normal, high-resolution karyotype. Typically, these patients have multiple congenital abnormalities suggestive of contiguous gene syndromes caused by segmental aneuploidy. This definition does not exclude any patient with normal IQ or mild MR, and is therefore broader than the moderate-to-severe MR groups studied previously [Koolen et al., 2004; Rooms et al., 2004].

Of the 51 patients tested (Group A), three showed subtelomeric copy number abnormalities. These demonstrated two of the three possibilities of simple deletion, simple duplication, or combined deletion/duplication.

Cases A40 and A45 showed simple deletions. Case A40 was a de novo intrachromosomal deletion of chromosome 4pter in a patient whose phenotype did not suggest Wolf-Hirschorn (MIM# 194190)/Pitt-Rogers-Danks syndrome (MIM# 262350). Case A45 showed a de novo 2.5 Mb intrachromosomal deletion of 1 pter in a patient with features suggestive of Cantu syndrome (MIM# 114620), which as yet has no assigned locus. The phenotype had little similarity to that of chromosome 1p36 deletion syndrome (MIM# 607872) [Heilstedt et al., 2003a, 2003b].

The combined deletion and duplication in case A9 is the result of the transmission of only one of the derivatives from the carrier mother, who has the balanced form of this cryptic translocation. The deleted region on chromosome 1p is 5–6.6 Mb, and the duplicate region on chromosome 17p is 1.8 Mb in size. These segmental aneuploidies contain approximately 150 and 50 known or hypothetical genes, respectively, and therefore would have a high likelihood of causing the observed phenotypic abnormality. Furthermore, finding this translocation is of crucial importance for future pregnancies, in which the risk of transmission of such small genome imbalances is very high.

Although it has been suggested that a careful, high-resolution microscopic analysis of chromosome ends will reveal the majority of subtelomeric abnormalities [Joyce et al., 2001], cases A9, A40, and A45 were not detectable on informed review, which suggests that the incidence of similar abnormalities is underestimated.

The incidence of subtelomeric abnormalities in this group justifies a review of any similar patients without a convincing etiological diagnosis. Importantly, the relevant MLPA probe locations give a starting point for mapping, as was done for patients A9 and A45. This is necessary for defining new syndromic loci or investigating phenotypic disparity in syndromes with established loci (as in cases A45 and A40, respectively).

Second, we used MLPA to identify anonymous chromatin in a group of six patients (Group B) with a single, incompletely characterized abnormal chromosome. Often these chromosomes are inherited from a parent with a balanced reciprocal translocation, and the donor chromosome can be identified from the parent's karyotype. However, this approach was not informative in this group, since all were de novo–presumably the result of a parental germ-line rearrangement. MLPA revealed a variety of abnormalities. Cases B1 and B2 showed additional chromatin derived from segmental, intrachromosomal duplication. Cases B3, B4, and B5 all showed that the additional chromatin was derived from interchromosomal translocation, and the donor chromosomes were identified. Furthermore, there was deletion of the subtelomeric region in the recipient chromosome in each case. This suggests that all of these abnormal chromosomes are derivatives of reciprocal translocations, which increases the risk of recurrence in any future pregnancies. Case B6 showed additional chromatin derived from interchromosomal translocation, and the donor chromosome was identified; however, in this case there was no apparent deletion of the subtelomeric region in the recipient chromosome. Again, MLPA provides a starting point for mapping the extent of the duplication/deletion involved.

Many studies have aimed to develop sets of subtelomeric probes that are nonpolymorphic but are located as near the telomeres as possible to minimize false-negative results. However, the inherent polymorphic copy number variation in several subtelomeric regions has frustrated these attempts, and the P036 and P070 sets still include several probes that show excessive normal variation (CV > 20%, Fig. 1). It may not be possible to develop a completely nonpolymorphic set of probes. Rather, a balance will probably have to be made between minimizing the chance of false negatives by using probes close to the telomere (i.e., within 0.5 Mb) and accepting the detection of low-frequency polymorphisms.

An analysis of a sample of our data (15 assays containing 44 peaks each) showed that 25 out of 660 P036 peak measurements (3.8%) and 29 out of 660 P070 peak measurements (4.4%) fall into our defined abnormal range (RPH < 0.70, RPH > 1.33). In contrast, for each individual subtelomeric region, only two out of 660 averaged peak measurements (0.3%) fall into the abnormal range. Thus, the strategy of using two sets of

TABLE 2. Comparison of Sub-telomeric Assay Techniques

Technique	All subtelomeric multiplex[a]	Samples run	Turnaround time (days)	Estimated relative cost[b]	Resolution (kb)[c]
MLPA	Yes	1–96	2	Low	~0.1
MAPH	Yes	1	2	Low	~0.1
QF-PCR	No	1–96	1	Low	~0.1
Multiprobe-FISH	Yes	1	1	High	~2
Metaphase CGH	Yes	1	2–3	High	~3000
BAC/CGH microarray	Yes	1	2–3	High	~50
Oligonucleotide microarray	Yes	1	2	High	~0.1
Genetic marker	No	1–96	1	Low	~0.1

[a]A single multiplex needs to cover all 39 unique subtelomeric regions.
[b]The cost estimate reflects that for consumables and the capacity for multisample testing within runs.
[c]Resolution is dependent on the size of the probe/primer and the potential density of separate, specific targets for each technique.

complementary probe sets largely overcomes the polymorphism problem, but caution has to be applied in distinguishing polymorphic from nonpolymorphic copy number changes. The borderline value for 4qsubtel in Case B6 (Fig. 8b) is an example of this, and verification of any apparent abnormalities with a specific FISH or quantitative PCR method is necessary. Testing parental samples to determine inheritance is also informative in this context. This subtelomeric MLPA is therefore a screening test rather than a stand-alone diagnostic test.

All of the subtelomeric assay techniques used in MR studies were recently reviewed [Rooms et al., 2005]. A comparison of MLPA with the other techniques is shown in Table 2. With the exception of the polymorphic genetic markers and quantitative real-time PCR (QF-PCR), all can be multiplexed in one test to cover all 39 unique subtelomeric regions. These techniques fall into one of two categories: those that are PCR-based, and those that are hybridization-based. The sensitivity of the techniques is affected by the size of the probes or primers used. Metaphase CGH has a resolution no higher than 3 Mb and is being superseded by microarray CGH, which is at least an order of magnitude higher in sensitivity. Short oligonucleotides of unique sequence are much less likely to give false-negative results than the much larger hybridization probes, which are actually collections of overlapping 100–400 bp probes. The technical complexity of the hybridization-based techniques and their higher relative costs present barriers to their widespread use. The PCR-based technique using genetic markers relies on polymorphisms, and multiple, closely linked loci are used to achieve near 100% informativeness. MLPA, on the other hand, targets nonpolymorphic loci and requires far fewer targets. In summary, the combination of robust multiplexing and high throughput makes MLPA a particularly useful technique for sophisticated cytogenetic analysis.

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