ORIGINAL ARTICLE

Multicolour FISH and quantitative PCR can detect submicroscopic deletions in holoprosencephaly patients with a normal karyotype

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Holoprosencephaly (HPE) is the most common structural malformation of the developing forebrain. At birth, nearly 50% of children with HPE have cytogenetic anomalies. Approximately 20% of infants with normal chromosomes have sequence mutations in one of the four main HPE genes (*SHH*, *ZIC2*, *SIX3*, and *TGIF*). The other non-syndromic forms of HPE may be due to environmental factors or mutations in other genes, or potentially due to submicroscopic deletions of HPE genes. We used two complementary assays to test for HPE associated submicroscopic deletions. Firstly, we developed a multicolour fluorescent in situ hybridisation (FISH) assay using probes for the four major HPE genes and for two candidate genes (*DISP1* and *FOXA2*). We analysed lymphoblastoid cell lines (LCL) from 103 patients who had CNS findings of HPE, normal karyotypes, and no point mutations, and found seven microdeletions. We subsequently applied quantitative PCR to 424 HPE DNA samples, including the 103 samples studied by FISH: 339 with CNS findings of HPE, and 85 with normal CNS and characteristic HPE facial findings. Microdeletions for either *SHH*, *ZIC2*, *SIX3*, or *TGIF* were found in 16 of the 339 severe HPE cases (that is, with CNS findings; 4.7%). In contrast, no microdeletion was found in the 85 patients at the mildest end of the HPE spectrum. Based on our data, microdeletion testing should be considered as part of an evaluation of holoprosencephaly, especially in severe HPE cases.

Holoprosencephaly (HPE) is the most common anomaly of the developing forebrain in humans. The prevalence of HPE is approximately 1 in 10 000 live births and 1 in 250 embryos.[1] HPE is associated with a wide spectrum of craniofacial anomalies ranging from lethal forms, such as alobar HPE and cyclopia, to less severe forms, such as lobar HPE in children with cognitive deficits and a virtually normal face.[2] A ''multiple hit'' hypothesis of genetic and environmental factors has been proposed to account for the extreme clinical variability that occurs even within the same family.[3] Numerous environmental factors/teratogens have been proposed as contributors to HPE, based on reports of maternal exposure during early gestation.[4 5] Several HPE associated teratogens are supported by animal models.[4] The most common genetic factors include various non-random cytogenetic abnormalities (trisomy 13, del(18p), del(7q36), del(13q32), del(2p21) and others) in up to 50% of live born infants with HPE.[6] Using this cytogenetic information, a positional candidate gene approach has led to the identification of mutations in the following genes: *sonic hedgehog* (*SHH)* on 7q36,[7] *ZIC2* on 13q32,[8] *SIX3* on 2p21,[9] and *TGIF* on 18p11.3.[10] Other genes that were suggested to be involved in the aetiology of HPE but have a very low rate or no point mutations published are *DKK1*, *PTCH*, *TDGF1*, *DISP1*, and *FOXA2*. Systematic mutation analyses of the four main genes in patients with non-syndromic HPE and normal karyotype identified sequence changes at a rate of about 24% (David *et al*, unpublished; Lacbawan and Muenke, unpublished).[9–18] However, the underlying aetiology remains unknown in the majority of non-syndromic HPE cases. Potential causes include (*a*) unknown teratogens, (*b*) mutations in regulatory elements of known HPE genes, (*c*) mutations in additional HPE genes, or (*d*) microdeletions that involve either all or part of an HPE gene. As no systematic investigations for microdeletions in HPE genes have been reported previously, we set out to use multicolour fluorescent in situ hybridisation (FISH) and real time quantitative PCR (qPCR) to detect submicroscopic rearrangements in a large cohort of patients with non-syndromic HPE. We detected microdeletions in each of the four main HPE genes (*SHH*, *ZIC2*, *SIX3*, and *TGIF*).

MATERIALS AND METHODS

Patients

Studies involving patients with HPE were approved by the institutional review boards at the University of Rennes and the National Human Genome Research Institute. Patient samples were independently collected by the Rennes HPE study group and the Medical Genetics Branch, National Institutes of Health. Of the combined collection of 424 karyotypically normal cases with no mutation in the major HPE genes, 339 patients had severe HPE (that is, central nervous system (CNS) findings consistent with HPE), whereas 85 had HPE microsigns including facial clefting, single maxillary central incisor, and others. In addition, we included 10 samples from patients with HPE who had known cytogenetic anomalies as controls for multicolour FISH and for qPCR experiments. They included two unrelated patients

Abbreviations: BAC, bacterial artificial chromosome; CNS, central nervous system; Ct, threshold cycle number; FISH, fluorescent in situ hybridisation; HPE, holoprosencephaly; LCL, lymphoblastoid cell lines; qPCR, quantitative PCR

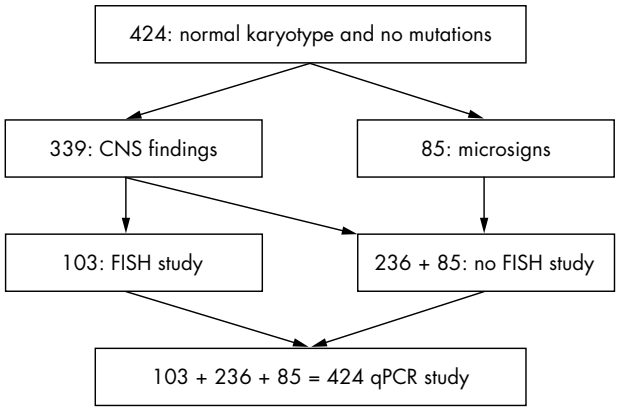

Figure 1 Description of the study.

with del(2)(p21), one with del(18)(p11.2pter), one with r(18)(p11.31q23), one with dup(18p), one with r(7)(p22q36), one with del(7)(q36qter), two unrelated patients with del(13)(q32), and one with t(2;10)(p21q26). For the FISH study, we used lymphoblastoid cell lines (LCL) from 103 patients with the severe CNS form, normal karyotype, and no detectable sequence mutations in the four major HPE genes. For the qPCR study, we screened the same 103 samples and an additional 321 DNA samples from unrelated patients with either severe HPE (with CNS findings; 236 samples) or HPE microsigns (85 samples) (fig 1).

FISH

For the FISH study, selected bacterial artificial chromosome (BAC) probes were obtained from the human RP11 library (BACPAC Resources, Children's Hospital Oakland Research Institute, Oakland, CA, USA; http://bacpac.chori.org/). A panel of six FISH probes containing four HPE genes and two candidate genes was selected from the NCBI (www.ncbi.nlm.nih.gov/), UCSC (www.genome.ucsc.edu/) and Ensembl (/www.ensembl.org/) databases: *SHH* on 7q36 (RP11-69O3), *TGIF* on 18p11 (RP11-113J12), *ZIC2* on 13q32 (RP11-12G12), *SIX3* on 2p21 (RP11-3H18), *DISP1* on 1q41 (RP11-455P21), and *FOXA2* (*HNF3β*) on 20p11 (RP4-788L20). To confirm that the desired gene sequences were present in the chosen BACs, we PCR amplified the first and last exons for each of the targeted genes (data not shown).

For the multicolour FISH experiments, three BACs were labeled by nick translation with one fluorescent dye each: *TGIF* with Cy3-dUTP (Amersham Biosciences, Buckinghamshire, UK) which yields a red signal after image processing; *ZIC2* with biotin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) secondarily detected with fluorescein isothiocyanate avidin DCS (Vector Laboratories Inc., Burlingame, CA, USA), which results in green; and *FOXA2* with Cy5-dUTP (Amersham Biosciences), which gives a blue colour. Three other BACs were labelled with two different dyes (*SHH* with Cy3-dUTP and Cy5-dUTP, magenta signal; *SIX3* with Cy3-dUTP and biotin-11-dUTP, yellow signal; and *DISP1* with biotin-11-dUTP and Cy5-dUTP, cyan signal).

FISH analysis of metaphase chromosomes from HPE patients was performed as described previously.[19] Scoring of metaphases and digital image acquisition were performed using a 100× objective lens mounted on a Leica DMRBE microscope (Leica, Wetzlar, Germany). The correct chromosomal localisation of each BAC probe was confirmed using standard FISH mapping on normal 46,XX chromosome spreads (fig 2). This panel of probes was applied on LCL

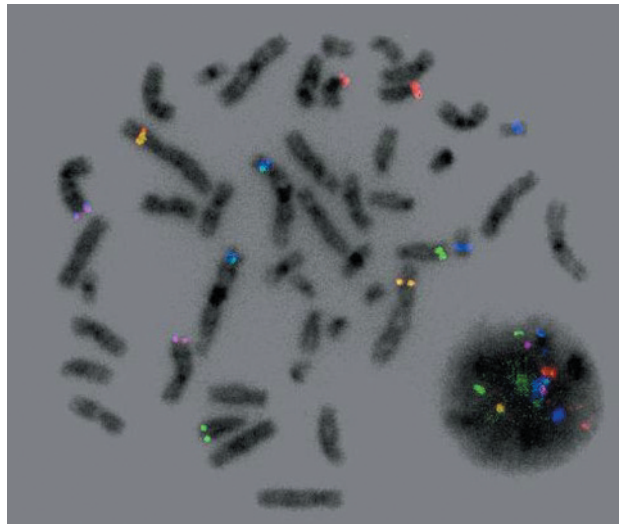

Figure 2 Correct chromosomal localisation of the six FISH probes. Each probe in the panel can be identified based on its unique colour and its chromosomal location. *DISP1* on 1q41 is cyan, *SIX3* on 2p21 is yellow, *SHH* on 7q36 is magenta, *ZIC2* on 13q32 is green, *TGIF* on 18p11 is red, and *FOXA2* on 20p11 is blue.

chromosome spreads of 10 positive control HPE patients with known rearrangements, and 103 coded patients with severe HPE (with CNS findings and normal karyotypes). At least 15 metaphases were analysed for each case. All 10 karyotypes of patients with known rearrangements hybridised as expected.

Real time qPCR reactions, *Taq*Man primers, and probes

Patient and control DNA was extracted from peripheral blood or LCL using a QIAamp DNA Blood Kit (Qiagen, www.qiagen.com) or by the classical phenol/chloroform method. The presence of microdeletions in the genes of interest was detected by real time quantitative PCR (qPCR) using the TaqMan assay system (Applied Biosystems, Foster City, CA, USA). For the *SHH*, *TGIF*, *SIX3*, *DISP1* and *FOXA2* genes, gene-specific *Taq*Man primers and probes (table 1) were designed following the instructions of the Assay by Design service (Applied Biosystems). The probes contained a 5′FAM reporter fluorophore and a 3′TAMRA quencher. During PCR amplification of the target sequence, the reporter fluorescent emission increased and was recorded. Each DNA sample was examined in triplicate for both the gene specific products and for *RNaseP*, an endogenous control gene. qPCR was carried out on either an ABI Prism 7900HT or an ABI Prism 7000 PCR machine (Applied Biosystems) in 96 well optical plates. Each PCR reaction contained 1× *Taq*Man Universal PCR Master Mix, 1× primer/probe *Taq*Man reaction assay, 100 ng DNA, and HPLC grade water to a final volume of 50 µl. All reactions on one plate were taken in aliquots from one PCR master mix. In addition to patient genomic DNA, each reaction plate contained the same known normal control genomic DNA sample (diploid for all targets) and control with no template (background). Thermal cycling conditions included a preliminary run of 2 minutes at 50°C and 10 minutes at 95°C. Cycle conditions were 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Primers and SYBR Green qPCR reactions

A second set of primers for the *SIX3*, *SHH*, *ZIC2, TGIF* genes and for *GAPDH* (internal reference; table 1) were designed using Primer Express software (Applied Biosystems). QPCR was carried out in 96 well optical plates essentially as

Table 1 Amplicons, primers, and probes

Gene	Forward primer (5′to 3′)	Reverse primer (5′ to3′)	*Taq*Man probe*
SIX3	cccaaccaccatctagcactac	catcagcgtttgactgtctgttg	accccaaaggacccc
SIX3	tcctgagcaaaaaggcaaagat	ctgaatgcatactgtggtgaagtg	SYBR Green
SHH	tctatccagggccctaactgtag	gccaatttgtcacgtccccttaaa	tcgccccaccctctc
SHH	cagtcaaggaacacagcaaagagat	tggtgagatggtcgatgcat	SYBR Green
ZIC2	tcagacggatgttttcagtctca	ggacgcagaacagcacagagt	SYBR Green
ZIC2	cgaaagagagaatgggtcaaaga	gcacatgcagagacctcacttc	SYBR Green
TGIF	ggaggactgacaggtctagaga	cacagagcccctcacttattcc	cacgcgctgtctgttg
TGIF	tgggatcagagcgtcctgtt	cagccgactctcccgtaact	SYBR Green
TGIF	ccggacctcaaccaggactt	tctctgcagcccgtttgagt	SYBR Green
DISP1	atttgggccagtgacctcaa	tgctctttgatgtccccttcc	cctgctccctaaattt
DISP1	gactttccaatcgactgaggtatca	gtttgtggctttggtctatgtgaaa	actgacagaaaatcc
FOXA2	gcctgaagccgtcgtctt	ccgcagatacctcctactacca	tcccggcccattatg
FOXA2	caccttcaggaaacagtcgttga	gcagcgctggcagaac	ccatccgccactcgct
GAPDH	ctccccacacacatgcacttac	cctagtcccagggctttgatt	SYBR Green

Primers were designed for two or three distinct loci from each target gene. Primer pairs for each amplicon, associated with SYBR Green or a TaqMan probe, are shown. SYBR Green associated primers were designed in the laboratory with Primer Express software; primers and their associated *Taq*Man probes were designed using the Primer by Design services from Applied Biosystems. *FAM-5′ to 3′-TAMRA.

described above, but in a final PCR reaction volume of 12 µl. Each PCR reaction contained 1× SYBR Green Universal PCR Master Mix, 10 pmol of each primer, 20 ng gDNA, and HPLC grade water to a final volume of 12 µl.

Quantitative PCR data analysis

Data evaluation was carried out using the ABI Prism sequence detection system and Microsoft Excel software (www.microsoft.com). Each sample was run in triplicate for the quantification of the HPE genes compared with the internal control gene (*RNaseP* or *GAPDH*). The threshold cycle number (Ct), which represents the PCR cycle number at which the detected fluorescence reaches a fixed threshold, was determined for all PCR reactions. Data analysis was performed only for samples with three amplifications and low standard deviation. We first confirmed equal amplification efficiencies for each target gene and endogenous control by creating a standard curve (log of gDNA dilution plotted against dCt) for each assay. Amplification efficiencies for all assays close to 100% were obtained and the difference in slope values of the standard curves between target and control genes was <0.1 for all assays.

These findings validate the use of the comparative Ct method (ddCt), previously described by Livak,[20] to calculate the target gene copy number from our qPCR results. Using the described calculations, the ratio of patient DNA copy number per cell divided by control DNA copy number per cell is $2^{(-ddCt)}$. A ratio about 1 for a diploid sample and about 0.5 for a haploid sample were obtained (fig 3). Standard deviations of ratios ($2^{(-ddCt)}$) were calculated for each gene. Ratios that were below a threshold equal to the average ratio value minus 2SD were interpreted as being deleted for the gene in question.

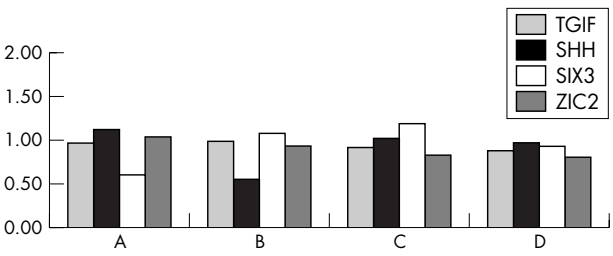

Figure 3 qPCR Results for patients with normal or deleted HPE gene loci. Patients C and D are normal with a DNA copy number per cell ratio equal to 1. Patients A and B show a ratio close to 0.5 for *SIX3* and *SHH* respectively, which reveals microdeletions for these gene loci.

RESULTS

FISH study on 103 lymphoblastoid cell lines

In total, 103 LCL from karyotypically normal HPE cases with the severe form were screened by multicolour FISH. We identified seven previously unknown microdeletions: one for *SHH*, one for *SIX3*, two for *TGIF* (fig 4), and three for *ZIC2*. In addition, 10 cell lines with known rearrangements were included as controls for the FISH experiments. All 10 controls were identified correctly with both methods. Interestingly, one HPE patient with cytogenetically known 18p duplication was found to have a deletion of the *TGIF* gene on the abnormal chromosome. Using a chromosome 18 centromeric probe (Alpha satellite (D18Z1) probe/red; Vysis), this patient was found to have an inversion/duplication of 18p with a submicroscopic deletion of *TGIF* (fig 5).

qPCR screening of 424 HPE DNA samples

Cytogenetically visible deletions in patients with HPE are more frequently found in 18p (containing *TGIF*), 13q32 (*ZIC2*), 7q36 (*SHH*), and 2p21 (*SIX3*) than in 1q41 (*DISP1*) or 20p11 (*FOXA2*). For this reason, and because we did not identify any microdeletions involving *DISP1* and *FOXA2* by FISH in our initial panel of samples from 103 patients, we chose not to test these genes by qPCR in our expanded study.

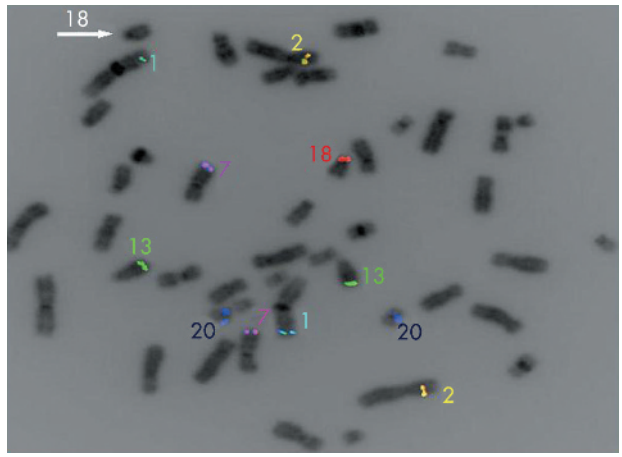

Figure 4 Multicolour FISH analysis for *TGIF* deletion. The HPE six probe panel of a case with deletion of the *TGIF* gene on chromosome 18 (white arrow) that was not detected by high resolution karyotype analysis. No other abnormalities were seen.

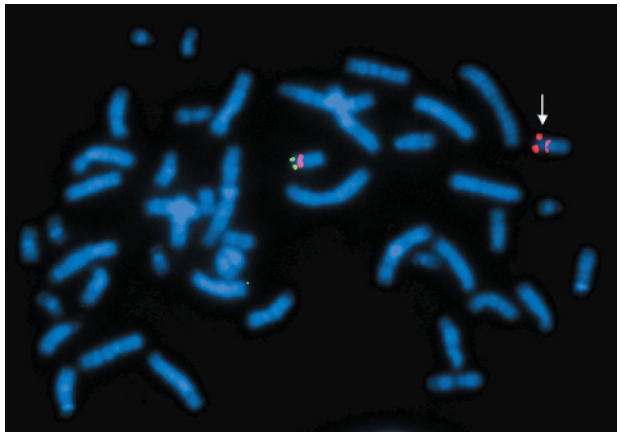

Figure 5 18p duplication and *TGIF* deletion. FISH analysis using a chromosome 18 centromeric probe (red) and a *TGIF* probe (green) shows inversion/duplication of 18p and a deletion of the *TGIF* gene on the same chromosome (white arrow). The other chromosome 18 is normal.

Based on the sensitivity and specificity of qPCR testing and the fact that this methodology is more appropriate for large scale screening, we proceeded with microdeletion testing by qPCR for the whole HPE patient cohort. All microdeletions previously detected by FISH on the 103 LCL were correctly confirmed by qPCR. Microdeletions identified by qPCR were confirmed by FISH or a second set of qPCR primers located in another part of the gene (table 1) when cell lines were not available.

In 424 samples included in this study, we found the following microdeletions: seven in *SHH*, four in *SIX3,* three in *ZIC2*, and two in *TGIF*. All microdeletions were found in 16 of 339 patients (4.7%) with severe HPE (with CNS findings who had a normal karyotype and were mutation negative). No microdeletion was present in 85 individuals who had HPE microsigns.

DISCUSSION

Based on our experience with patients who have HPE and the fact that genomic rearrangements have now been reported in many genes, we hypothesised that small submicroscopic deletions could lead to a partial or total deletion of one of the reported HPE genes. In this study, we developed and compared multicolour FISH and qPCR for the identification of microdeletions for the four main HPE genes and two candidate genes. One advantage of cytogenetic analysis and multicolour FISH is that it allows the detection of somatic chromosomal mosaicism, which has been described in some patients with HPE. A major disadvantage of FISH analysis, however, is that the average FISH probe size in our panel is between 100 and 150 kb, which is larger than the targeted HPE genes, and potentially can lead to false negative results. This may explain why one microdeletion could appear positive by qPCR but negative by FISH, as qPCR permits the analysis of very small sequences, ranging from 50 to 150 bp, allowing the detection of deletions of individual exons. Other advantages of qPCR include the ability to study HPE cases where only DNA is available and no chromosomes can be obtained (for example, spontaneous abortions). Lastly, real time quantitative PCR is less time consuming than FISH. However, base pair changes within the primer or *Taq*Man probe annealing sites may yield false positive results. In addition, DNA quality is critical for qPCR analysis; we had best results when DNA was column extracted from blood or LCL (for example, using the QIAamp DNA Blood kit or QIAamp DNA Mini kit, respectively). In summary, qPCR is more applicable for large patient cohorts, as shown in our study.

Using qPCR and/or multicolour FISH, we were able to demonstrate that microdeletions in *SHH, SIX3, ZIC2*, and *TGIF* were present in 16 of 339 patients (4.7%) with CNS findings consistent with HPE, normal karyotype, and no mutation. In contrast, no microdeletion was found in individuals who are on the mild end of the HPE spectrum, (those who have structurally normal brain findings and HPE associated facial anomalies). It is of interest that the incidence of microdeletions detected in HPE patients (4.7%) is similar or somewhat higher than the mutation frequency in some reported HPE genes (*TGIF*, *SIX3*; 1–3%), but smaller than that in *SHH* and *ZIC2* (8–10%). Recently, large scale polymorphisms were described in normal subjects[21 22] and published online (The Centre for Applied Genomics Database of Genomic Variants; http://projects.tcag.ca/variation) but did not involve the loci tested in our study. Consequently, based on these data, we believe that microdeletions in HPE genes are not present in normal subjects.

Although no deletions were found in two HPE candidate genes (*DISP1* and *FOXA2*), the methods applied in our study could be easily used to test other HPE susceptibility genes for submicroscopic rearrangements. To determine the size of the deletions and potential involvement of neighbouring genes, qPCR with primer sets extending from the vicinity of the deleted gene to either side could be employed.

Based on our results, a study for submicroscopic deletions in patients with non-syndromic HPE should be considered as part of the routine laboratory evaluation, in addition to high resolution chromosomal and mutation analysis. Positive results in any of these studies will help to better understand the aetiology of HPE and aid in recurrence risk counselling for families.

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