Published in final edited form as:

Am J Med Genet. 1998 February 26; 76(1): 51–57.

Molecular Cytogenetic Analysis of Patients With Holoprosencephaly and Structural Rearrangements of 7q

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Abstract

The holoprosencephaly (HPE) sequence is a malformation complex with abnormal midline cleavage of the embryonic forebrain. HPE is genetically heterogeneous with at least 6 different chromosome regions containing genes involved in the expression of the phenotype. *HPE3*, recently identified as the human Sonic hedgehog gene, is localized to 7q36. We have used fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR) amplification in 5 cell lines from patients with HPE (3 cases), HPE and sacral agenesis (1 case), and microcephaly (1 case) to further define the structural rearrangements of the long arm of chromosome 7 in each case. All cell lines demonstrated loss of material in the critical region of *HPE3* at band 7q36, which includes the Sonic hedgehog gene. We report here the analysis of these patient cell lines. Am. J. Med. Genet. 76:51–57, 1998.

Keywords

holoprosencephaly; brain malformation; chromosome 7; fluorescence in situ hybridization

INTRODUCTION

Holoprosencephaly (HPE) sequence is a developmental field defect involving the forebrain and midface, with an estimated incidence of approximately 1/16,000 individuals [Roach et al., 1977]. The embryonic forebrain fails to divide into cerebral hemispheres, resulting in a single central ventricle. Facial malformation often coincides with the brain malformation and even can "predict the brain" [DeMyer et al., 1963]. DeMyer and Zeman [1963], classified the degrees of HPE into alobar, semilobar, and lobar types. At the mild end of the

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spectrum are malformations such as microcephaly, absence or hypoplasia of the corpus callosum, and absence or hypoplasia of the olfactory bulbs and tracts.

At least 6 different chromosome regions have been implicated as containing genes involved in HPE. These include 2p, 3p, 7q, 13q, 18p, and 21q [Muenke, 1994], The Human Gene Mapping 11 Conference catalogued 4 putative HPE genes which were designated as follows: *HPE1* (21q22.3); *HPE2* (2p21); *HPE3* (7q36); and *HPE4* (18pter-q11) [Frézal and Schinzel, 1991].

A critical region for *HPE3* on chromosome 7 was described by Gurrieri et al. [1993] with boundaries flanked by markers D7S392 (TM 196) and D7S292 (pJ71). *HPE3* was subsequently shown to be linked to D7S22 at 7q36 by Muenke et al. [1994] in families with autosomal dominant (AD) HPE. Belloni et al. [1995] further characterized 2 sisters with HPE and terminal deletions of 7q36 resulting from abnormal segregation of a balanced reciprocal translocation, t(7;9)(q36;q34), in a mother with mild craniofacial HPE findings. Most recently, the human Sonic hedgehog gene was mapped to 7q36 and found to be mutated in families with AD HPE [Belloni et al., 1996; Roessler et al., 1996].

We report our findings using 24 markers by fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR) amplification on cell lines from 5 phenotypically abnormal individuals, 3 with HPE, 1 with HPE and sacral agenesis, and 1 patient with microcephaly and mild craniofacial abnormalities. All 5 patients had structural rearrangements involving 7q from 7q35 to 7qter. All carried deletions of the critical region of 7q36, defined by markers D7S392-D7S292, which contains the Sonic hedgehog gene.

MATERIALS AND METHODS

Patients and Cell Lines

The clinical descriptions and karyotypes of the patients are listed in Table I. G-banded metaphase analysis was performed on all 5 cell lines by standard cytogenetic methods. All lines except V-1 are nontransformed fibroblast cells. V-1 was received as a fixed cell pellet obtained from cord blood. Cell lines GM00657, GM10064, and GM7216 were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ). Patient F-1 was previously described [Morichon-Delvallez et al., 1993]. The fifth patient (V-1) was seen at Vanderbilt University. This patient was an infant girl, the fifth child born to a 38-year-old mother. Delivery was by cesarean section performed because of hydrocephalus and oligohydramnios. Multiple congenital anomalies including microcephaly, hypotelorism, single nostril, micrognathia, short neck, and ambiguous genitalia were noted at birth. A head ultrasound study demonstrated HPE. The infant died 47 minutes after birth. An autopsy was denied. Chromosomes studied from cord blood with GTG-banding demonstrated a 46,XX,der(7)t(3;7)(p22;q35) karyotype (Fig. 1), resulting in partial trisomy of 3p and partial monosomy of 7q.

Molecular Studies

Genomic DNA was prepared according to standard methods using a cesium chloride gradient [Crkvenjakov and Byus, 1974], Primer pairs for PCR experiments were obtained

from Research Genetics (Huntsville, AL) or American Type Culture Collection [Rockville, MD]. The primer sequences, allele sizes, and loci order have been previously reported (Table II). Amplification of genomic DNA was performed according to standard procedures with 1 μCi radiolabeled ^{32}P (Amersham, Arlington Heights, IL) added to a 100 μl master mix of primers and reagents in a 10X stock solution containing 10 pmole/μl of each primer. PCR amplificaiton was programmed as 40 seconds (94°C) for denaturation, 40 seconds (55°C) for annealing, and 80 seconds (65°C) for extension for 30 rounds. Equal amounts of amplified DNA were loaded into the wells of a 6% polyacrylamide/7 M urea sequencing gel. Separated radioactive products were exposed to film overnight. Autoradiographs were scored for the presence or absence of heterozygous alleles.

FISH studies were performed on dividing cells from all patients. A minimum of 10 cells was scored for each probe. Probes included human inserts contained in yeast artificial chromosomes kindly provided by Dr. Eric Green. Cosmid probes HTY146–7, HTY146–14, and HTY146–49, which map to the subelomeric region of 7q (D7S427), were provided by Dr. Robert Moyzis. Commercial probes included the Williams Critical Region probe (chromosome 7), DiGeorge Critical Region probe (chromosome 22), the “coatasome” chromosome 7 paint and centromere probes (Oncor, Gaithersburg, MD), and a whole chromosome 13 paint (Cambio, Cambridge, England). The Williams Critical Region probe contains cosmid clones for locus D7S427 as part of the probe mixture. Plasmid probe pMP4 from EN2 was provided by Dr. Alexandra Joyner, D7S392 (TM196) by Dr. Lap-Chi Tsui, and D7S22 was a gift from Dr. Alec Jeffreys.

YAC probes were prepared from either total yeast DNA or Alu-PCR amplification with oligonucleotides CL1, CL2, and CL3 [Lengauer et al., 1990] obtained from Research Genetics. Cosmid and plasmid probes were prepared following standard protocols [Sambrook et al., 1989], FISH was performed following standard methods [Pinkel et al., 1986]. Probes were nick translated with either biotin-dUTP or digoxigenin-dUTP. Detection was accomplished using either avidin conjugated with FITC or a series of antidigoxigenin antibodies conjugated with fluorophores, FITC, rhodamine, or Cy3. All cells were viewed with a Leitz Aristoplan fluorescent microscope with a triple pass filter and enhanced with Adobe Photoshop III software.

The genetic map including plasmid, cosmid, YAC, and microsatellite markers on chromosome 7q is shown in Table II according to the Second International Workshop on Human Chromosome 7 Mapping, 1994 [Tsui, 1995] and Belloni et al. [1995].

RESULTS

The FISH and PCR results are summarized in Table II. All 5 cell lines contain deletions for the critical region of *HPE3*. The breakpoint in GM00657 occurred between TCRB and D7S505 and everything distal to the breakpoint in 7q36 was lost and not translocated to chromosome 18 as previously reported [Punnett et al., 1979]. Signals for the subtelomeric cosmid, 146–14, were present on the normal chromosome 7 and not on the der(7) or any other chromosome in all cells analyzed (Fig. 2).

Cell line GM10064 showed one breakpoint at 7q35 and a second breakpoint at 7q36 between loci D7S22 and D7S427. Signals for D7S427 (Williams Critical Region probe and cosmid 146–14) were observed on 13p (Fig. 3a,b), indicating a complex translocation involving the der(7). Therefore, our results indicate that 13q12.3→13qter was translocated to 7q35, 7q35–36 was lost, and 7q36→7qter was translocated to distal 13p.

The breakpoint for cell line GM7216 occurred in 7q36 between loci D7S798 and D7S637 and chromatin distal to the breakpoint was lost from the cell. The breakpoint for cell line F-1 occurred at 7q36 within locus D7S798. FISH with the DiGeorge Critical Region probe indicated that most of the long arm of chromosome 22 was translocated to 7q36 (Fig. 4a). However, contrary to the published karyotype, 7q36 was not reciprocally translocated to chromosome 22, and resulted in a deleted chromosome 22. Investigations with the Williams Critical Region probe, cosmid 146–14, and the chromosome 7 paint probe failed to demonstrate signals from 7q on the derivative chromosome 22 (Fig. 4b). In the original report of this karyotype, the authors indicated approximately 50% mosaicism for the translocated chromosome 22 in all cells examined. We estimated that the deleted chromosome 22 was lost in 70–80% of the cells analyzed and resulted in partial monosomy for a small segment of euchromatin of 22q between the centromere and the DiGeorge probe locus, DS22S75.

Cells from a fixed pellet of V-1 were analyzed by FISH. The karyotype indicated a der(7) chromosome with a breakpoint at 7q35. A single fluorescent signal was observed only on the normal chromosome 7 for all markers tested, demonstrating a deletion of 7q36 including the *HPE3* critical region.

DISCUSSION

In this report we describe the molecular analysis, utilizing PCR amplification and FISH with plasmid, cosmid, and YAC probes, of 5 patient cell lines with deletions of 7q36 and a phenotype of HPE, microcephaly, or sacral agenesis. Three of the cell lines are contained in the NIGMS Human Genetic Mutant Cell Repository and are widely distributed to researchers around the country. We have revised the karyotypes in 2 of these cell lines (Table II). Cell line GM00657 does not appear to carry a reciprocal translocation as observed in G-banded metaphase analysis [Punnett et al., 1979] or by Southern blot hybridization [Gurrieri et al., 1993], Our studies indicate a terminal deletion of 7q from 7q35–36 to the terminus.

A complex translocation was identified in GM10064, so that in this cell line there is trisomy for 13q12.3→13qter, monosomy for 7q35–36, and disomy for 7q36 to 7qter with the terminal chromosome 7 sequences translocated to 13p. While possible, we do not think these findings are culture induced for the following reasons. These nontransformed fibroblast lines were received and processed at a low passage number and the karyotypes performed on all lines corresponded to those provided by the NIGMS repository. Additionally, the band 7q36 is composed of G-band-negative chromatin and is difficult to distinguish separately from the G-negative chromatin of 18q21 or the satellite of 13p.

A deletion from 7q36 (D7S798) to 7qter and mosaicism for partial monosomy of 22pter→q11.2 were observed in cell line F-1. The patient from whom F-1 was obtained demonstrated sacral agenesis as well as HPE. Reported linkage with markers from 7q indicates gene for AD sacral agenesis maps to the HPE region on 7q and resides between D7S396 (not on our map but centromeric to D7S550) and the telomere, an interval estimated to be less than 10 Mb [Lynch et al., 1995]. Our data indicate that F-1 has haploinsufficiency of the region which contains this putative gene associated with sacral agenesis. Partial monosomy of chromosome 22pter→q11.2 has not been consistently associated with sacral agenesis [Schinzel, 1984], which provides strength for the hypothesis that the chromosome 7 aberration is responsible for this phenotype.

Recently, the human development gene Sonic hedgehog, mapped to the *HPE3* critical region [Belloni et al., 1996], was found to be mutated in 5 of 30 families with AD HPE [Roessler et al., 1996]. The human Sonic hedgehog gene (*SHH*) is one member of a family of genes related to the Drosophila segment polarity gene hedgehog (*hh*) [Echelard et al., 1993]. *Shh* is expressed in the notochord, floor plate, and zone of polarizing activity [Johnson et al., 1994], Targeted gene disruption in the mouse (*Shh*−/−), generated by homologous recombination in embryonic stem cells, showed that *Shh* plays a critical role in the patterning of vertebrate embryonic tissue including the brain, spinal cord, axial skeleton, and limbs. In particular, morphological defects of the forebrain region in homozygous mouse mutants produce the phenotype of cyclopia and a proboscis or single nostril, which are features of the HPE spectrum [Chiang et al., 1996], A role for *Shh* disruption in caudal regression has been demonstrated in the zebrafish *no tail* mutant as well as in the mouse [Schulte-Merker et al., 1994]. Therefore, a deletion or disruption of *SHH* may be responsible for the phenotype in our patient with HPE and sacral agenesis.

Belloni et al. [1996] reported that *SHH* was not disrupted in affected members of 2 families with translocations involving 7q36, and in fact the translocation breakpoints were estimated to lie 15–250 kb proximal to *SHH*. These authors proposed a “position effect” leading to aberrant *SHH* expression caused by the disruption of cis-acting regulatory elements. Our map would indicate haploinsufficiency of *SHH* in all 5 cell lines and most likely reduced expression of the Sonic hedgehog gene. This information would not explain why the patients from whom these cell lines were obtained expressed a phenotype of HPE while individuals with the 7q deletion syndrome and typically more extensive deletions usually do not have HPE. Perhaps more restricted breakpoints within 7q36 lead to aberrant expression of *SHH* due to a positional effect as proposed by Belloni et al. 1996, or there may be an imprinting effect with disruption of an imprinting domain as suspected for Prader-Willi and Angelman syndromes on chromosome 15 [Lalande, 1994]. Another consideration for cell lines GM10064, GM7216, and V-1 is the interaction of partial monosomy of 7q and the dosage effect of partial trisomies of 13q and 3p, since both trisomies have been associated with the HPE phenotype [Roach et al., 1977; Kurtzman et al., 1987; Berry et al., 1990]. Analysis of the Sonic hedgehog gene in these cell lines is underway. Further analysis of patients with structural cytogenetic rearrangements as reported here will help to elucidate the role of developmental genes, like Sonic hedgehog, in the etiology of a wide range of genetic defects.

ACKNOWLEDGMENTS

We thank Mark Stewart for discussion and technical assistance, and Kristen Bennett and Connie Sutton for their clerical help.

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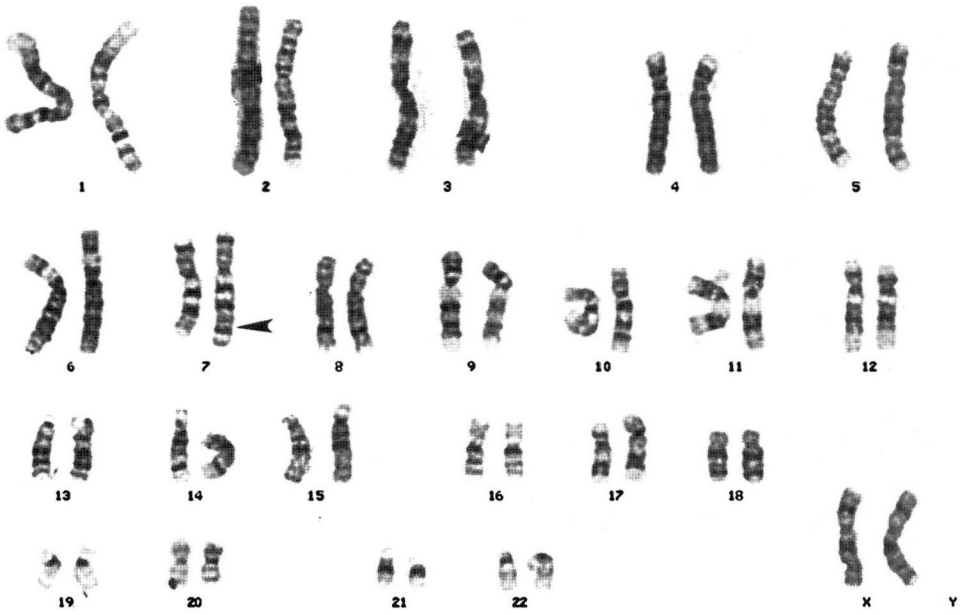

Fig. 1.

G-banded karyotype of cell line V-1, 46,XX,der(7)t(3;7)(p22;q35). The arrowhead points to derivative chromosome 7.

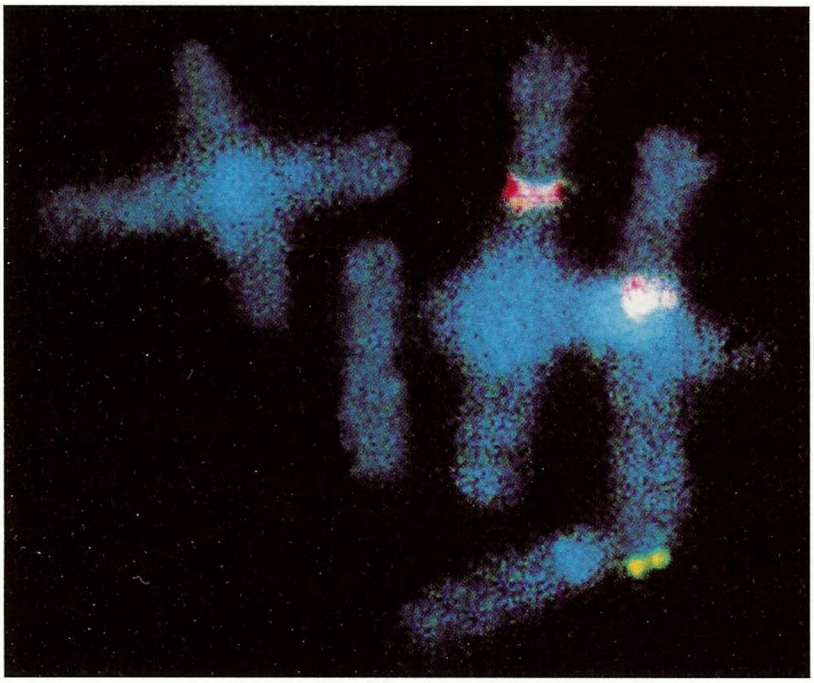

Fig. 2.

Partial FISH metaphase from cell line GM00657. Red signals indicate hybridization to the centromeres of the normal chromosome 7 and der(7). Green signals show hybridization of the cosmid 146–14 to the normal chromosome 7 only.

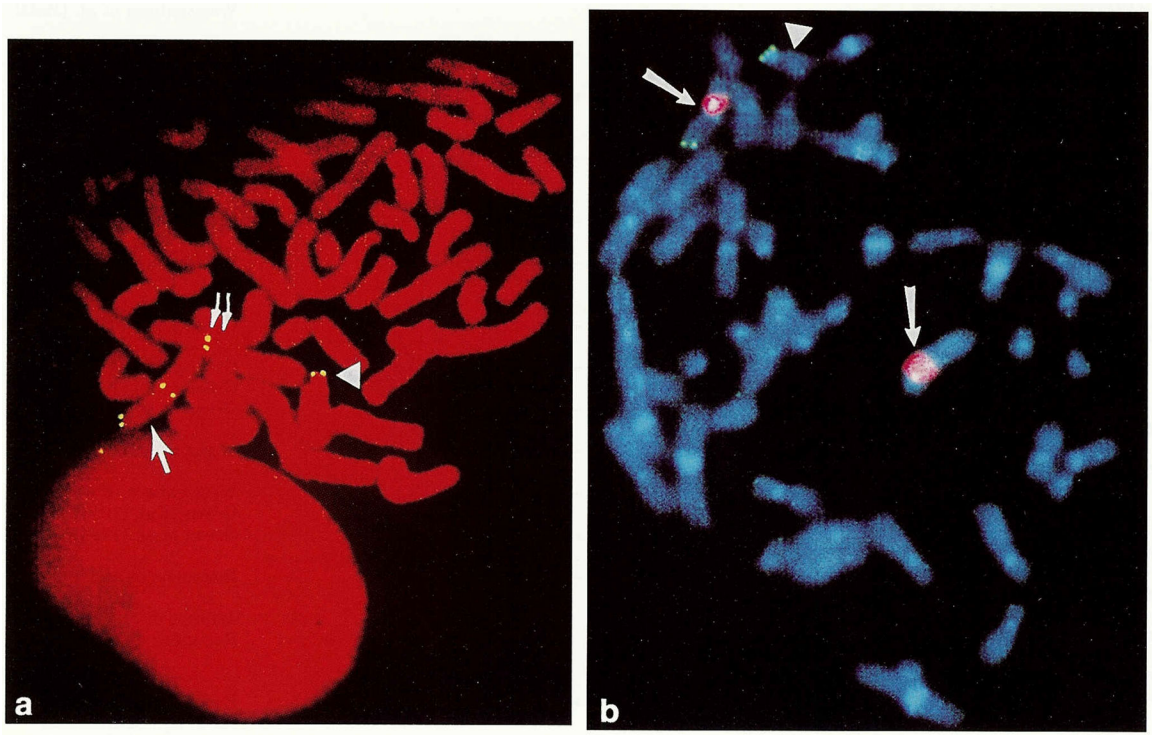

Fig. 3.

a: GM10065 metaphase hybridized with the Williams Critical Region probe (Oncor). The arrowhead indicates the der(13) chromosome with FITC signals for probe D7S427 on 13p. The single arrow points to the normal 7 with signals for both the Elastin gene (*ELN*) and marker probe D7S427. Double arrows points to the der(7) with signals only for *ELN* at 7q11.23. **b:** Metaphase from GM10064 hybridized with probe 146–14 (green signals). Red signal is the chromosome 7 centromere. Arrowhead indicates the der(13). Arrows point to the normal 7 and der(7).

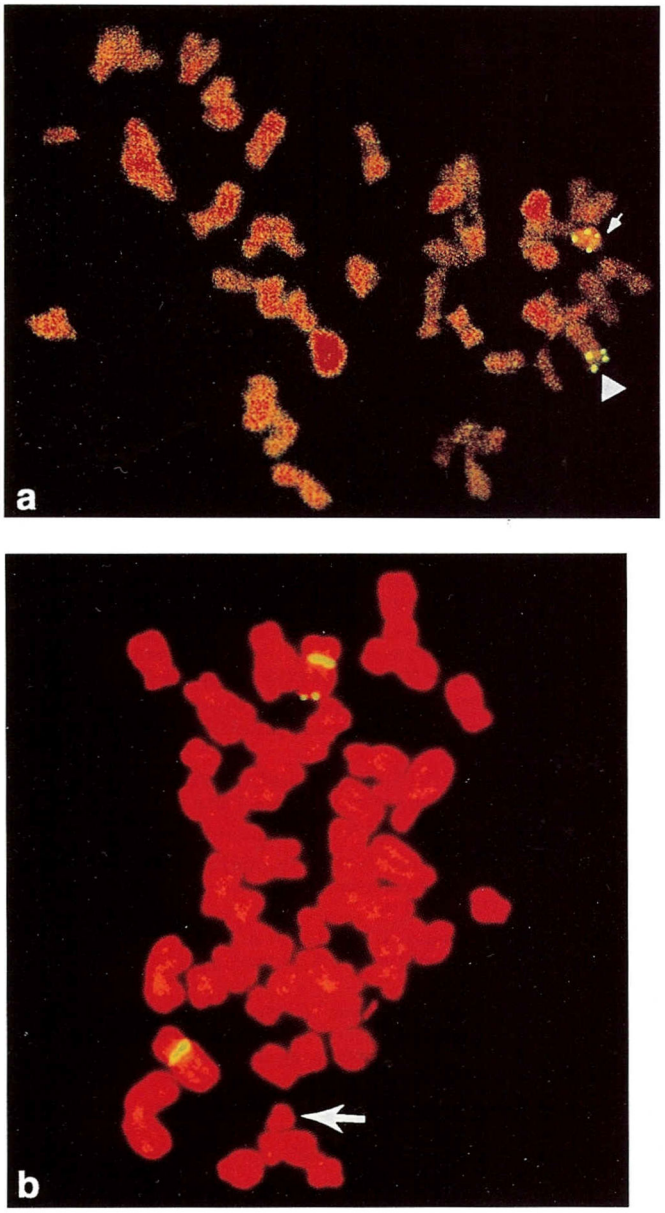

Fig. 4.

a: Cell line F-1 metaphase hybridized with the DiGeorge Critical Region probe (Oncor). Arrowhead indicates the der(7) with most of 22q translocated to 7q36. FITC signals are for D22S75, at 22q11.2, and marker probe, D22S39, at 22q13. The arrow points to the normal chromosome 22. **b:** Metaphase from cell line F-1 hybridized with cosmid 146–14. Normal chromosome 7 with signals for the centromere and 146–14. The der(7) demonstrates signals for the centromere only. Signals for 146–14 are not observed on the del(22) (arrow).

TABLE I.

Clinical Data and Original and Revised Karyotypes of Cell Lines

Cell line	Clinical data	Original karyotype	Revised karyotype	Cell type
GM00657	One-month-old white male with cleft palate, microcephaly, and developmental delay	46,XY,t(7;18)(q34;q12.2)mat	46,XY,der(7)t(7;18)(q35;q12.2)mat,del(18)(q12.2)	Fibroblasts
GM10064	Female newborn with HPE	46,XX,der(7)t(7;13)(q34;q12.3)pat	46,XX,der(7)t(7;13)(q35;q12.3)pat,der(13)t(7;13)(q36;p13)	Fibroblasts
GM7216	25-week female fetus with HPE and cyclopia	46,XX,der(7)t(3;7)(p21.3;q36)	NR *	Fibroblasts
F-1	23-week male fetus with HPE and sacral agenesis	45,XY,t(7;22)(q36;q11), −22/46,XY,t(7;22) (q36;q11)	45,XY,der(7)t(7;22)(q36;q11.2), −22/46,XY,der(7)t(7;22) (q36;q11.2),del(22)(q11.2)	Fibroblasts
V-1	Newborn female with HPE	46,XX,der(7)t(3;7)(p22;q35)	NR *	Leukocytes from cord blood

* NR = no revision.

Am J Med Genet. Author manuscript; available in PMC 2019 October 03.

TABLE II.

Markers Used in This Study and Deletion Map of Cell Lines *

Marker	Map position	GM00657	GM10064	GM7216	F-1	V-1	References
D7S500	7q32–33						
AFM198zh8		+		+	+		Weissenbach et al. [1992]
D7S684	7q34						
AFM312wb5		+	+	+	+		Gyapay et al. [1994]
TCRB	7q35						
VB6–7		+	+	+	+		Scherer et al. [1994]
D7S505	7q36						
AFM199zd4		−	−	+	+		Weissenbach et al. [1992]
yac 1249		−	−	+	+		Green et al. [1994]
yac 3729				+	+		Green et al. [1994]
D7S642	7q36						
AFM074wc12		−	−		+		Gyapay et al. [1994]
D7S483	7q36						
AFM074xg5		−	−	+	+		Weissenbach et al. [1992]
D7S798	7q36						
yac 3401		−	−	+	+		Green et al. [1994]
AFM205va3		−	−	+	−		Gyapay et al. [1994]
D7S637	7q36						
AFM211xc3		−	−	−			Gyapay et al. [1994]
yac 131				−			Green et al. [1994]
yac 849		−	−	−	−	−	Green et al. [1994]
EN2	7q36						
pMP4		−	−	−			Logan et al. [1989]
D7S550	7q36						
AFM274xh4		−	−	−	−		Weissenbach et al. [1992]
yac 3501					−		Green et al. [1994]
yac 2979		−	−	−	−	−	Green et al. [1994]
D7S392	7q36						

Am J Med Genet. Author manuscript; available in PMC 2019 October 03.

Marker	Map position	GM00657	GM10064	GM7216	F-1	V-1	References
pTM196	HPE3	–	–	–	–	–	Rommens et al. [1988]
D7S22	Critical						
pλgt3	Region			–		–	Jeffreys et al. [1988]
D7S292						–	Rommens et al. [1988]
pJ71							
D7S427	7q36						
wcr		–	+	–	–	–	Ewart et al. [1993]
cos 146–7				–	–	–	Riethman et al. [1993]
cos 146–14		–	+	–	–	–	Riethman et al. [1993]
cos 146–49				–	–	–	Riethman et al. [1993]

*
wcr = Williams Critical Regional Probe (Oncor, Inc.);

+ = two copies of the marker present; – = a single signal for the marker present.