

## Mapping of balanced chromosome translocation breakpoints to the basepair level from microdissected chromosomes

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### Abstract

The analysis of structural variants associated with specific phenotypic features is promising for the elucidation of the function of involved genes. There is, however, at present no approach allowing the rapid mapping of chromosomal translocation breakpoints to the basepair level from a single chromosome. Here we demonstrate that we have advanced both the microdissection and the subsequent unbiased amplification to an extent that breakpoint mapping to the basepair level has become possible. As a case in point we analysed the two breakpoints of a t(7;13) translocation observed in a patient with split hand/foot malformation (SHFM1). The amplification products of the der(7) and of the der(13) were hybridized to custom-made arrays, enabling us to define primers at flanking breakpoint regions and thus to fine-map the breakpoints to the basepair level. Consequently, our results will also contribute to a further delineation of causative mechanisms underlying SHFM1 which are currently unknown.

**Keywords:** breakpoint mapping • chromosome translocation • monogenic disease • unbiased amplification • oligonucleotide arrays • split hand/foot malformation

### Introduction

The elucidation of the genetic architecture of complex diseases is one of the predominant issues in current human genetics. Genome-wide association studies have identified multiple genetic variants associated with complex human diseases. However, these variants explain only relatively small increments in risk and only a small proportion of familial clustering. Therefore, strategies to explain the remaining, 'missing heritability' are required [1, 2]. So far structural variations, such as copy number variants or copy neutral variations (*i.e.* inversions and translocations) [3], have not been appropriately included in association studies yet and may therefore account for some of the unexplained heritability. Interest in the analysis of copy number variants has recently increased due to an improvement in the methods for their detection [4]. In con-

trast, the analysis of copy neutral variations has been neglected to a large extent, although the detailed analysis of rare, balanced chromosomal rearrangements associated with specific phenotypic features may facilitate the detection of rare and low frequency variants. However, their detailed analysis has been hampered because easy and fast methods to pinpoint the breakpoints of a cytogenetically visible translocation to the basepair (bp)-level were missing.

Traditionally, chromosome breakpoints were mapped with fluorescence *in situ* hybridization employing locus-specific probes. Yet these are laborious and time-consuming efforts, often yielding results with an insufficient resolution. With the advance of array technologies [5] more efficient approaches were introduced. If derivative chromosomes are hybridized to a whole-genome array the exact content of this derivative chromosome is elucidated and, importantly, breakpoints can be mapped with ease. For example, if both derivative chromosomes from a reciprocal translocation are differentially labelled and hybridized onto DNA arrays plotting of the fluorescence ratio of probes ordered along the chromosomes can reveal the breakpoint flanking region, whereas breakpoint spanning probes usually show intermediate ratios. This approach has been

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termed array painting [6]. Technologies allowing the generation of libraries consisting only of material from the respective derivative chromosome are central to such powerful approaches. For array painting initially small numbers of flow sorted chromosomes ( $n = 500$ ) were hybridized to large insert genomic clone DNA microarrays [6, 7] or also to oligonucleotide arrays designed to tile breakpoint regions at extremely high resolution [8].

However, flow sorting of chromosomes requires special, very expensive equipment, not readily available in the majority of the laboratories. Furthermore, it can only be applied when the physical properties of the derivative chromosomes allow them to be flow sorted. An alternative to flow-sorting is microdissection of chromosomes (e.g. [9–12]). Baxck *et al.* collected 4–15 chromosome copies by microdissection and hybridized the amplification products to arrays consisting of ~3500 BAC and PAC clones to map breakpoints with a one Mb resolution [9].

Here we show that amplification products from microdissected chromosomes yield reliable results on array platforms with tiling path resolution, so that flanking primer pairs to sequence breakpoints can be determined. This strategy makes rare copy neutral structural variants amenable to high-resolution analyses and may therefore contribute to the elucidation of some monogenetic diseases but also to components of the missing heritability of complex diseases. In order to demonstrate the feasibility we applied this strategy to a carrier of a balanced t(7;13) translocation and split hand foot malformation (SHFM).

## Materials and methods

### Laser microdissection and amplification of the derivative chromosomes

Cytogenetic studies were performed with PHA stimulated lymphocytes according to standard cytogenetic procedures. The cells were kept in 3:1 methanol:acetic acid and stored at  $-20^{\circ}\text{C}$  until microdissection.

Cells were dropped onto a PET membrane covered microscope slide (Zeiss, Vienna, Austria) and stained with Giemsa according to standard protocols. Isolation of the derivative chromosomes was carried out using a laser microdissection and pressure catapulting system (Zeiss). The derivative chromosomes were selected and directly catapulted into the cap of a 200  $\mu\text{l}$  Eppendorf tube containing 10  $\mu\text{l}$  digestion mix.

We performed whole genome amplification of the derivative chromosomes according to our recently published protocol [13]. In brief, we employed the GenomePlex Single Cell Whole Genome Amplification Kit (#WGA4; Sigma-Aldrich, Munich, Germany) according to the manufacturer's instructions. The usefulness of this kit for the amplification of microdissected chromosomes was recently confirmed by Hockner *et al.* [14]. Here we added some modifications. In a final volume of 10  $\mu\text{l}$ , the chromosomes were centrifuged at  $20.800 \times g$  for 10 min. at  $4^{\circ}\text{C}$ . After proteinase K digest, the DNA was fragmented and libraries were prepared. Amplification was performed by adding 7.5  $\mu\text{l}$  of  $10\times$  Amplification Master Mix, 51  $\mu\text{l}$  of nuclease-free water and 1.5  $\mu\text{l}$  Titanium Taq DNA Polymerase (#639208; Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). Samples were amplified using an initial denaturation of  $95^{\circ}\text{C}$  for 3 min.

followed by 25 cycles, each consisting of a denaturation step at  $94^{\circ}\text{C}$  for 30 sec. and an annealing/ extension step at  $65^{\circ}\text{C}$  for 5 min. After purification using the GenElute PCR Clean-up Kit (#NA1020; Sigma-Aldrich), DNA concentration was determined by a Nanodrop spectrophotometer (ND-1000; PEQLAB Biotechnologie GmbH, Erlangen, Germany). Amplified DNA was stored at  $-20^{\circ}\text{C}$ .

### Array painting

Array-comparative genomic hybridization was carried out using a whole genome oligonucleotide microarray platform (Human Genome CGH 244A Microarray Kit) and a tiling oligo array platform covering the breakpoint regions (Chr7: 95184920–97684920/Chr13: 96918402–99418402) with a resolution of 125 bp (both Agilent Technologies, Santa Clara, CA, USA). The tiling oligo array was designed using the eArray software (<https://earray.chem.agilent.com>). As a reference DNA, commercially available male DNA was used (Promega, Madison, WI, USA). A total of 250 ng reference DNA and 250 ng of the respective amplified derivative chromosomes were pooled and hybridized against each other. Samples were labelled with the Bioprime array CGH genomic labelling system (#18095011; Invitrogen, Lofer, Austria) according to the manufacturer's instructions. Briefly, 500 ng of the derivative chromosome 7/reference DNA and the derivative chromosome 13/reference DNA were differentially labelled with dCTP-Cy5 or dCTP-Cy3 (GE Healthcare, Piscataway, NJ, USA). Further steps were performed in accordance with the manufacturer's protocol (version 6.0). Slides were scanned using a microarray scanner (G2505B) and images were analysed using CGH Analytics software 3.4.40 (both from Agilent Technologies).

### Breakpoint sequencing and mutation analysis

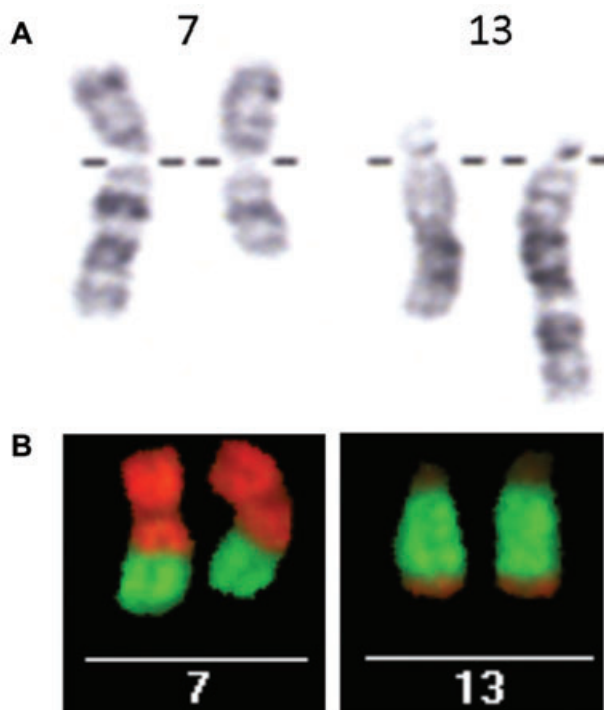
Genomic DNA was extracted from peripheral blood cells by standard methods. To map the breakpoint down to base pair level, multiple primers adjacent to the breaks were used to generate chromosome 7–chromosome 13 junction fragments for sequencing.

For the mutation screening the entire coding regions of the *DLX5*, *DLX6* and *SHFM1* genes were amplified by polymerase chain reaction. Fragments were verified on a 1% agarose gel and subsequently sequenced in both forward and reverse direction. Results were aligned and compared with the reference sequences for *DLX5* (NM\_005221.5), *DLX6* (NM\_005222.2) and *SHFM1* (NM\_006304.1). All primers and PCR conditions are available on request.

Sequencing was performed with the ABI BigDye Terminator Cycle Sequencing Kit (v3.1, #4337457; ABI, Vienna, Austria) according to the supplier's protocol and was analysed on an ABI3130 genetic analyser (ABI).

### Expression analyses of the *DLX5*, *DLX6* and *SHFM1* genes

RNA was isolated from lymphoblastoid cell lines using the PAXgene blood RNA system in accordance with the manufacturer's protocols (PreAnalytiX GmbH, Hombrechtikon, Switzerland). For cDNA synthesis we used the Omniscript RT Kit (#205113; Qiagen, Hilden, Germany) with oligo dT primers. The cDNA was subsequently used for relative expression analyses of the *DLX5*, *DLX6* and *SHFM1* genes. All primers and PCR conditions are available on request.



**Fig. 1** (A) G-banded partial karyotype showing the translocation  $t(7;13)(q21;q32)$ . (B) Hybridization of the amplification products of a single  $der(7)$  translocation chromosome (labelled in Cy3.5; red) and of a single  $der(13)$  translocation chromosome (labelled in FITC; green) to a normal metaphase spread demonstrating partial hybridizations to chromosomes 7 and 13. Complete metaphase spreads after hybridization with the  $der(7)$  and  $der(13)$  translocation chromosomes are shown in Fig. S1a–d.

## Results and discussion

Here we describe our method for the fast mapping of translocation breakpoints with basepair resolution. The  $t(7;13)(q21;q32.1)$  translocation (Fig. 1A) was observed in a 9-month-old male patient with the clinical diagnosis of SHFM1. SHFM, also known as ectrodactyly, is a congenital limb malformation involving the central rays of the autopods and presents with syndactyly, median clefts of hands and feet, and aplasia and/or hypoplasia of phalangeal, metacarpal and metatarsal bones [15, 16]. The disease locus was mapped to 7q21.3–q22.1 through the analysis of cytogenetic abnormalities such as deletions, translocations and inversions in this region in patients with SHFM [17–19] and was designated as SHFM1 (OMIM 183600). In fact, this 7q21 region had been implicated in SHFM1 in many other reports, *e.g.* [20–22]. Thus, the translocation breakpoint on chromosome 7 in our patient is consistent with the diagnosis of SHFM1.

After the patient's parents had given written informed consent, we performed array CGH with genomic DNA from this patient to rule out any gains and losses, especially close to the translocation

breakpoints. However, we observed a balanced profile (data not shown), confirming that this translocation was indeed balanced.

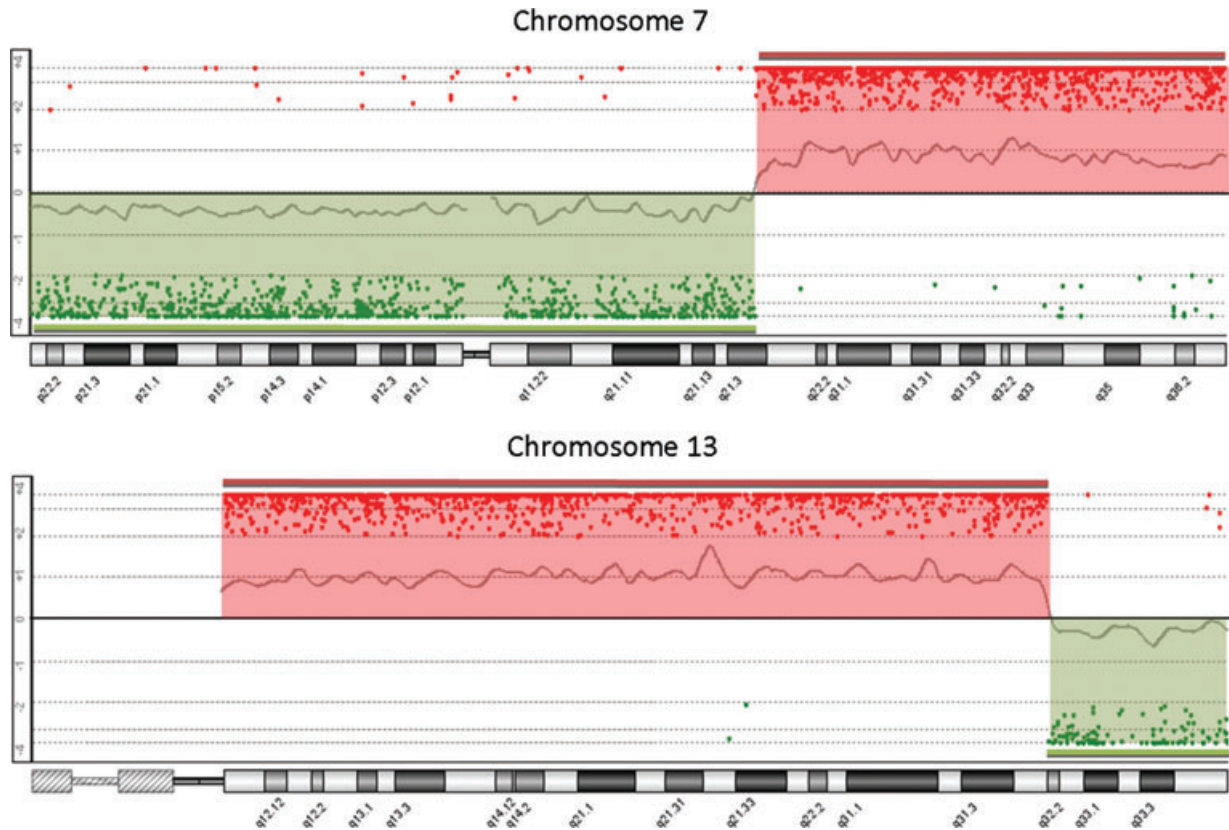
We generated lymphoblastoid cell lines of the affected child and the parents and prepared metaphase spreads. As expected, the parents were not carriers of this translocation, so that it had occurred *de novo*. Subsequently, we laser microdissected single translocation chromosomes and performed an unbiased amplification of single translocation chromosomes using a commercially available amplification kit (*i.e.* GenomPlex library technology, #WGA4-Kit; Sigma-Aldrich). For a successful amplification a single microdissected chromosome was sufficient. As a control we hybridized amplification products to normal metaphase spreads. These hybridizations confirmed that we had indeed isolated and amplified only material of the respective translocation chromosome and allowed us to assess the quality of the amplification product (Figs 1B and S1a–d). To achieve good hybridization signals as shown in Fig. 1B and in the Fig. S1a–d, the amplification product of a single microdissected chromosome was sufficient.

This enabled us to select the best six laser-microdissected chromosomes of each derivative chromosome. These were pooled and hybridized on a 244K array (Fig. 2). Based on the obtained hybridization pattern, we could already locate the chromosome 7 breakpoint to chromosomal region 96350310–96498328 and the chromosome 13 breakpoint to 98146050–98213431. We used this information to design an extremely high-resolution tiling oligoarray where each breakpoint region was covered with a probe density of one probe/125 bp. This high-density custom-made array enabled us to pinpoint the breakpoint on the derivative chromosome 7 to a 3.3 kb region (Chr7: 96463274–96466595) and the breakpoint on the derivative chromosome 13 even to a 130 bp region (Chr13: 98184265–98184394 (Fig. 3).

This information was sufficient for the design of flanking primer pairs, which we used in a final step to sequence the breakpoints. The exact location of the breakpoints is [hg 19] on chromosome 7 at position 96466302 (chromosome band 7q21.3) and on chromosome 13 at position 98184040 (chromosome band 13q32.1). There was no gene or transcriptional active region close to the chromosome 13 breakpoint (Fig. S2). The chromosome 7 breakpoint is located between genes *SHFM1* and *DLX6* and did not disrupt any presently known transcriptional active region (Fig. 4).

We checked for any characteristic features in DNA sequences around the breakpoints compared between chromosomes 7 and 13, for example, sequence similarity that may explain the occurrence of this reciprocal translocation. However, there was no evidence for long homologies or microhomologies making non-homologous end joining the most likely mechanisms for formation of this rearrangement [23].

Three potential candidate genes, *DLX5*, *DLX6* and *SHFM1* (also *DSS1*, deleted in the Split hand/foot 1 region), have been suggested to be involved in pathogenesis of SHFM1 because of their roles in limb development in mice [19]. For example, double knockout of *Dlx5* and *Dlx6* in mice resulted in typical SHFM [24]. However, and as in our case, these three candidate genes did not seem to be interrupted directly by any of the previously reported human chromosomal rearrangements [19, 25].



**Fig. 2** Hybridization of the der(7) and der(13) chromosomes to a high-density oligonucleotide consisting of about 244,000 probes. This hybridization suggested that the chromosome 7 breakpoint is between region 96350310 and 96498328 and the chromosome 13 breakpoint between region 98146050 and 98213431.

Due to the importance of *DLX5* and *DLX6* for limb development [24] and the previously suggested involvement of the *SHFM1* gene in SHFM1 we sequenced the entire coding region of these genes, however, a mutation was not observed. This confirms previous reports that no gene mutations associated with SHFM1 have been reported to date [19]. Previously it was suggested that disruption of distant cis-acting regulatory elements or positional effects may result in aberrant expression of *SHFM1*, *DLX5* and *DLX6* [19, 26]. Such a putative cis-acting regulatory element is likely tissue specific and may be active only in osteoblasts because our expression analysis of *DLX5*, *DLX6* and *SHFM1* did not show significant differences in the lymphoblastoid cell lines of the patient compared to his parents. Alternatively, a gene not yet identified may exist in the SHFM1 region. Detailed genomic analysis of the 7q21.3 region as shown here will contribute to an improvement of our understanding of SHFM1.

More importantly, we have shown here that chromosome breakpoints can now be mapped with ease with basepair resolution. The growing awareness that high-resolution tools are needed for the characterization of copy-neutral chromosomal rearrangements is also reflected in other efforts. For example, shotgun sequencing of flow-sorted derivative chromosomes using 'next-generation' (Illumina/Solexa) multiplex sequencing-by-synthesis technology

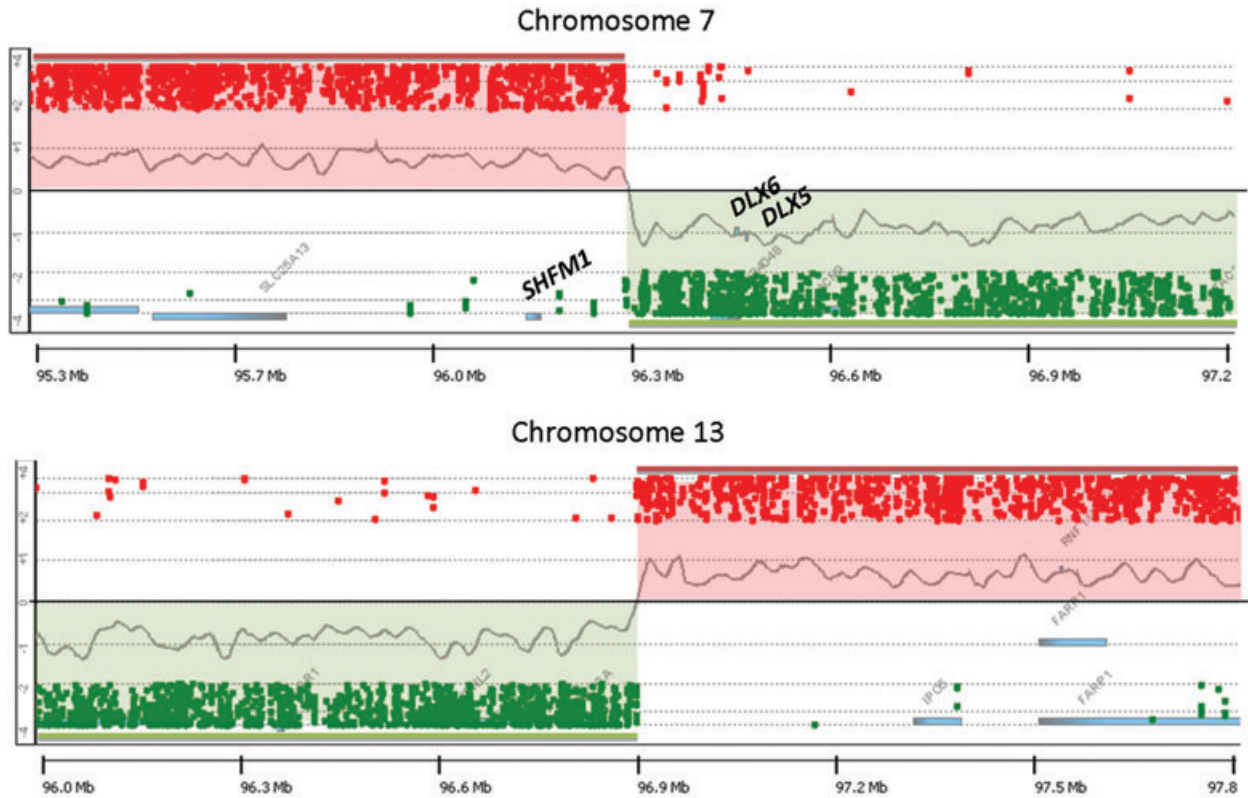
was recently described as another promising approach for breakpoint mapping [27]. Furthermore, the use of a massively parallel paired-end sequencing approach to characterize chromosome rearrangement breakpoints with a resolution sufficient for subsequent PCR amplification and Sanger sequencing of junction fragments was reported [28]. However, the authors noted that sequencing costs can be reduced significantly by construction of libraries from flow-sorted derivative chromosomes [28]. To this end Weise *et al.* microdissected six chromosomal regions from metaphase spreads and performed high-throughput sequencing after amplification. The results suggest that sequence reads of sufficient quality can be obtained from as few as six chromosomal fragments [29].

In summary, the abovementioned strategies make rare copy neutral structural variants amenable to high-resolution analyses and may therefore contribute to the elucidation of some components of the missing heritability of complex diseases.

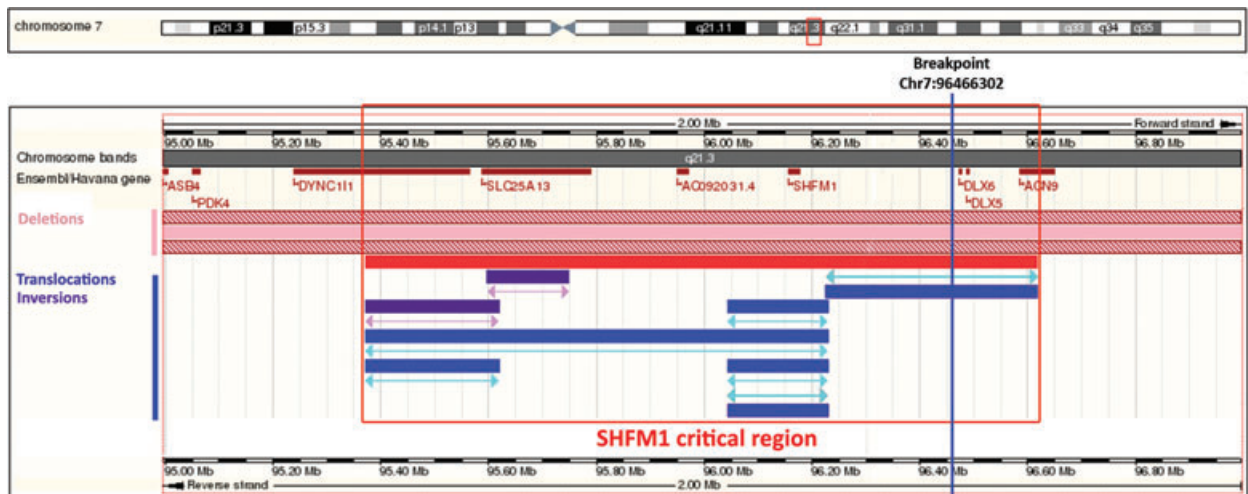
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**Fig. 3** Hybridization of the der(7) and der(13) chromosomes to custom made arrays of the respective breakpoint region with a probe spacing of one oligonucleotide every 125 bp.



**Fig. 4** Exact localization of the chromosome 7 breakpoint between genes *SHFM1* and *DLX6*. The red frame indicates the critical region of the *SHFM1*-syndrome based on cases with deletions, inversions and breakpoints. Pink bars indicate patients with deletions, violet bars indicate inversions and blue bars translocation regions which were not mapped to the basepair level. Patient data were retrieved from the Decipher database (<https://decipher.sanger.ac.uk>), the data in this image are based on the Ensembl Genome Browser, Release 57, hg19).

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## Conflict of interest

The authors confirm that there are no conflicts of interest.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1** Metaphase and respective karyotypes showing the hybridization patterns of a single microdissected der(7) translocation chromosome (**a–b**) and of a single der(13) translocation chromosome (**c–d**).

**Fig. S2** Exact localization of the chromosome 13 breakpoint. (The data in this image are based on the Ensembl Genome Browser, Release 57, hg19.)

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