RESEARCH ARTICLE

MLPA Screening Reveals Novel Subtelomeric Rearrangements in Holoprosencephaly

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Communicated by Jean-Louis Mandel

Holoprosencephaly (HPE) is the most common developmental brain anomaly in human, associated with a wide spectrum of presentations. The etiology is heterogeneous, due to environmental and genetic factors. Out of 12 cytogenetic candidate loci previously reported, eight were subtelomeric, including the loci in which two of the four major HPE genes were identified (SHH and TGIF). Recently, we reported that these two genes could be mutated or microdeleted. Therefore, we hypothesized that subtelomeres screening in HPE patients could refine the known subtelomeric candidate loci and identify novel ones. In this study, 181 samples, 72 fetuses and 109 live-born infants, with HPE and a normal karyotype, and 10 patients deleted for SHH or TGIF (3.5 Mb from subtelomeres) were screened for subtelomeric rearrangements using the multiplex ligation probe-dependent amplification (MLPA) method with two kits. Quantitative PCR was performed when discrepancies were observed between these two kits. We found that known SHH and TGIF microdeletions on 7q and 18p, encompassed their subtelomeric region (3.5 Mb) and were often associated with cryptic gains. Out of the 181 samples, we detected rearrangements in known candidate HPE loci (1q, 20p, and 21q) as well as in other novel subtelomeric locations (1p, 5q, 8p, 17q, 18q, 22q, and Xq) and in the subcentromeric 15q. We also found associations between cryptic subtelomeric gain and loss that may be inherited from a parental balanced translocation, which is helpful for genetic counseling. These findings reinforce the multihit origin for HPE and contribute to the explanation of the wide phenotypic spectrum described in this developmental disorder. Hum Mutat 0, 1–9, 2007. © 2007 Wiley-Liss, Inc.

KEY WORDS: holoprosencephaly; subtelomeres; MLPA; FISH; quantitative PCR

INTRODUCTION

Holoprosencephaly (HPE; MIM# 236100) is the most common forebrain developmental anomaly in humans. This phenotype results from a partial or complete failure of the forebrain to divide into two separate hemispheres and ventricles. The HPE clinical spectrum of central nervous system abnormalities ranges from alobar (single median ventricle) to semilobar (incomplete separation of two ventricles), and lobar (complete separation but continuity across the cortex). These cerebral defects are often associated with facial anomalies whose severity generally correlates with brain defects [DeMyer et al., 1964] including cyclopia, anophthalmia, hypotelorism, cleft palate, midline cleft lip, or palate, flat nose, and single central maxillary incisor, sometimes without mental retardation.

Cytogenetic defects in HPE live births led to the identification of 12 HPE candidate loci [Roessler and Muenke, 1998]. Based on the minimal critical regions, four major HPE genes have been identified: HPE3 (7q36) (Sonic Hedgehog, SHH; MIM# 600725) [Belloni et al., 1996; Dubourg et al., 2004; Roessler et al., 1996]; HPE5 (13q32) (ZIC2; MIM# 603073) [Brown et al., 1998, 2001];

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

Received 17 July 2006; accepted revised manuscript 5 June 2007.

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Grant sponsors: Groupement d’Intérêt Scientifique (GIS) Institut des Maladies rares; Projet Hospitalier de Recherche Clinique (PHRC) Région Bretagne.

DOI 10.1002/humu.20594
Published online in Wiley InterScience (www.interscience.wiley.com).
HPE2 (2p21) (SIX3; MIM# 603714) [Laflamme et al., 2004; Pasquier et al., 2000; Wallis et al., 1999], and HPE4 (18p11.3) (TGIF; MIM# 602630) [Aguiella et al., 2003; Gripp et al., 2000]. However, no candidate gene has been identified from the eight remaining candidate loci: HPE1 (21q22.3), HPE6 (3p24–pter), HPE7 (13q12–q14), HPE8 (14q13), HPE9 (20p13), HPE10 (1q42–qter), HPE11 (5pter), and HPE12 (6q26–qter).

Resulting from these findings, HPE molecular diagnosis was first based on the sequencing of the four main HPE genes (SHH, SIX3, ZIC2, and TGIF). More recently, we demonstrated that testing these four genes for microdeletions (fluorescent in situ hybridization [FISH], real-time quantitative PCR, and quantitative multiplex PCR of short fluorescent fragments [QMPSF]) was also an efficient approach in the evaluation of HPE patients [Benavidez et al., 2006a, 2006b].

To date, HPE etiologies can be summarized as follows: approximately 45% were chromosomal HPE, diagnosed on standard karyotype; up to 15% were related to evident environmental factors (maternal diabetes during early pregnancy) or seen in multiple malformation syndromes (25 HPE-associated syndromes, including Smith-Lemli-Opitz, Rubinstein-Taybi, and Pseudotrisomy 13 [Muenke and Beachy, 2001]). Out of the 40% remaining HPE cases, called isolated HPE (nonchromosomal and nonsyndromic), sequencing and gene dosage on the four main HPE genes identified molecular anomalies in up to 25%. Consequently, still 75% of isolated HPE have a genetic and/or environmental etiology to be determined.

Our group focuses on the genetic etiologies of isolated HPE, based on a large collection of DNA samples located in Rennes, France, and our strategy aims at identifying or refining the size of chromosomal loci that could contain new HPE candidate genes.

The present study was based on the observation of previous data [Roessler and Muenke, 1998] showing that 8 out of 12 HPE candidate loci were found on recurrent karyotype anomalies that included subtelomeres, out of which SHH in 7q36 and TGIF in 18p11.3 were identified. Moreover subtelomeric microarrangements have been implicated in developmental disorders such as mental retardation [Koolen et al., 2004]. Consequently, we hypothesized that subtelomeric screening of HPE patient samples with normal karyotype could identify microarrangements in the eight subtelomeric HPE loci previously described or in other subtelomeric regions.

Until 2004, the only commercially available technique to perform this screening was subtelomeric FISH analysis. However, this approach required sequential testing of subtelomeres and was expensive and time consuming; moreover, it required the availability of live cells from the patients (e.g., fresh blood, cultured cells, or immortalized cells) to prepare chromosome spreads. Recently, another PCR-based method, multiplex ligation-dependent probe amplification (MLPA), was successfully used in several studies to identify subtelomeric rearrangements [Koolen et al., 2004; Northrop et al., 2005]. The “selective adaptor ligation, selective amplification” (SALSA) MLPA kit applied to subtelomeric studies allows simultaneous testing of all the subtelomeres, requires small quantities of genomic DNA, and thus is easier and less expensive than FISH.

We describe here the first study that systematically screened DNA samples for subtelomeric gains or losses in HPE patients. We used a combination of two SALSA MLPA kits, P036B and P070 “human telomeres” from MRC Holland with 6-FAM labeled probes. Both kits P036B and P070 were used concomitantly to detect deletions or gains. The two MLPA mixes contained probes for each subtelomeric region except for the short arms of the acrocentric chromosomes. For the latter, target sequences were localized on the subcentromeric region of the q arm, in one of the first genes located close to the centromere. We chose to screen the patients with these two kits because they both analyze the same subtelomeric regions but with different subtelomeric targets. Consequently, we hypothesized that any subtelomeric deletion or gain found on both kits could be considered as confirmed.

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We used the MLPA DNA detection and quantification protocol from MRC-Holland [www.mlpa.com], as specified in the kit. Briefly, all experiments started with 100 ng of DNA sample. Ligation and PCR reactions were realized on a PTC-200 thermocycler (MJ-Research, Watertown, MA). A total of 10 µl of the ligation product was PCR-amplified with 35 cycles. Electrophoresis of the amplification product was performed on a 16 capillaries ABI-3130xl Genetic Analyzer with ROX-500 internal size standard and POP7 polymer (Applied Biosystems, Foster City, CA).
MLPA Analysis

Each MLPA experiment tested 16 samples including patients and normal male pools used as a reference in the analysis process. All the 16 electrophoresis runs obtained from the capillary analyzer (.fsa files) were imported in GeneMarker Software (Softgenetics LLC, State College, PA). This software allowed visualizing, normalizing and comparing the electrophoretic profiles based on size standard and signal strength. Referring to patient gender, 46 peaks were obtained for females (44 autosomal peaks and two peaks corresponding to the pseudo-autosomal regions of X and Y chromosomes) and 48 peaks for males (adding two peaks from the Yp arm) (Fig. 1).

Finally, for each sample tested, the software superimposed the normalized peak profiles of a patient and of the male pool reference and compared the peak sizes, calculating a ratio. The resulting ratios were approximately 1.0 for every wild-type peak, 0.5 for heterozygous deletions, and 1.5 for heterozygous gains.

Confirmation Experiments

Subtelomeric FISH. When the results from MLPA kit P036B and P070 were different, FISH analysis using sets of Vysis probes (Vysis, Downers Grove, IL) was carried out if a cell line was available. Fixed chromosomes suspensions were prepared from cultured peripheral blood lymphocytes. Slide preparation and hybridization were performed using a standard protocol. A Leica DRMXX2 fluorescence microscope (Leica Microsystems, Wetzlar, Germany), equipped with appropriate filters, was used for visual examination of the slides. Images were captured using a CoolSnap ES camera (Roper Scientific GmbH, Ottobrunn, Germany) and analyzed with Metaview and Metamorph software (Molecular Devices Ltd, Wokingham, United Kingdom). For two positive samples, cultured cell lines of the parents were analyzed, to confirm the cytogenetic mechanism hypothesized on the defects identified in the proband (Fig. 2).

Duplex quantitative PCR. When live cells were not available for chromosomal preparation and FISH analysis, we tested DNA with duplex quantitative PCR [De Rosa et al., 1999; Pastore et al., 1996] to resolve discrepant results found with the two MLPA kits. We designed several duplex PCR assays using primers for an endogenous control gene, HMBS, and for the subtelomeric target gene showing a gain or loss with one of the MLPA kits in order to confirm its CNV. Sequences flanking the MLPA probes hybridization loci were selected using the University of California, Santa Cruz (UCSC) Genome Browser (http://www.genome.ucsc.edu/) primers pairs were chosen on DNA sequences presenting with no SNPs, no repeated sequences (RepeatMasker: http://www.repeatmasker.org/) and no local similarity with other human genomic sequences (BLAST: http://www.ncbi.nlm.nih.gov/BLAST/). Primers were designed using Primer premier software (Premier Biosoft International, Palo Alto, CA) (Supplementary Table S1; available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat). All genes were coamplified with the HMBS primer pair. Duplex PCR were performed in a 25-µl reaction mixture containing 10% DMSO, 0.2 mM each dNTP 1.5 mM MgCl₂, 1.5 U DNA Taq polymerase, 3.5–7.5 pmol of each primer, and 100 ng genomic DNA. Using a PTC-200 (MJ-Research), PCR cycling conditions were an initial step of denaturation at 94°C for 5 min, followed by 24 cycles of denaturation at 94°C for 10 sec, annealing at 55°C for 15 sec, and extension at 72°C for 20 sec, and then a final step of extension at 72°C for 5 min.

The same duplex PCR mix was applied to normal control DNA pool and HPE patient DNA. PCR products were run on a 3% agarose gel containing SYBR-safe dye (Invitrogen Corporation, Carlsbad, CA). Gel imaging was realized with Biocapt 11.01 for

FIGURE 1. The two charts represent MLPA results for female samples (the two Y-specific probes are not amplified). The first chart represents a normal result, all subtelomeric probes having a ratio close to 1.0 when compared to normal pools results. The second chart revealed a 7q telomere deletion (ratio = 0.5) associated with a gain on one 7p telomere (ratio = 1.5).
Windows™ software (Vilber Lourmat, Eberhardzell, Germany), avoiding signal saturation. Pictures were subsequently analyzed using Image Quant 5.2 software (Molecular Dynamics, Sunnyvale, CA) to determine peak sizes for each PCR fragments. Using normal control DNA, PCR primer amounts were modified so that the two PCR fragments had equivalent intensities on the gel (same reaction kinetics). Patients and control DNA PCR peak sizes were normalized based on HMBS peak size. For one target gene, the ratio of patient normalized peak size above control DNA normalized peak size was calculated. Ratios below 0.75 or above 1.25 confirmed respectively deletion or gain.

7q and 7p breakpoint determination. Patients with known SHH microdeletion in 7q36 and confirmed to be deleted in 7q telomere with MLPA were further screened by semiquantitative duplex PCR to detect the breakpoint location toward the centromeric direction. We selected different genes regularly spaced toward the centromeric direction and starting from SHH: DPP6, ARP3B (HUGO-approved symbol ACTR3B), XRCC2, MLL3, PRKAG2, CUL1, and ARHGEF5. Panels of duplex semiquantitative PCR were designed to localize the breakpoint locus as described above. The same approach was done to delineate two 7p gains identified in this study; four genes were screened from the telomere toward the centromeric direction: CARD11, PSCD3, GLCCI1, and PHF14 (Fig. 3).

RESULTS

The 10 positive controls were already known to have a deletion of SHH or TGIF, and we hypothesized that they could be used as “positive” controls for subtelomeric deletions: as the two deleted genes were located 3.5 Mb far from their respective telomeres, we postulated that the deletion would encompass the whole subtelomeric region including these two genes; accordingly, all the 10 expected deletions were correctly identified by subtelomeric MLPA. This technique revealed also an associated gain in another subtelomeric region in five of these 10 controls (Table 1).

Subtelomeric aberrations were also detected in eight patients out of the 181 cases with no known anomaly (4.4%), showing either a single anomaly or an association between a deletion and a gain; additionally, these rearrangements were very heterogeneous, encompassing 10 different subtelomeric regions (Table 2).

Analysis of Normal Controls

We analyzed 100 DNA samples from healthy volunteers with MLPA kits P070 and P036B. Out of these results, we detected two anomalies with both kits: a gain in 11p subtelomere and a loss in 3p subtelomere. We also found a gain in 15q subtelomere for two patients only with the P070 kit, and a gain in Xp subtelomere gain for two patients only with the P036B kit; these 15q and Xp gains were confirmed by quantitative PCR.

We compared our results with the TCAG Database of Genomic Variants (The Center for Applied Genomics, Toronto, ON, Canada; http://projects.tcag.ca/variation) that compiled data from genome variation studies in healthy normal volunteers [Iafrate et al., 2004; Locke et al., 2006; Redon et al., 2006; Sebat et al., 2004; Sharp et al., 2005; Wong et al., 2007]. No CNV encompassing 11p subtelomeric probes have been reported to date in the database. The 3p loss found with the two MLPA probes (P036B and P070 kits) was described as a CNV region (18 gains and one loss out of 270 controls). CNV also overlapped the sequence of the 15q probes of the P070 kit (gene ALDH1A3); nevertheless the database reported three losses among 269 controls, but no gain as in our normal controls. We also found two gains in Xp subtelomere for SHOX gene (kit P036B) with no reported CNV. On the opposite PPP2R3B gene (Xp locus in kit P070) was normal in our control group whereas the database reported gains (3 out of 270 controls).

Analysis of Positive Controls

First, we tested the 10 positive controls known to have SHH or TGIF deletions, two HPE genes located in 7q36.3 and 18p11.3,
respectively, and 3.5 Mb far from their respective telomeres. The MLPA probes, tested in 7q and 18p, were located 0.2 Mb from these telomeres but were also deleted, demonstrating that the deletion encompassed the region located between the HPE gene and the relevant gene targeted by MLPA probe (approximately 3.5 Mb).

TABLE 1. Rearrangements and Phenotypes Observed in 10 Patients With SHH or TGIF Known Deletions

<table>
<thead>
<tr>
<th>Known deletion</th>
<th>HPE locus</th>
<th>Rearrangement</th>
<th>HPE phenotype</th>
<th>Fetus (F) or live-born infant (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHH del7q32-qter</td>
<td>HPE 3</td>
<td>del 7q</td>
<td>Alobar</td>
<td>F</td>
</tr>
<tr>
<td>SHH microdeletion</td>
<td>HPE 3</td>
<td>del 7q</td>
<td>Alobar</td>
<td>L</td>
</tr>
<tr>
<td>SHH microdeletion</td>
<td>HPE 3</td>
<td>del 7q</td>
<td>Semilobar</td>
<td>L</td>
</tr>
<tr>
<td>SHH microdeletion</td>
<td>HPE 3</td>
<td>del 7q/dup7p</td>
<td>?</td>
<td>L</td>
</tr>
<tr>
<td>SHH microdeletion</td>
<td>HPE 3</td>
<td>del 7q/dup8p</td>
<td>Alobar</td>
<td>F</td>
</tr>
<tr>
<td>SHH microdeletion</td>
<td>HPE 3</td>
<td>del 7q/dup8p</td>
<td>Semilobar</td>
<td>F</td>
</tr>
<tr>
<td>SHH microdeletion</td>
<td>HPE 3</td>
<td>del 7q/dup1q</td>
<td>?</td>
<td>F</td>
</tr>
<tr>
<td>SHH microdeletion</td>
<td>HPE 3</td>
<td>del 18p</td>
<td>Alobar</td>
<td>F</td>
</tr>
<tr>
<td>TGIF del18p</td>
<td>HPE 4</td>
<td>del 18p</td>
<td>Microform</td>
<td>L</td>
</tr>
</tbody>
</table>

TABLE 2. Rearrangements and Phenotypes Observed in Eight Patients With No Previously Known Abnormalities Before MLPA Subtelomeric Study

<table>
<thead>
<tr>
<th>HPE locus</th>
<th>Rearrangement</th>
<th>HPE phenotype</th>
<th>Fetus (F) or live-born infant (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPE 10</td>
<td>dup1q</td>
<td>Familial recurrence of HPE</td>
<td>L</td>
</tr>
<tr>
<td>HPE 1/HPE 9</td>
<td>del21q/dup20p</td>
<td>Familial, alobar HPE</td>
<td>F</td>
</tr>
<tr>
<td>Unknown</td>
<td>del5q/dup17q</td>
<td>Isolated, alobar HPE</td>
<td>L</td>
</tr>
<tr>
<td>Unknown</td>
<td>dup15q subcentromeric</td>
<td>Isolated, alobar HPE</td>
<td>F</td>
</tr>
<tr>
<td>Unknown</td>
<td>del18q/dupXq</td>
<td>Microform: absence of corpus callosum, facial anomalies</td>
<td>L</td>
</tr>
<tr>
<td>Unknown</td>
<td>del1p</td>
<td>Familial history of HPE; microform: cleft lip and palate, absence of corpus callosum, mental retardation</td>
<td>L</td>
</tr>
<tr>
<td>Unknown</td>
<td>del1p</td>
<td>Microform: single central maxillary incisor, choanal atresia, mental retardation</td>
<td>L</td>
</tr>
<tr>
<td>Unknown</td>
<td>del22q</td>
<td>Microform: septal agenesis, absence of corpus callosum, midline clefting, diabetes insipidus, mental retardation, seizures</td>
<td>L</td>
</tr>
</tbody>
</table>

FIGURE 3. Semiquantitative PCR and localization of the 7q and 7p breakpoints. a: This graph represents semiquantitative duplex PCR (agarose gel electrophoresis and Image Quant 5.2 integrated electrophoretogram profiles) of CARD11 and HMBS used as endogenous control, on a normal control (C) and a patient (P) DNA. Based on integrated and normalized peak sizes the CARD11 peak was 1.5 times higher in the patient, confirming the gain in 7p22.3 including this gene. b: This graph represents semiquantitative duplex PCR (agarose gel electrophoresis and Image Quant 5.2 integrated electrophoretogram profiles) of ARP3β/ACTR3B and HMBS used as endogenous control, on a patient sample (P) and a normal DNA sample pool (C). Based on integrated peak sizes, the ARP3β peak was 0.5 times lower in the patient, confirming the deletion in 7q36.2 including this gene. c: Breakpoints determination in 7q and 7p: rearrangement location was refined for five 7q deletions and the two 7p gains and is represented by black circles on the chromosome ideogram. To determine 7q breakpoint regions, several genes starting from SHH toward the centromere direction were tested by the semiquantitative method. Most of the rearrangements are located at the telomeric end of the 7q36.1 cytoband in a hotspot covering about 1 Mb. The locus for the two 7p gains also encompassed a short region of 1.5 Mb.
Moreover, out of the eight positive controls with a 7q deletion, five had an associated gain on another telomere (Table 1): two on 7p, two on 8p, and one in 1q (HPE10 locus). Gains in 8p were detected with only one of the two MLPA kits (P036B) and we performed semiquantitative PCR to definitely confirm their presence. When both parents’ metaphases spread were available, targeted FISH was also performed and balanced translocations could be found between two parental chromosomes (Fig. 2). Further studies to delineate the size of the 7q microdeletions were carried out on five patients known to have a SHH deletion previously identified by QMPE. This allowed us to determine, in four out of five patients, a 7-Mb to 8-Mb subtelomeric deletion with a 1-Mb critical hot spot for rearrangements located in the telomeric end of cytoband 7q36.1 (Fig. 3). For the two patients with a 7p gain associated with the 7q deletion, the gain encompassed approximately 7 Mb as well, with a border located in the middle of 7p22.1 cytoband. This symmetry between gain and defect sizes and banding patterns explained the misdiagnosis in the middle of 7p22.1 cytoband. This symmetry between gain encompassed approximately 7 Mb as well, with a border located in the middle of 7q36.1 (Fig. 3). For the two patients with a 7p gain associated with the 7q deletion, the gain encompassed approximately 7 Mb as well, with a border located in the middle of 7p22.1 cytoband. This symmetry between gain and defect sizes and banding patterns explained the misdiagnosis with the karyotype.

Compared with the TCAG database, none of the two HPE genes (SHH, TGIF) are reported to have CNV in normal subjects to date. Concerning the three gains associated with a 7q deletion, MLPA probes for 1q and 7p did not overlap with CNV from normal controls, but sequences from 8p probes matched with CNV (3 gains out of 270 healthy volunteers). For this 8p gain, we realized subtelomeric FISH in the mother of a proband with 7q deletion and 8p gain and observed a balanced translocation between 7q and 8p (Fig. 2); consequently, we concluded that the 8p gain in the proband was related to an unbalanced translocation and not from an associated CNV.

Analysis of 181 HPE Cases With Normal Karyotype

Subtelomeric aberrations were detected and confirmed in 8 out of 181 (4.4%) HPE cases (Table 2). Two patients had abnormalities in three previously described HPE loci: one patient had 1q gain (HPE10) and another patient had a 21q deletion and a 20p gain (HPE1 and HPE9 loci, respectively). For the six other patients, a gain and/or a loss of chromosomal material was detected in novel subtelomeric regions, not previously reported as being a candidate HPE loci: 1) a 5q deletion associated with a 17q gain; 2) an 18q deletion associated with a 1q gain; 3) a 15 subcentromeric gain; 4, 5) an lp deletion in two unrelated patients; and 6) a 22q deletion.

Part of these anomalies was detected with only one of the two MLPA kits and we performed semiquantitative PCR to definitely confirm their presence. Three gains (15q subcentromeric, 17q, and 20p) and one loss (1p) were only detected using the P036B kit and not with the P070 kit; on the other hand, one deletion in 22q subtelomere was found only with the P070 kit.

We also found anomalies, detected by only one MLPA kit, that could not be confirmed by semiquantitative PCR, even if the sequence used for the primers design was close to the target sequence of the MLPA probe. We found four false-positive cases with kit P036B (9q gain (1 case), 16p deletion (2 cases), and 22q gain (1 case)), and one false-positive case with kit P070 (17q gain).

Comparing with the TCAG database, subtelomeres 1q, 5q, 17q, and 18q had no CNV matching with the MLPA probe sequences. Subtelomeres 20p, 21q, and 22q had rare CNV (1 gain out of 270 controls for 21q; 4 losses out of 270 controls for P070 kit probe for 20p; 1 gain out of 270 controls for P036B kit probe for 22q). On the opposite, the database contained a high rate of CNV for the 15q subcentromeric probes, with up to 228 CNV out of 270 controls (204 gains and 24 losses).

Associated Phenotypes

In the positive control group, six out of 10 patients were fetuses and the pregnancy was terminated based on prenatal diagnosis of a severe HPE (alobar or semilobar HPE). Three of the four live-born infants also had a severe HPE but the one with an 18p deletion had only HPE microforms (Table 1).

Among the eight patients with microdeletions that did not involve SHH or TGIF, the phenotypes were less severe except for two fetuses (one with alobar HPE and 15q subcentromeric gain, and another with lobar HPE and 21q deletion with 20p gain). The six other live-born infants had microform or lobar HPE (Table 2).

DISCUSSION

MLPA screening based on the P036B and P070 kits was performed on 181 HPE DNA samples and 10 "positive" controls. All 10 positive controls (six fetuses and four live-born infants, mostly with severe phenotypes), with SHH or TGIF known subtelomeric deletions, were deleted until the telomere based on the MLPA study; furthermore, five (four fetuses and one newborn) also had an associated gain previously missed using HPE genes-specific dosage methods. Among the 181 patients, eight had subtelomeric rearrangements (4.4%); they had less severe phenotypes than the positive control patients and were mostly live-born infants (6/8) rather than fetuses (2/8).

MLPA Method Results

MLPA revealed to have a low false-positive rate (Table 3). Out of the DNA samples tested in this study (normal control samples, control patients, and patients, n = 291) with the two kits, subtelomeric anomalies (n = 38) were identified in 29 patients (10%). Among these patients, five were false positive, as their rearrangements were not confirmed by quantitative PCR. To discuss false-positive results, we had to be very careful with the genomic distances between two MLPA targets when comparing the results for one subtelomere; recent studies [Iafrate et al., 2004; Redon et al., 2006; Sebat et al., 2004; Wong et al., 2007] demonstrated that CNV could involve regions encompassing no more than one gene or two; consequently, a normal result for one kit associated with a deletion or a gain in the second kit can also be the real genomic state of this subtelomere and not a false positive. Therefore, we designed the quantitative PCR as close as possible to the MLPA target that was deleted or duplicated. In final, we concluded on a low false-positive rate of 1.7% for the MLPA technique.

The possibility of large scale CNV should be considered, mostly for the new candidate loci identified in this study. The TCAG variations database summarizes human large-scale genomic variants with information about frequency and their relation to genes. Based on this CNV database, the 15q subcentromeric gain including the CYFIP1 gene could be an example of such CNV polymorphism. Finally, only the study of the recurrence of such rearrangements in HPE, and their absence in a control cohort of normal individuals, would help to differentiate a common CNV from a potential pathologic rearrangement.

That is why we purposely tested both kits on 100 DNA samples from normal volunteers and found six with subtelomeric anomalies. Two anomalies were found by the two kits; four were found by one kit only and secondarily confirmed by quantitative PCR. These regions should be considered as new CNV and added
to the database. But the most important was that none of the variations found among this normal group was found in the HPE group, and conversely, giving a higher probability for the rearrangements found in the HPE group to be involved in the pathology.

This study did not allow the determination of false-negative samples because the only commercial alternative to MLPA is subtelomeric FISH, and we did not have cell lines for most of our samples to do such a comparison. Other molecular methods could be used, such as QMPSF or microsatellites, but they are not commercially available or would need parental study.

All positive controls were correctly detected even if SHH or TGIF deleted genes were located 3.5 Mb away from their respective telomeres and subtelomeric MLPA probe loci. As this MLPA approach detected cryptic anomalies associated in five out of eight 7q36 deletions, the technique may therefore be considered as a method of choice to complete the specific dosage of HPE genes that we previously described (FISH, qPCR, or QMPSF) for a diagnostic purpose [Bendavid et al., 2006a, 2006b].

To conclude, MLPA is a robust tool, cost effective, and easier to use for large groups of patients than subtelomeric FISH in a diagnosis (or research) laboratory. Effectively, the cost of subtelomeric MLPA is 15 times less expensive than subtelomeric FISH. Furthermore, in our experience, MLPA could easily screen 13 patients' samples per run in 48 hours, whereas a screening with subtelomeric FISH needs 15 different multicolor FISH preparations per patient, making it difficult to test more than two patients in a 48 hour lapse of time. In the near future, subtelomeric FISH should be used only as a second-line test, orientated by the proband MLPA results. On the opposite, the advantage of the cytogenetic approach is the qualitative chromosomal description of rearrangements. Consequently FISH must be applied to the parents in order to detect a balanced anomaly that cannot be detected by MLPA.
Patient Results

The microdeletions involving SHH or TGIF genes in our 10 positive control patients (previously detected by FISH or qPCR) always encompassed the genomic material from the genes up to the telomeres, delineating a minimal deleted region 3.5-Mb-long. MLPA results also demonstrated that half of the 10 positive controls had associated cryptic subtelomeric gains, maybe inherited from one of the parents carrying a balanced translocation; this could be a great help in the genetic counseling considering the high rate of recurrence observed when a parent has a balanced translocation.

This is the first study showing so many associated gains and losses involving demonstrated HPE loci like SHH, known HPE loci (del12q–dup20p) but also unknown HPE loci (del18q–dupXq, del5q–dup17q). It is worth noticing that none of the patients tested had any concomitant association between a subtelomeric defect and another genetic anomaly already known to cause HPE (point mutation in HPE genes or microdeletion in ZIC2 or SIX3).

So, except for SHH or TGIF deletions, we still do not know what is the most prone to cause the HPE phenotype between the deleted and duplicated regions; these findings could be an interesting argument toward the multiple-hit hypothesis previously described by Ming et al. [2002] and give another explanation to the wide phenotypic spectrum of HPE.

The two unrelated live-born infants with a 1p deletion were included in the cohort of patients because they had microforms of HPE; the first one had a familial history of HPE (the mother already had a termination of pregnancy for HPE found by ultrasonography) and presented with cleft lip and palate, absence of corpus callosum, and mental retardation; the second patient presented with single central maxillary incisor, choanal atresia, and mental retardation. Deletion in 1p36 has been frequently described in several cases of mental retardation [Campbell et al., 2002; Rooms et al., 2004] and this region was also associated with the “1p deletion syndrome” associated with hypotonia and developmental delay (usually severe), growth abnormalities (growth retardation, microcephaly, obesity), and craniofacial dysmorphism with a large anterior fontanelle, prominent forehead, deep-set eyes, flat nasal bridge and midface hypoplasia, ear asymmetry, a pointed chin, and oro-facial clefting [Slavotinek et al., 1999]. So, the two 1pter deletion cases raised the question of the phenotypic variability in HPE as previously reported [Lazar et al., 2004]: even if severe forms of the disease can share the same genomic defect with milder forms, testing reciprocally microforms sharing phenotypic traits with other syndromes may identify genomic anomalies that may not specific of HPE; moreover, a mutation found in a mild form that could be also associated with a severe form in another proband underly the influence of modifying genes or environment.

Increasing the number of patients tested should lead to detect recurrent rearrangements and minimal critical regions where the search for candidate genes will be focused. Further studies should be done with a whole-genome approach using array comparative genomic hybridization comparative genomic hybridization (CGH) or quantitative PCR to precisely determine the breakpoints, at the exonic level.

Such a recurrence was observed in the 7q subtelomere known to contain SHH and our semiquantitative PCR study allowed the evaluation of an average 7-Mb deletion, with a 1-Mb hotspot of recombination in the telomeric end of the 7q36.1 cytoband. This hotspot is close to the 7q36.2 cytoband, which appears to be a region very poor in genes but containing many repetitive short interspersed nuclear elements (SINE) sequences that could be involved in recombinant chromosomes.

Taking into account these data, the HPE diagnosis strategy should be performed in successive steps: after clinical characterization of HPE, a cytogenetic approach can identify the chromosomal forms of HPE; if the karyotype is normal, known HPE genes are screened by sequencing and gene dosage methods (QMPSF) for SHH, ZIC2, SIX3, and TGIF; then, MLPA subtelomeric testing is systematically realized either in a candidate gene loci approach, either to find an associated duplication to a known deletion (inherited from a parental balanced translocation). To date, this strategy should be realized prior to array CGH (Fig. 4), the cost of which is higher than MLPA. Nevertheless, clinicians should be aware that any MLPA subtelomeric or array CGH finding in their patients, is only descriptive and needs further investigations before being definitely involved in the phenotype emergence.

In summary, this study performed for the first time a systematic subtelomeric screening in 181 HPE patients based on MLPA. Using this method, we were able to identify nine new potential candidate loci for HPE and to find submicroscopic anomalies in previously known HPE loci (HPE 1, HPE 9, and HPE 10). For 7q deletions and 7p gains, we demonstrated that the rearrangement sizes could encompass up to 7 Mb, meaning that several genes could be involved. Additionally to these potential contiguous gene syndromes, we also observed associations between submicroscopic gain and losses. All these findings enhance the hypothesis of a multigenic origin in HPE and give an explanation to the wide phenotypic spectrum of this developmental disease.

Acknowledgments

We are grateful to Dr. Bassem R. Haddad from Georgetown University, Washington, DC, for helpful discussions and suggestions and for critical review of the manuscript. We thank the IFR 140 Microscopy Core Facility located in UMR 6061 CNRS and Stéphanie Dutertre for helping to set up the FISH imaging.
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