ORIGINAL INVESTIGATION

A new locus for split hand/foot malformation with long bone deficiency (SHFLD) at 2q14.2 identified from a chromosome translocation

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Abstract Split hand/foot malformation (SHFM) with long bone deficiency (SHFLD) is a distinct entity in the spectrum of ectrodactylous limb malformations characterised by associated tibial a/hypoplasia. Pedigrees with multiple individuals affected by SHFLD often include non-penetrant intermediate relatives, making genetic mapping difficult. Here we report a sporadic patient with SHFLD who carries a de novo chromosomal translocation t(2;18)(q14.2;p11.2). Characterisation of the breakpoints revealed that neither disrupts any known gene; however, the chromosome 2 breakpoint lies between *GLI2* and *INHBB*, two genes known to be involved in limb development. To investigate whether mutation of a gene in proximity to the chromosome 2 breakpoint underlies the SHFLD, we sought independent evidence of mutations in *GLI2*, *INHBB* and two other genes (*RALB* and *FLJ14816*) in 44 unrelated patients with SHFM, SHFLD or isolated long bone deficiency. No convincing pathogenic mutations were found, raising the possibility that a long-range *cis* acting regulatory element may be disrupted by this translocation. The previous description of a translocation with a 2q14.2 breakpoint associated with ectrodactyly, and the mapping of the ectrodactylous *Dominant hemimelia* mouse mutation to a region of homologous synteny, suggests that 2q14.2 represents a novel locus for SHFLD.

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Introduction

Ectrodactyly or split hand/foot malformation (SHFM) has a birth prevalence of ∼1 in 18,000 (Czeizel et al. 1993) and presents in a variable fashion. The archetypal form involves deficiency of the central rays of the hands and feet, characterised by median clefts associated with syndactyly of the remaining digits and aplasia and/or hypoplasia of the phalanges, metacarpals and metatarsals (MIM 183600). Alternatively oligo- or monodactyly may occur, often in association with hemimelic deficiency of the distal long bones of the upper or lower limb, with the radius and tibia being more frequently involved. This variation may be found in the same pedigree or even on different limbs of a single individual.

Isolated SHFM is genetically heterogeneous and five loci have been mapped: *SHFM1* on chromosome 7q21 (MIM 183600), *SHFM2* on Xq26 (MIM 313350), *SHFM3* on chromosome 10q24 (MIM 600095), *SHFM4* on chromosome 3q27 (MIM 605289), and *SHFM5* on chromosome 2q31 (606708). Of these, only intragenic mutations in the

TP73L gene encoding the P63 protein (on 3q27) have been identified as a relatively rare cause of SHFM (Ianakiev et al. 2000; van Bokhoven et al. 2001). *TP73L* mutations more frequently cause EEC syndrome (MIM 129900), which includes ectrodactyly, ectodermal dysplasia and cleft lip/palate (Celli et al. 1999; de Mollerat et al. 2003a). More commonly, complex chromosome rearrangements seem to underlie SHFM. The 7q21 locus is associated with both deletions and duplications, candidate genes are *DLX5*, *DLX6* and *DSS1* (Scherer et al. 1994; Crackower et al. 1996), the 10q24 locus is thought to involve rearrangements of the *FBXW4* gene encoding DACTYLIN (Ianakiev et al. 1999; Sidow et al. 1999; de Mollerat et al. 2003b) and 2q31 is associated with deletions of the *HOXD* cluster, although the *DLX1* and *DLX2* genes are also candidates (Del Campo et al. 1999; Goodman et al. 2002).

Split hand/foot malformation associated with tibial a/hypoplasia appears to be a distinct syndrome that has been termed split hand/foot malformation with long bone deficiency (SHFLD) (MIM 19100), or alternatively SHFM type II (Zlotogora 1994) and is particularly disabling owing to the associated tibial deficiency which often requires below-knee amputation. Approximately 50 families and more than 100 sporadic cases of SHFLD have been described: the largest study reported 6 families and reviewed a further 31 with more than one affected member (Majewski et al. 1985) and found the phenotype to be very variable in severity, ranging from tetramonodactyly or transverse hemimelia to hypoplastic halluces. In most cases SHFLD follows an autosomal dominant pattern of inheritance. In some families, however, all affected individuals belong to the same generation suggesting autosomal recessive inheritance, although parental consanguinity has been noted in only one instance (Emami-Ahari and Mahloudji 1974). Furthermore, there is evidence for X-linked recessive inheritance in one family (Kapur et al. 1982).

Significantly, when "pure" familial SHFM occurs, penetrance is at least 96% whereas in families in which at least one individual has additional long bone malformations (SHFLD), transmission is often unpredictable with penetrance among offspring of affected individuals being around 66%; several pedigrees have been described in which related SHFLD cases are connected via multiple unaffected intermediate relatives (Majewski et al. 1985; Zlotogora 1994). Although both SHFM and SHFLD are likely to result from similar developmental mechanisms, the marked difference in inheritance patterns suggests that these malformations are distinct entities. This complicates the classification of sporadic cases of SHFM as it is unclear whether a given case is SHFM, or SHFLD with mild expressivity.

Recently, the first two specific loci for SHFLD have been proposed at chromosomes 1q42.2-q43 and 6q14.1, based on genome wide linkage analysis of a single large family (Naveed et al. 2007). Here we report a patient with SHFLD and who carries a de novo chromosomal translocation t(2;18)(q14.2;p11.2). Initially, two pieces of evidence suggested that the 2q14.2 breakpoint might signal a locus for SHFLD. First, the report of a patient with a t(2;4)(q14.2;q35) translocation associated with holoprosencephaly and ectrodactyly suggested a common aetiology (Corona-Rivera et al. 2000). Second, deletions of the short arm of chromosome 18 are relatively frequent and are not associated with limb abnormalities while confirmed deletions of 2q14.2 are extremely rare (Schinzel 2001). We therefore characterised the breakpoints of the translocation and found that the 2q14.2 breakpoint lies between two genes, *INHBB* and *GLI2* that have both been implicated in limb development. Moreover the breakpoint coincides with the homologous region of the mouse to which the *Dominant hemimelia* (*Dh*) limb malformation mutant has been mapped (Hughes et al. 1997). We therefore screened the four genes (*GLI2*, *INHBB*, *RALB* and *FLJ14816*) in proximity to the 2q breakpoint for mutations in 44 patients with limb malformations including SHFM, SHFLD or isolated long bone deficiency; however, no clearly pathogenic mutation was found. The conserved synteny observed in a wide range of vertebrates across this region raises the possibility that a long-range *cis* acting regulatory region may be disrupted by this translocation.

Methods

Permission to undertake this work was obtained from the Oxfordshire Research Ethics Committee (reference: C99.181) and local institutional review boards. We ascertained the translocation case during a cohort study of patients requiring surgery for limb malformations. We screened an additional panel of 44 patients with SHFM or SHFLD. Of these ten were recruited from the Oxford study and included one case with SHFLD, seven with SHFM and two with long bone deficiency. All cases were sporadic except for one SHFM. The panel also included 25 patients ascertained during a previous study and in whom mutation analysis of *TP73L* was negative (de Mollerat et al. 2003a), and 6 additional cases. These 31 patients included 17 cases of SHFLD (7 familial and 10 sporadic), 13 cases of SHFM (2 familial and 11 sporadic) and a single case of long bone deficiency. Some of the SHFLD cases do not have tibial deficiency but rather a/hypoplasia of another long bone, additionally four of the sporadic SHFM cases are syndromic including three with features overlapping EEC syndrome and one with Karsch-Neugebauer syndrome (MIM 183800). Three further sporadic cases of SHFLD ascertained during a separate study (Celli et al. 1999) were also screened.

Fluorescence in situ hybridisation (FISH) mapping of the breakpoint used bacterial artificial chromosomes (BACs) obtained from Invitrogen. The chromosome 2 BACs were RP11-81B17, RP11-41C17, RP11-85B19, RP11-438012, RP11-56H11, RP11-78E20, RP11-1105G24 and RP11-416H1; the chromosome 18 BACs were RP11-341P8, RP11-119J17, RP11-24G5 and RP11-976N2 (http://www.genome.ucsc.edu/cgi-bin/hgGateway). The centromere of chromosome 18 was visualised using a specific biotinylated probe (Cambio) and detected with Texas Red. Probe generated from BAC clones was labelled with digoxygenin and detected with fluorescein.

To isolate the translocation breakpoint, single copy probes from the breakpoint spanning BAC RP11-56H11 were synthesised and hybridised to Southern blots of patient and control DNA. Initially, a 1.7 kb *Bsr*GI breakpoint fragment was identified and this enabled further localisation on Southern blots by looking for additional breakpoint fragments. Having localised the der(18) breakpoint to a 55 bp region, the breakpoint fragment was obtained by inverse PCR. Briefly, a *Bam*HI fragment was identified as spanning the breakpoint. Genomic DNA from the patient was digested with *Bam*HI, diluted to 10 ng/µl and T4 ligase was added to promote intramolecular ligation. Religated DNA was subjected to digestion with a second enzyme (*Tsp*45I) predicted to linearise the fragment and PCR was conducted using the primer pair: 5′-CCATCCGTAGTCCCATGCTGAGG-3′ and 5′-TAGGGAATGGAAGGCGATGGCTCC-3′. DNA sequencing revealed 570 bp of unknown sequence, which was shown to originate from chromosome 18 by BLAST analysis. PCR with primers located centromeric to the 2q breakpoint and telomeric to the 18p breakpoint was employed to isolate the reciprocal breakpoint from the der(2). The primer pair used to amplify the der(2) breakpoint was 5′-AGTCTTCACACAATCCAGCCACC-3′ and 5′-AAGGATAGATTGGCAGGGCATC-3′ and the primer pair for the der(18) breakpoint was 5′-GCACCTCGTTCTGTAAGCAGTCC-3′ and 5′-CCATGCAGAGAAACATTAGCGTC-3′.

Array comparative genomic hybridisation (array CGH) was performed using a human 185 K genome-wide oligonucleotide array (Agilent Technologies) according to manufacturer's instructions. In brief, genomic DNA from the translocation patient and from a sex-matched reference were separately double-digested using the restriction endonucleases *Alu*I and *Rsa*I (Promega) and purified using Microcon centrifugal filter devices (Millipore Corporation). A total of 1.5 µg of the digested products was differentially labeled by the random priming method using the fluorophores Cy3-dUTP and Cy5-dUTP (Perkin Elmer) and cohybridised to the array for 48 h at 65°C in a rotating oven. The hybridised arrays were washed and scanned using an Agilent Microarray Scanner. The image data were extracted using Agilent Feature Extraction software version 8.5 and the data analysed using Agilent CGH Analytics software version 3.4 (*z*-score method setting). These data have been submitted to the DECIPHER database (http://www.sanger.ac.uk/PostGenomics/decipher/).

The entire open reading frames of *INHBB*, *GLI2*, *RALB* and the predicted gene *FLJ14816* were screened in the patient panel. Amplification was carried out in 30 µl reactions containing 20 ng of DNA, $1\times$ Gold Buffer II (Applied Biosystems), 1.5 mM $MgCl_2$, primers at 0.5 µM, and dNTPs at 200 µM (final concentrations), with 0.75 U of AmpliTaq Gold (Applied Biosystems) and 0.1 U of *Pwo* DNA polymerase (Roche). PCR consisted of an initial incubation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s, and extension at 72°C for 30 s, with a final extension for 10 min. The product was denatured at 95°C for 5 min, followed by cooling at 1.5°C/min to allow heteroduplex formation. Denaturing high performance liquid chromatography (DHPLC) analysis was carried out as described previously on a Wave 3500HT (Transgenomic). Fragments showing abnormal elution profiles were sequenced on the ABI PRISM 3100 DNA sequencer, employing Big Dye Terminator mix (Applied Biosystems). Sequence changes were confirmed by restriction digest.

In order to identify non-coding evolutionarily conserved regions, we first determined the orthologous regions in mouse, chicken and puffer fish (*Takifugu*) genomic sequences. These sequences were compared using VISTA (http://www.genome.lbl.gov/vista/index.shtml), and noncoding regions which showed conservation above 75% over a window of 100 bp were selected. We compared selected regions with human genome-wide studies (Bertone et al. 2004) and sites of RNA polymerase II preinitiation complex (PIC) binding (Kim et al. 2005). By all these methods we identified a positive signal 61,100 bp 5′ of the first known exon of *GLI2*. We designed oligonucleotide primers complementary to this sequence and sequence in exon 1, and used them for reverse transcriptase PCR with RNA isolated from fibroblasts from unaffected individuals. We found that one of these regions (primer pairs 5′-CCGCGCCAGCCAAGGTGGGATG-3′ and 5′-AGCTACCGCTGCTGCTGCCGCTGCA-3′) is spliced to the previously identified first exon of *GLI2* suggesting that this locus represents a previously unrecognised exon of the human *GLI2* transcript (Genbank Accession no. EF547128).

Results

The patient, a male currently 11 years old, was the only child born to healthy parents with no family history of limb malformations. The pregnancy was normal and he was born

at 39 weeks of gestation. The limb abnormalities were apparent at birth and assessed in detail at the age of 6 years. The features were characteristic of SHFLD (see legend of Fig. 1). He had normal intelligence and was otherwise well, except that he developed a stricture of the urethral meatus, possibly attributable to balanitis xerotica obliterans that required a circumcision at the age of 2 years and was associated with secondary renal damage.

The G-banded karyotype revealed a de novo t(2;18)(q14.2;p11.2) translocation in all cells analysed from a peripheral lymphocyte culture (Fig. 2a). Further characterisation of the translocation breakpoints was carried out by FISH. Four BAC clones from 2q and four from the 18p breakpoint region were selected from the human genome sequence (see section "Methods") and hybridised to metaphase chromosomes from the patient. Three of the 2q probes hybridised proximally to the 2q breakpoint on the der(2), while the remaining clone hybridised distally (data not shown), indicating that the breakpoint lay within a 1.5 Mb interval. Four BACs located within this interval were selected for a secondary round of hybridisation, one of these (RP11-56H11) hybridised to the normal chromosome 2 and to both the der(2) and der(18) indicating that it spanned the breakpoint (Fig. 2b). These results confirmed that the 2q breakpoint lay within band q14.2. Of the chromosome 18 BACs, two hybridised proximally and two hybridised distally to the breakpoint; however, due to the success of the chromosome 2 hybridisation this was not further pursued. None of the clones hybridised with a reduced or absent signal on the derivative chromosomes, providing no evidence for a deletion associated with the translocation. This was further confirmed by array CGH of the patient's DNA and a reference

DNA to a high-density human 185 K genome-wide oligonucleotide array (average feature separation of ∼16 kb). At this resolution, there were no consistent deviations from two genome copies identified in association with the translocation; in addition, no novel copy number variations were observed elsewhere in the genome (data not shown).

Further characterisation of the der(18) breakpoint by Southern analysis and subsequently by inverse PCR (see section "Methods") led to identification of the breakpoint at 120,960,699 bp on chromosome 2 and at 10,368,893 bp on chromosome 18 (Fig. 3) (all nucleotide positions refer to the hg18 assembly). The breakpoint is very clean without the deletion of any nucleotides and the net addition of a single thymidine at one of the breakpoints (arbitrarily shown on the der(18) in Fig. 3b). Analysis of the normal sequences at the two breakpoints showed that both are unique, with no significant similarity except for a shared 3 bp TCA motif, indicating likely repair of the breaks by non-homologous end joining.

Neither breakpoint disrupts a known gene (Fig. 4). The 2q14.2 breakpoint lies between *INHBB* (135 kb away) and *GLI2* (305 kb away) and within 172 kb of *RALB*, whilst the 18p11.22 breakpoint lies between *APCDD1* (76 kb away) and *VAPA* (419 kb away). As outlined in the Introduction, we already favoured a more likely contribution of the 2q14.2 breakpoint to the SHFLD: the precise breakpoint mapping reinforced this for three reasons. First, several promising candidate genes lie near the 2q14.2 breakpoint. *GLI2* is a mediator of Hedgehog signalling and *Sonic hedgehog* is a key regulator of normal limb patterning as *Shh*-null mice limb buds arrest in development soon after formation (Chiang et al. 1996). *INHBB* is a member of the

Fig. 1 Clinical phenotype and radiographic appearance of upper limbs (postoperative) (**a**, **b**) and lower limbs (preoperative) (**c**, **d**) of the translocation patient aged 6 years. **a**, **b** Appearance following bilateral release of syndactyly of the ulnar digits. Both hands exhibit oligodactyly, which is more severe on the radial side. On the *right hand* the first ray is entirely absent, all phalanges of the second digital ray are absent, the third ray has a single phalanx and the phalanges of the fourth and fifth rays are deformed. On the *left side*, the head of the first metacarpal is abnormal and the phalanges are absent. Rudimentary proximal pha-

langes are present on the second and third digital rays and those of the fourth and fifth rays are deformed. The long bones of the forearm appear unaffected. **c**, **d** A single toe is present on each foot, there is also bilateral shortening of the tibiae, more marked on the right side, associated with a short, thick and bowed fibula leading to bowing of the leg and inversion of the foot. Additionally, bilateral patellar dislocation and distal flaring of the metaphases of the femur is present. *L* left side; *R* right side
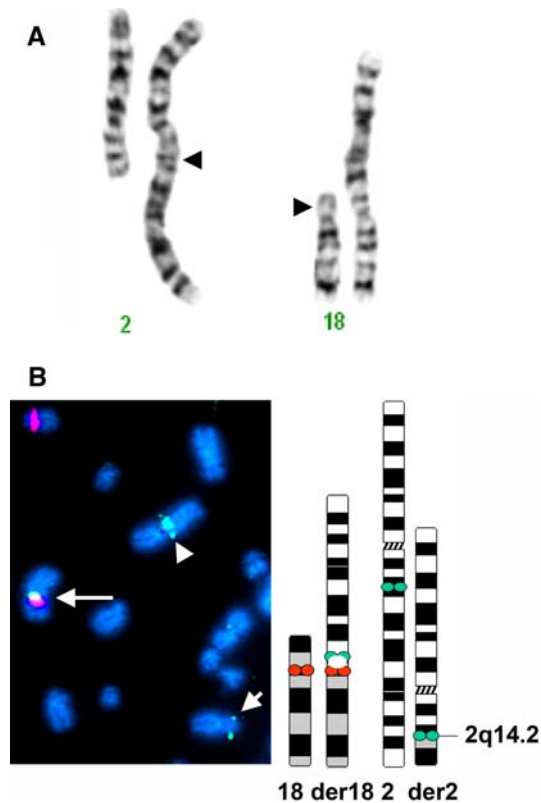
Fig. 2 a Partial G-banded karyotype of the patient showing a t(2;18)(q14.2;p11.2) translocation. *Arrowheads* indicate the position of the breakpoints. **b** Fluorescence in situ hybridisation with bacterial artificial chromosomes (*BAC*) clone RP11-56H11 from 2q14.2. The centromere of chromosome 18 is visualised as a red signal and the BAC as a green signal. Overlapping signal on der(18) appears *white*. Note hybridisation of the BAC to the normal chromosome 2 (*arrowhead*), der(2) (*short arrow*) and der(18) (*long arrow*). The BAC signal present both on der(2) and der(18) indicates that the clone spans the chromosome 2 translocation breakpoint

Fig. 3 DNA sequences from the two breakpoints of the t(2;18) (q14.2;p11.22). **a** Chromatograms showing breakpoint sequences compared with normal at the chromosome 2 and 18 breakpoints. **b** Alignment of the two normal and two breakpoint sequences. *Red capital* and *blue lower case letters* represent sequence from chromosomes 2 and 18, respectively; sequence on the derivative chromosomes is in *bold*. An extra thymidine residue (in *black bold italic*) is present at the breakpoint junction and the short homology between the chromosome 2 and 18 breakpoints at this junction is *underlined*

TGF-β superfamily, a large group of extracellular proteins involved in many aspects of development; in the chick, *Inhbb* is expressed during joint formation and ectopic application leads to abnormal chondrogenesis and joint fusion (Merino et al. 1999). *RALB* plays a role in mediating FGF signalling in *Xenopus* (Lebreton et al. 2003) and is expressed in the developing limb bud in mice (Zhao and Rivkees 2000). Second, the patient with a t(2;4)(q14.2;q35) translocation described by Corona-Rivera et al. (2000) had holoprosencephaly as well as ectrodactyly; holoprosencephaly is known to result from heterozygous mutations of *GLI2* (Roessler et al. 2003), suggesting that the 2q14.2 breakpoint in this patient was close to the *GLI2* gene. Third, the homologous chromosomal bands in the mouse are 1E2 for the 2q14.2 breakpoint and bands 18E1 and 17E1 for the 18p11.2 breakpoint. Interestingly, the spontaneous mouse mutant *Dh*, for which the underlying mutation has not been identified, maps to band 1E2. *Dh* exhibits partial or complete loss of the tibia and preaxial digits, a pattern
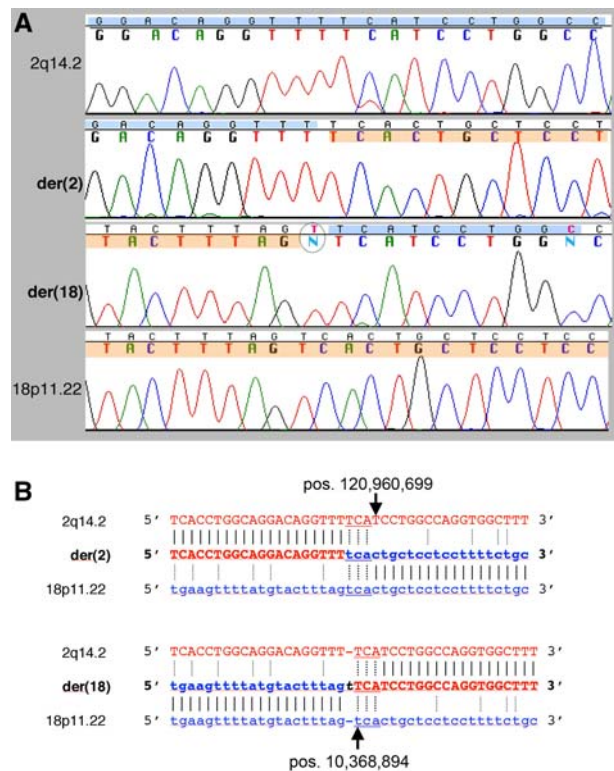
remarkably similar to the proband (Lettice et al. 1999). *Dh* was genetically indistinguishable from *Gli2* in 561 meioses, although no *Gli2* mutation was identified in *Dh* mice (Hughes et al. 1997).

To establish whether mutations in any of the genes in proximity to the chromosome 2 breakpoint (*GLI2*, *INHBB*, *RALB* or a fourth potential transcript, *FLJ14816*) underlie the ectrodactylous phenotype, we undertook mutation analysis by heteroduplex screening using Wave DHPLC in a panel of 44 SHFM, SHFLD and long bone deficiency patients (see section "Methods"). Analysis of *GLI2* revealed three non-synonymous sequence changes in the patient panel. A 2159G>A (R720H) substitution was absent in 456 normal chromosomes but this could be discounted as a pathogenic mutation because the similarly affected mother did not show the change. A 4332_4333GC>AT double nucleotide change encoding adjacent M1444I and L1445F substitutions in *cis* could also be discounted as pathogenic, both because it was inherited from the unaffected parent, and because the same double substitution
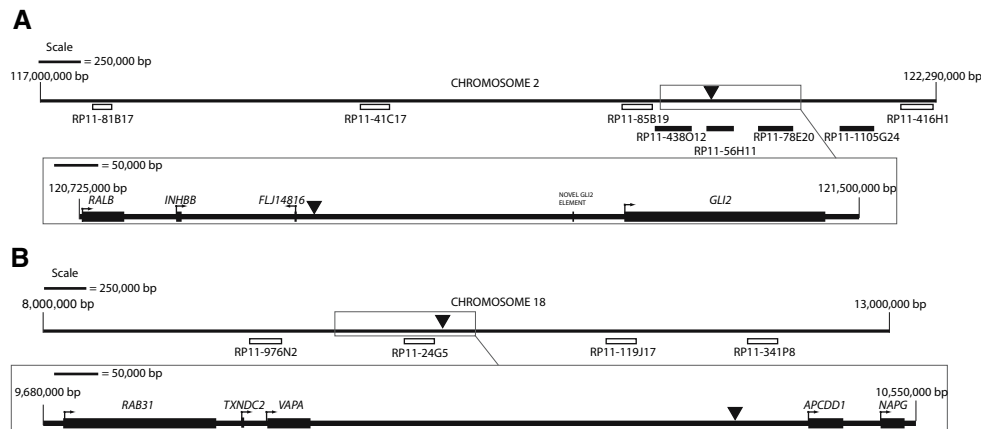
Fig. 4 Genomic maps of the regions around the 2q breakpoint (**a**) and the 18p breakpoint (**b**). BACs used during the first (*open rectangles*) and second (*filled rectangles*) rounds of hybridisation are shown, the breakpoints are represented as *arrowheads*. The boxes enclose the regions shown at higher scale in the corresponding *lower panels*, including the genes surrounding the translocation breakpoints. The direction of transcription is represented by the *arrows*. The conserved region thought to represent the transcription start of *GLI2* also shown (**a**)

was identified in 2 out of 348 normal chromosomes. Finally, a 3349G>T (V1117L) substitution was absent in 566 normal chromosomes. The alteration was not seen in the unaffected mother and the unaffected father's DNA was unavailable for analysis, so we could not determine whether or not this change had arisen de novo. However, valine 1117 is not a highly conserved residue in the human GLI proteins nor in GLI2 proteins from other species. Analysis of *INHBB* and *RALB* did not reveal any coding changes in the patient cohort. Screening of a putative transcript (*FLJ14816*) located ~20 kb from the breakpoint on chromosome 2 and predicted to comprise a single exon containing an open reading frame of 177 amino acids, was also negative.

To determine whether the translocation identified in this report affects the transcription levels of the four candidate genes in proximity to it, we screened these genes in the patient for informative polymorphisms. The proband was heterozygous for a novel T/C polymorphism in the 3′ untranslated region (UTR) of *INHBB* (1956T>C) (ss69374874) and for a known polymorphism (774T>A) (rs1065518) in the 3′ UTR of *RALB*. We amplified these transcripts from cDNA generated using RNA isolated from a fibroblast cell line derived from the patient. Amplification products were digested with a restriction endonuclease to discriminate between the alleles. Neither *RALB* nor *INHBB* showed a major difference in the expression levels of the normal and translocated alleles (data not shown). We could not amplify the predicted *FLJ14816* transcript from cDNA derived from patient fibroblasts or an immortalised B-cell line, and neither the coding sequence nor the untranslated regions of *GLI2* contained any informative polymorphisms in the proband.

Discussion

Although the limb represents a classical developmental system and the mechanisms of limb development have been described in considerable detail (Capdevila and Belmonte 2001; Tickle 2003), the pathogenesis of SHFLD/SHFM remains relatively poorly understood (Duijf et al. 2003). However, the primary defect appears to involve proximo-distal outgrowth, whereas antero-posterior and dorso-ventral polarity is usually maintained. Proximo-distal outgrowth involves inductive interactions between the apical ectodermal ridge (AER) and the underlying progress zone (PZ). Failure to maintain the normal function of the AER has been described in some models of SHFM including the *Dac* mutant mouse, a model for SHFM3 (Crackower et al. 1998), and in *Tp73l* mutant mice (Yang et al. 1999; Mills et al. 1999). The induction of cell death in the AER by treatment with retinoic acid has also been shown to result in ectrodactyly (Sulik and Dehart 1988). Given that hemimelia may also accompany ectrodactyly, it is interesting that the posteriorly placed zone of polarising activity (ZPA) is also involved in AER maintenance. Clearly the identity of novel loci and/or mechanisms that disrupt limb development would be of considerable medical and biological importance. Some insight has been gained from the *Dh* mouse in which *Fgf4* and *Fgf8* are ectopically expressed in the anterior AER and this is associated with a reduction of the anterior mesenchyme (Lettice et al. 1999).

Here we have identified a novel chromosome translocation associated with SHFLD, and characterised its breakpoints in detail. Based on the previous description of a translocation with a 2q14.2 breakpoint associated with ectrodactyly, and the mapping of the *Dh* mouse mutant to a region of conserved synteny, we propose that 2q14.2

represents a novel locus for SHFLD. This SHFLD region contains several promising candidate genes, but despite screening these genes in a panel of patients we did not find any convincingly pathogenic mutations. There are several potential explanations for this. First, the clustering of 2q14.2 breakpoints and SHFM could be a coincidence. We believe that this is unlikely given the additional evidence implicating this region in ectrodactyly (see sections "Introduction" and "Results"). Second, we may have missed rare pathogenic mutations because of patient selection or insensitivity of DHPLC screening. We cannot completely exclude this possibility, which can be investigated in future studies. Third, the limb malformation patients may have genomic rearrangements in this region of chromosome 2. This possibility may be further investigated in the future. Fourth, the translocation may affect regulation of a nearby gene. We found no significant difference in the transcription levels of *INHBB* or *RALB* in patient fibroblasts; however, such an alteration may only be evident in temporal and/or tissue-specific developmental context. Additionally, we were unable to assay the affect of the translocation on *GLI2* or *FLJ14816*. Therefore, misregulation of one or more candidate genes in this region during limb development remains a possible cause of malformations. The importance of regulatory mutations is well recognised (Kleinjan and van Heyningen 2005) and there are several examples of translocations that affect regulation of specific genes over distances of up to 1 Mb. For example, a translocation 1 Mb away from *MAF* is associated with cataract, ocular anterior segment dysgenesis and coloboma (Jamieson et al. 2002), microdeletions 900 kb proximal to *POU3F4* cause X-linked deafness (de Kok et al. 1996), a deletion 1.2 Mb away from *FOXC1* is associated with glaucoma/autosomal dominant iridogoniodysgenesis (Davies et al. 1999) and rearrangements up to 950 kb away from *SOX9* are associated with campomelic dysplasia (Pfeifer et al. 1999). Identifying the regulatory sequences involved remains a major challenge, although there have been some notable successes, one being the ZPA regulatory sequence (ZRS) which lies 1 Mb upstream of *SHH* (Lettice et al. 2002). Interestingly, several other SHFM loci may also represent regulatory mutations: recent reviews of mapped SHFM patients suggest that 79% of *SHFM1*, 15–29% of *SHFM3* and 100% *SHFM5* patients have chromosome abnormalities (Elliott and Evans 2006; Everman et al. 2006). Despite considerable efforts, the precise mechanisms of altered gene dosage remain to be defined at all these loci.

As discussed above, difficulties in associating *cis* regulatory elements with the genes they control often confounds efforts to implicate them in human disease. It seems likely that chromosomal breakage during evolution may be constrained by the requirement of *cis* regulatory elements to remain physically linked to the genes they act upon. A recent study has identified syntenic blocks on a whole-genome scale by generating multiple alignments of the human, mouse, chicken and frog genomes (Ahituv et al. 2005). Syntenic blocks are defined as chromosomal segments in which all sequences are in the same order and orientation in the species analysed, the minimum size of blocks included by Ahituv et al. (2005) was 200 kb. These evolutionarily conserved synteny blocks may delimit borders for distant *cis* regulatory regions and therefore conserved noncoding regions within them may harbour regulatory elements for the gene(s) in each block. A search of the synteny blocks reported by Ahituv et al. (2005) reveals that the 2q14.2 translocation breakpoint reported here occurs at position +1.04 Mb in a block that extends over 1.77 Mb in the human genome. This block is conserved in the mouse and chicken genomes, and is classified as within the largest 10% of such blocks, consistent with the presence of long-range *cis* regulatory elements. We conclude that any of the nine genes within this block (*PTPN4, EPB41L5, FAM11B, RALB, INHBB, FLJ14816, GLI2, TFCP2L1, CLASP1*), may be potentially affected by a regulatory mutation within the novel SHFLD region identified in this report.

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