Molecular characterization of breakpoints in patients with holoprosencephaly and definition of the HPE2 critical region 2p21


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Holoprosencephaly (HPE) is a common developmental defect involving the brain and face in humans. Cytogenetic deletions in patients with HPE have localized one of the HPE genes (HPE2) to the chromosomal region 2p21. Here we report the molecular genetic characterization of nine HPE patients with cytogenetic deletions or translocations involving 2p21. We have determined the parental origin of the deleted chromosomes and defined the HPE2 critical region between D2S119 and D2S88/D2S391. As a first step towards cloning the HPE2 gene which is crucial for normal brain development we have constructed a YAC contig which spans the smallest region of deletion overlap. Several of these YACs could be identified which span three different 2p21 breakpoints in HPE patients. These YACs narrow the HPE2 critical region to less than 1 Mb and are now being further analyzed to identify the gene causing holoprosencephaly on chromosome 2.

INTRODUCTION

Higher intellectual functioning requires normal development of the forebrain structures. To date, several genes are known to be expressed within the mammalian forebrain during early embryogenesis, suggesting a critical role of their gene products in the formation of this region (for reviews see 1,2). The identification of genes involved in human central nervous system (CNS) formation can be accomplished through the study of patients with abnormal brain development. Holoprosencephaly (HPE) is such a malformation complex, which results from incomplete cleavage of the forebrain during early embryonic development. HPE is a common birth defect with a prevalence of 1:16 000 among live born infants (3).

The etiology of HPE is heterogeneous with known associations to teratogenic agents and genetic factors (4–6). A genetic basis for HPE is supported by the occurrence of familial HPE, HPE as part of genetic syndromes, and in combination with various chromosome anomalies (7,8). Specifically, variable interstitial deletions have been seen within the short arm of chromosome 2 in HPE (9–13). To date, all of the reported cytogenetic deletions involving 2p21 are associated with signs of the HPE spectrum. Therefore, we postulated that this region contains an HPE gene, designated HPE2, necessary for normal brain development (7). Here we define the HPE2 minimal critical region (MCR) and present a contig of yeast artificial chromosomes (YACs) covering this region. Several of these YAC clones span the 2p21 breakpoints in three HPE patients which are assumed to disrupt HPE2 gene-specific sequences. These YACs should prove crucial in the process of the cloning and characterization of the HPE2 gene.
RESULTS

Clinical and cytogenetic characterization of HPE patients with 2p21 deletions or translocations

As a first step towards further molecular genetic characterization of the HPE2 critical region, we established lymphoblastoid cell lines from six HPE patients with variable cytogenetically visible deletions involving 2p21 and three HPE patients with rearrangements involving 2p21 (Table 1). The clinical findings in these patients represent the wide spectrum of the holoprosencephaly sequence from the most severe form with cyclopia and alobar holoprosencephaly as seen in fetus 823 to mild phenotypic expression with hypotelorism and midline dental anomalies in a 7 year old male patient (471) with general developmental delay (Fig. 1).

Mapping of region-specific microdissection probes

More than 50 clones from two region-specific microdissection libraries of the human chromosome 2 (14, 15) were used as probes for dosage blot analysis of HindIII-digested DNA from HPE patients with variable 2p21 deletions. We could demonstrate that 12 of these clones were deleted in the smallest 2p21 deletion of patient 002. Subsequently, sequence analysis of these 12 microclones was used to generate sequence tagged sites (STSs; Table 2).

Generation of somatic cell hybrid clones from the smallest deletion and PCR screening

To separate the normal from the deleted chromosome 2 we generated hamster–human somatic cell hybrid clones. The HPE cell line 002 with the smallest visible deletion was fused to Chinese hamster ovary mutants of the Urd-A complementation group which selectively retains the human chromosome 2 in uridine-depleted medium (16). Cytogenetic analysis by GTG-banding and PCR screening of the somatic cell hybrids with the 2p21 specific STS primers identified two clones (SCH002-10 and SCH002-11) with the chromosome del(2) only but not the normal homolog. The 12 generated STSs (Table 2) did not yield PCR products from DNA templates derived from these two hybrid clones. In addition, available polymorphic microsatellite markers from the short arm of chromosome 2 were analyzed by PCR using DNA from both the hybrids with the del(2) chromosome (SCH002-10 and SCH002-11) or with the normal chromosome 2 (SCH002-5, SCH002-9 and SCH002-12) only. D2S119 was amplified in the three hybrid clones with the normal chromosome 2 but found to be deleted in the two somatic cell hybrids with the del(2) chromosome (Fig. 2). In contrast, polymorphic markers flanking D2S119 were present in all five hybrid clones (D2S177, D2S288, D2S391), thus confirming the presence of the human chromosome 2 with or without the 2p21 deletion in the respective somatic cell hybrids.
Table 1. Karyotype and phenotype correlation of the six holoprosencephaly patients

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Karyotype</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>002</td>
<td>46,XX, del(2)(p21-p22)</td>
<td>Holoprosencephaly, microcephaly, hypotelorism</td>
<td>present study</td>
</tr>
<tr>
<td>1187</td>
<td>46,XY, del(2)(p21-p22)</td>
<td>Holoprosencephaly, microcephaly, hypotelorism</td>
<td>present study</td>
</tr>
<tr>
<td>1187</td>
<td>46,XY, del(2)(p22.2)</td>
<td>Holoprosencephaly, microcephaly, hypotelorism</td>
<td>present study</td>
</tr>
<tr>
<td>519</td>
<td>46,XX, inv dup(2)(p21-q24 p13)</td>
<td>Holoprosencephaly, microcephaly, hypotelorism</td>
<td>present study</td>
</tr>
</tbody>
</table>

Table 2. The generated PCR primers for the 2p21 specific STSs are listed with their sequence, the expected size of the amplified PCR product and the primer specific annealing temperature

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequence (5' to 3')</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2S119</td>
<td>5'-GAAGTCTCTTTAAAGATGAT-3'</td>
<td>367</td>
<td>60</td>
</tr>
<tr>
<td>D2S391</td>
<td>5'-GCTGCTGCTGTTTACCTCTG-3'</td>
<td>180</td>
<td>60</td>
</tr>
<tr>
<td>D2S139</td>
<td>5'-TTCATGCTGCTGTTTACCTCTG-3'</td>
<td>161</td>
<td>60</td>
</tr>
</tbody>
</table>

Determination of the deletion extent and the parental origin of the deleted chromosomes

To define the origin of the deleted chromosome and the extent of the six 2p deletions on a molecular level, we tested DNA of the HPE patients and their parents with polymorphic markers from the vicinity of D2S119 (Fig. 3). Our data demonstrate the presence of two D2S119 alleles in LCL 518 and of two D2S391 alleles in LCL 002. This defines the smallest region of deletion overlap between the telomeric deletion breakpoint of LCL 518 and the centromeric deletion breakpoint of LCL 002. Since this HPE MCR in 2p21 is flanked proximally by D2S288/D2S391 and distally by D2S119.

In addition, we analyzed our PCR results for a possible parental origin effect. Maternal origin of the deleted chromosome 2 was observed in patients 471 and 735. However, loss of paternal sequences could be demonstrated for patients 002, 683 and 823 (Fig. 3), suggesting that parental origin of the deleted sequences is not critical in the disease etiology.
To determine the order of the YACs, the 2p21 specific STSs (Table 2) were amplified by PCR of YAC DNA. This analysis determined the location of six STS loci within the newly defined HPE2 MCR: centromere–D2S288/D2S391–D2S1489–D2S1488–D2S1487–D2S1486, D2S1485–D2S1484–D2S119–telomere.

Identification of 2p21 breakpoint spanning YACs

To further narrow the HPE2 MCR we hybridized YACs from this contig to chromosome preparations of HPE patients with balanced translocations involving 2p21 (Fig. 5). YACs from both ends of the contig hybridized to opposite sides of the translocation breakpoints (Fig. 4b) placing the breakpoints within the defined HPE2 MCR. Subsequently, several YACs could be identified which span all three 2p21 breakpoints (Fig. 4c and d). The intensity of the hybridization signals present on both the normal and the respective derivative chromosomes 2 suggests: (i) the position of the translocation breakpoints relative to each other, and (ii) that the breakpoint spanning YACs overlap only partially. Hybridization with YAC 957b10 (1.18 Mb) results in approximately equal signals on both sides of all three translocations, placing them in the middle portion of this YAC. In contrast, the smallest YAC 746d4 (800 kb) spans the breakpoint of 519 with the larger signal telomeric to the breakpoint (Fig. 4c). However, when the same YAC is hybridized to chromosomes of 567 or 1187, no signal can be detected proximal to the respective 2p21 breakpoints (data not shown). This may indicate that the latter two breakpoints are at the centromeric end or just outside of the genomic insert in 746d4. The suggested order of the translocation breakpoints in 567 and 1187 centromeric to 519 is further supported by FISH of YACs 913g4, 919e8, 919c10 and 921b6, which consistently results in bigger hybridization signal on the centromeric side of the breakpoints when hybridized to chromosomes of 519 in comparison with 567 and 1187 (Fig. 6).

In addition, a new polymorphic marker, D2S1761 (Research Genetics), could be amplified in DNA of all breakpoint spanning YACs thus placing it within the HPE2 MCR between D2S119 and D2S288/D2S391. As expected, D2S1761 was found to be deleted in DNA of HPE patients with 2p21 deletions (471, 823, 002 and 735) when compared with parental DNA (Fig. 3).

DISCUSSION

Previous reports of cytogenetically visible 2p21 deletions in sporadic cases with holoprosencephaly (9–13) led to the hypothesis that this region contains HPE-related gene sequences (7). Here we present molecular genetic data from nine patients with holoprosencephaly and cytogenetic anomalies in 2p21. Using single copy probes from two microdissection libraries we were able to identify 12 STSs and two additional polymorphic markers D2S119 and D2S1761 within the deletion. PCR analysis of six HPE patients with cytogenetically visible 2p deletions established the borders of the newly defined HPE2 MCR between the centromeric deletion side of patient 002 and the telomeric deletion side of patient 518 flanked by the polymorphic markers D2S288/D2S391 and D2S119. The genetic distance of these two flanking markers was estimated to be 5 cM (17).

In addition, both maternal as well as paternal origins of the deleted chromosomes 2 in the HPE patients with 2p21 deletions could be demonstrated, arguing against major imprinting effects on chromosome 2 involved in the pathogenesis of holoprosencephaly.

Construction of a YAC contig spanning the minimal critical region

STS information, available through the Genethon server, was used to select YACs from the CEPH mega YAC library, positive for STS information, available through the Genethon server, was used to identify additional YACs from within the HPE2 critical region using available Alu-PCR hybridization and fingerprint data of the Genethon database (Fig. 4a).

Figure 3. Results of the radioactive PCR with polymorphic markers from the vicinity of the HPE2 MCR using genomic DNA from HPE patients and their parents. The black or white bars represent the presence of both parental alleles in the respective child, the deletion of one parental allele is labeled as +, uninformative regions are marked as striped bars. The origin of the deleted chromosome is indicated by a black bar for the paternal, or white for the maternal chromosome, respectively. For patient 518 both parental alleles were present along the gray bar, D2S288 and D2S391 were not informative.

Figure 2. PCR amplification of D2S119 from DNA of hamster human somatic cell hybrids. The clones SCH002–5, SCH002–9 and SCH002–12 contain the D2S119 allele and thus the normal chromosome 2. The hybrid clones SCH002–10 and SCH002–11 retained the del(2) chromosome of HPE patient 002 and are deleted for D2S119. The two negative controls contain no DNA (−) or hamster DNA (CHO).
Figure 4. Analysis by fluorescent in situ hybridization (FISH) with YACs on metaphase spreads from patient 002 (a) with the smallest visible 2p21 deletion and all three patients with 2p21 breakpoints 567 (b), 519 (c) and 1187 (d). (a) The hybridization signals of YAC 853f1 appear on the short arm of the normal chromosome 2 (*). The del(2) chromosome below with the short arm pointing to the left does not show any hybridization signal (arrow). Thus, this YAC appears to be deleted on the del(2) chromosome of patient 002. (b) Hybridization with YACs 941e7 (green) and 738a5 (red) from both sides of the generated YAC contig on chromosomes of patient 567 shows both signals on the normal chromosome 2 (*), but YAC 941e7 (green) maps centromeric (bold arrow) and YAC 738a5 (red, small arrow) telomeric to the 2p21 breakpoint, placing this breakpoint within the HPE2 critical region. (c) Analysis of the signal intensity of YAC 746g4 after hybridization to chromosomes of patient 519 demonstrates, that this YAC spans this 2p21 breakpoint with the majority of the YAC insert mapping distal to the breakpoint. (d) YAC 919e8 is spanning the 2p21 breakpoint of patient 1187. Normal chromosomes 2 are indicated by an asterisk (*), der(2) chromosomes by bold arrows, and der(1) chromosomes (b, c) by small arrows.

FISH analysis with CEPH Mega YACs known to be positive for STS markers of chromosome 2 identified several YACs which were deleted within the del(2) chromosome of LCL002. The order of these YACs within the newly defined HPE2 MCR was determined by PCR analysis with the 2p21 specific STSs. Thus, six of the STSs were located between the flanking polymorphic markers D2S119 and D2S288/D2S391. The remaining six STSs (D2S1481, D2S1482, D2S1483, D2S1490, D2S1491, D2S1492),
Figure 5. Schematic diagram of the chromosomal rearrangements in three HPE patients with 2p21 breakpoints. The gray shadowed regions indicate chromosome 1 material for patients 567 and 1187 and the part of 2q from patient 519 which was inverted and inserted into 2p21.

known to be deleted in LCL002, were not present in any of the tested YACs forming this YAC contig. Our PCR results with D2S119 demonstrate that the telomeric side of the smallest cytogenetic visible deletion of LCL002 exceeds the distal end of the HPE2 MCR, suggesting that those six remaining markers are located within chromosomal band 2p21 but distal to D2S119.

The YACs from this contig have subsequently been hybridized to chromosomes of three patients with translocations involving chromosomal band 2p21. We could identify six YACs which span the 2p21 breakpoints of all three HPE patients. Based on our FISH mapping data and the observed signal intensity of the hybridized YACs on the resulting derivative chromosomes, the translocation breakpoints of patients 567 and 1187 are suggested to map centromeric of the breakpoint in patient 519. The putative HPE2 gene is expected to be part of the genomic insert of those breakpoints spanning YACs which narrow the HPE2 critical region to less than 1 Mb.

Our results now allow us to test HPE patients with normal karyotypes for submicroscopic 2p21 deletions using D2S1761 and other polymorphic marker from the vicinity of the HPE2 MCR and HPE families with known autosomal-dominant (AD) transmission for linkage to these markers. Although several AD HPE families were recently linked to markers from the vicinity of the putative HPE3 gene in 7q36 (18), AD HPE families not linked to HPE3 may have an alteration in the HPE2 gene in 2p21. As a next step towards understanding early embryonic brain development, we are now isolating candidate genes from the HPE2 MCR in order to generate a transcriptional map which should contain the putative HPE2 gene.

MATERIALS AND METHODS

Patients

Nine families with HPE children participated in this study. Two of these had been reported previously: patients 002 (10) and 735 (13). At the time of this reevaluation, informed consent was obtained in accordance with the standards set by local institutional review boards. Medical records, clinical photographs and head CTs/MRIs were reviewed when available.

Southern blot analysis

DNA was extracted from lymphocytes or established lymphoblastoid cell lines (LCLs) of HPE patients, parents and normal
control subjects by routine methods. Microdissection clones from the two region-specific microdissection libraries (14,15) have been used for Southern blot analysis of HindIII-digested human genomic DNA. Probe labeling and Southern hybridization were performed according to routine methods (19).

Sequencing, generation of STS-specific primers and PCR

DNA sequencing of the microclone inserts was done using the Sequenase Kit V. 2.0 (USB) following the manufacturers recommendations. Primer pairs were chosen by computer analysis of these insert sequences with the program (Generunner V. 3.00, Hastings Software, Inc.). Each PCR reaction contained a final volume of 50 µl, 50 ng genomic DNA, 200 µM dNTP, 25 pmol of each primer, 1% formamide, 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% w/v gelatin, and 1 U Taq polymerase (Perkin Elmer Corp.). Conditions of the PCR reactions were as follows: (i) 92°C 45 s; (ii) primer-specific annealing temperature 1 min; (iii) 72°C 30 s; for 30 cycles. The PCR products were separated on a 4% Nusieve agarose gel. For the radioactive PCR this protocol has been adapted using a modified dNTP mixture with 200 µM each of dATP, dGTP, dTTP, 2.5 µM dCTP and 15 µCi α-32P-dCTP. The amplified PCR products were denatured, and separated on a 7% polyacrylamide gel. The gels were dried and exposed to autoradiography film (Kodak or Fuji). Genome Database Identification (GDB ID) of the generated 2p21 specific STSs are as follows: G00–434–124, –127, –130, –133, –137, –140, –143, –148, –151, –154, –159, –165.

Somatic cell hybrids

The human lymphoblastoid cell line 002 with the smallest cytogenetically visible deletion del(2)(p2101–p2109) was fused to UrdA–Chinese hamster ovary cell mutants, which are defective in the pyrimidine biosynthesis pathway, using 50% polyethylene glycol 1500. After 24 h fusion the cells were grown in medium deficient for uridine, which selects against unfused hamster ovary cells (16).

Preparation of YACs and FISH

Total yeast DNA was prepared and labeled with biotin or digoxigenin-dUTP by nick translation. Metaphase chromosome spreads were prepared from lymphoblastoid cell lines by standard methods. Fluorescent in situ hybridization (FISH) and analysis were done as described previously (20,21).

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