Balanced translocation in a patient with craniosynostosis disrupts the SOX6 gene and an evolutionary conserved non-transcribed region

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Abstract

Introduction: Craniosynostosis is a congenital developmental disorder involving premature fusion of cranial sutures resulting in an abnormal shape of the skull. Significant progress in understanding the molecular basis of this phenotype has been made for a small number of syndromic craniosynostosis forms. Nevertheless, in the majority of the ~100 craniosynostosis syndromes as well as in non-syndromic craniosynostosis the underlying gene defects and pathomechanisms are unknown. Here we report on a male infant presenting at birth with brachycephaly, proptosis, midfacial hypoplasia and low set ears. Three-dimensional cranial computer tomography showed fusion of the lambdoid sutures and distal part of the sagittal suture with a gaping anterior fontanelle. Standard chromosome analysis revealed a de novo balanced translocation t(9;11)(q33;p15). Mutations in the genes for FGFR2 and FGFR3 as well as the P252R mutation in FGFR1 were excluded.

Methods: Mutation screening was done by DNA sequencing. Translocation breakpoints were mapped by FISH and subsequently cloned using a PCR-based strategy. Functional analysis of the breakpoint regions was based on evolutionary comparison of the sequence content and RT-PCR experiments.

Results: The breakpoint on chromosome 11p15 disrupts the SOX6 gene, a gene known to be involved in skeletal growth and differentiation processes. SOX6 mutation screening of another 104 craniosynostosis patients revealed one missense mutation leading to the exchange of a highly conserved amino acid (p.D68N) in a single patient and his reportedly healthy mother. The breakpoint on chromosome 9 is located in a region without any known or predicted genes but, interestingly, disrupts patches of evolutionary highly conserved non-genic sequences and may thus lead to a dysregulation of flanking genes on chromosome 9 or 11 involved in skull vault development.

Conclusions: The present case is one of the very rare reports of an apparently balanced translocation in a patient with syndromic craniosynostosis and reveals novel candidate genes for craniosynostoses and cranial suture formation.
Introduction

Craniosynostosis is the premature fusion of one or more cranial sutures and is a relatively common birth defect with a prevalence of one per 2,100 to 3,200 births [1]. The etiology of craniosynostosis is heterogenous and it often occurs as an isolated feature (non-syndromic), although it can also be associated with other malformations as is the case in about 100, mostly autosomal-dominant, syndromes [2]. Clinically, the premature fusion of calvarial bones variably results in cranial and facial asymmetry, brachy- or turricephaly, mid-face hypoplasia, hypertelorism with exorbitism, and, depending on the timing and type of affected sutures, neurological complications.

Our current knowledge on the pathomechanisms of craniosynostosis and on suture biology is mainly based on the identification of genes and gene defects in a few autosomal-dominant craniosynostosis syndromes. Dominant-negative mutations, causing a ligand-independent activation of fibroblast growth factor receptors 1 – 3 were found in different syndromes, including Pfeiffer-, Crouzon-, Jackson-Weiss-, Apert- and Muenke syndrome [2][3]. Mutations in the MSX2 gene, encoding a homeobox transcription factor, were identified in a family with craniosynostosis Boston type, and loss-of-function mutations in the transcription factor gene TWIST were identified in a subset of patients with Saethre-Chotzen syndrome [2][3]. Nevertheless, the molecular basis of many syndromic craniosynostosis forms is still unknown. This also applies to non-syndromic craniosynostoses, which are sporadic in most cases, account for approx. 80 % of all craniosynostoses and involve both environmental and genetic risk factors (Review: Cohen, 2000). Thus the identification of novel candidate genes is of major importance for the molecular understanding of the aetiology and pathomechanisms of these disorders.

Here we report on a male infant with a de novo balanced translocation t(9;11)(q33;p15) and a complex craniofacial dysostosis, including craniosynostosis and distinct facial features. Cloning of both breakpoint regions revealed that the translocation disrupts the SOX6 gene on chromosome 11p15, and a region riddled with highly conserved non-genic sequences (CNGs) on chromosome 9. This is one of the very rare reports of an apparently balanced translocation in a patient with syndromic craniosynostosis. The present study adds novel genes to the current list of candidate genes that may be involved in craniosynostoses and cranial suture formation in general.
Patient, Materials, and Methods

Patient

The patient was born after an uneventful pregnancy to non-consanguineous parents (31 year-old mother and 38 year-old father) of German origin. At birth (week 38+3) length was 51 cm (50th centile), weight 2670 g (< 10th centile) and occipito-frontal circumference (OFC) 33 cm (just above 10th centile). The neonate presented with a flat midface, flat supraorbital ridges, a high forehead, downslanting palpebral fissures, low set and posteriorly rotated ears, a wide anterior fontanelle and muscular hypotonia. There were no associated features of hand or feet such as syndactyly, brachydactyly or broad first rays. The suspicion of bilateral lambdoid and posterior sagittal craniosynostosis was supported radiographically (Figure 1). Results of brain, cardiac and renal ultrasound studies, of routine neonatal laboratory screening including thyroid function, and of eye and ear examination gave normal results. A conventional cytogenetic analysis on G-banded metaphases of peripheral lymphocytes of the patient and his parents revealed the karyotype 46,XY,t(9;11)(q33;p15) de novo in the patient. Successful release surgery of the lambdoid and posterior sagittal suture was performed at the age of 5.5 months.

The anthropometric data remained within normal range: now at 5.5 years of age length is 107 cm (10th centile and within parental target centile range), weight is 17 kg (25th centile), and OFC is 51 cm (25th – 50th centile). There is mild developmental delay of speech (on average 5-word sentences, attends normal kindergarten with 1 additional hour per week for speech therapy). Repeated hearing exams have yielded normal results. Family history and parental examination were unremarkable with respect to craniosynostosis or other skeletal features.

In order to exclude mutations in the two genes known to be mutated in Crouzon syndrome we screened the complete coding sequence of the \( \text{FGFR2} \) and \( \text{FGFR3} \) genes. No mutation was found which could account for the phenotype (data not shown). In addition, we excluded the P252R mutation in the \( \text{FGFR1} \) gene.

Cytogenetic and fluorescence in situ hybridisation (FISH) studies

Cytogenetic analysis was conducted on G-banded chromosomes of cultured peripheral blood lymphocytes. Initial FISH experiments were performed with YAC clones from the regions of interest. Fine mapping of breakpoints was performed with smaller BAC and PAC clones. DNA samples were prepared according to standard protocols and were labeled with either biotin-16-dUTP or digoxigenin-11-dUTP, by nick translation. Immunocytochemical detection of probes was performed as described elsewhere [4]. Chromosomes were counterstained with 4’6-diamino-2-phenyl-indole (DAPI). Metaphases were analyzed with a Zeiss epifluorescence microscope.

Molecular investigations

Genomic DNA was extracted from peripheral blood lymphocytes. For mutation screening all 18 coding exons of the \( \text{FGFR2} \) and \( \text{FGFR3} \) genes were amplified by PCR using intronic primers from \( \text{FGFR2} \) (sequences can be obtained on request) and \( \text{FGFR3} \) [5]. Direct cycle sequencing was carried out by using the PE Big Dye Terminator sequencing kit on an ABI377 and an ABI3730 automatic sequencer with gene-specific primers. PCR amplification and Southern blot analysis were performed according to standard protocols. As probes for the Southern blot analysis we used three different PCR fragments, a 417 bp PCR fragment (probe 1; fw-primer 5’-ttactgaggaatggagata-3’; rev-primer 5’-gcgccgtttggatagctc-3’), a 402 bp fragment
(probe 2 ; fw-primer 5′-gagaataggtagctatccatg-3′; rev-primer 5′-cagtgccatcagcatcaag-3′), and a 422 PCR fragment (probe 3 ; fw-primer 5′-gaagatagtgcaggttgctat-3′; rev-primer 5′-aacttcttactgttgtggtc-3′) of the SOX6 gene (see Figure 3). Annotated genes in the breakpoint regions of chromosome 9 and 11 were taken from the UCSC Genome Bioinformatics Server (http://www.genome.ucsc.edu/) as of August 2005.
Results

Cytogenetic and fluorescence in situ hybridisation studies

Analysis of the G-banded metaphase chromosomes of the patient revealed an apparently balanced translocation involving the long arm of chromosome 9 and the short arm of chromosome 11, 46,XY,t(9;11)(q33.1;p15.3) (Figure 2A). The karyotypes of the parents were normal. Whole chromosome painting confirmed this result and showed that there was no microscopically visible exchange with other chromosome material (data not shown).

Breakpoint mapping using FISH

The breakpoints were mapped by FISH with a set of BAC and PAC clones covering the chromosomal subregions. In the patient BAC clone RP11-451E16 from chromosome 9q33.1 and BAC clones RP11-71F16 and RP11-417O17 from chromosome 11p15.2 showed split signals and therefore span the breakpoints (Figure 2B,C and data not shown).

To refine the breakpoints further we performed Southern blot analysis of DNA from the patient and a control. Hybridization with probe 2 from BAC clone RP11-417O17 revealed a 4.3 kb MspI fragment not present in control DNA (Figure 3A). This enabled us to locate the breakpoint on chromosome 11. To clone the junction fragment we performed PCR reactions with a combination of a common forward primer located immediately 5´ to probe 2 and 17 different reverse primers (M1-M17), flanking every putative MspI site in the sequence of the chromosome 9 BAC clone RP11-451E16 on the 3´ side (Figure 3B). The combination with primer M14 generated a PCR product of 4 kb exclusively in the patient´s DNA but not in DNA from the control. Cloning and subsequent sequencing of this junction fragment revealed the exact position of the breakpoint on derivative chromosome 11 (Figure 3C,D). Amplification and sequencing of the junction fragment from the derivative chromosome 9 revealed an identical breakpoint (data not shown). The breakpoint regions do not contain any repeat structure or the presence of repetitive sequence elements, which would explain the recombination event.

Molecular structure of the breakpoint regions

On chromosome 11 the breakpoint is located between exons 6 and 7 of the SOX6 gene. Thus, the translocation disrupts SOX6, leaving the 201 kb long 5´ part of the gene intact. Exons 2 to 6 encode 259 out of 808 amino acids of SOX6, including the leucine zipper and most of the Q-box at the N-terminal region. On chromosome 9 the breakpoint falls into an intergenic region of approximately 1 Mb without any annotated, verified gene. Interestingly, the region is riddled by short sequences of 300 to 600 bp in length, which are highly conserved in evolution (up to 90 %) down to chicken (Figure 5). RT-PCR experiments with RNA from different murine tissues, including complete mouse embryos at stages E15.5, E17.5, E18.5 and skull from (P0) newborn mice, did not reveal that any of these elements is transcribed (results not shown). Therefore, the sequences most probably represent CNGs (conserved non-genic sequences). Since SOX6 is a major player in cartilage development and has been suggested as a factor in mesenchymal differentiation, SOX6 represents a candidate gene for craniosynostosis. We therefore screened the DNA from 104 patients with different craniosynostoses of sagittal and/or coronal sutures, in whom changes of the most common mutation hot spots in the FGFR1, -2, and -3 genes were excluded, for mutations in SOX6 by amplification of exons 2-16 (including the complete coding region) and subsequent sequence analysis of the PCR products. In
addition to several known polymorphisms, we identified a heterozygous missense mutation at position 263 in the SOX6 cDNA (NM_033326) (c.263G>A) in a 7 year old boy with complex craniosynostosis involving the coronal and sagittal suture. The mutation resulted in an amino acid exchange from aspartate to asparagine (p.D68N) (Figure 4). The mutation affected a highly conserved amino acid in the N-terminal half of SOX6 (Figure 4). The mutation was absent in 200 chromosomes from healthy individuals and 206 chromosomes from patients with various forms of craniosynostosis. Nevertheless, we also detected the mutation in a heterozygous state in the DNA from the patient’s apparently healthy mother. She was not available for further clinical examination, but denied any signs of craniosynostosis in her childhood.
Characterisation of breakpoints in patients with apparently balanced chromosome rearrangements has proven a valuable tool in the identification of disease genes [6][7]. Nevertheless analysis is not always straight forward for two reasons.

Firstly, the association of craniosynostosis and balanced chromosomal translocation in a single patient could be a random coincidence. Interestingly, some years ago Turleau et al. [8] described a patient with a *de novo* interstitial deletion of chromosome 9q32-q34, who shows a very similar craniofacial phenotype compared to our patient, with brachycephaly, a flat midface, a high forehead, downslanting palpebral fissures and low set ears. The deletion most likely included the breakpoint region identified in our patient and supports the involvement of chromosome region 9q33.1 in craniosynostosis.

Secondly, position effects may occur and influence expression of genes in a distance from the breakpoint [9][10]. Thus, a thorough evaluation of the breakpoint regions and the affected genes is necessary. In the present study we have analysed the breakpoints in a male patient with an apparently balanced *de novo* chromosome translocation t(9;11)(q33.1;p15.3) and a complex craniofacial dysostosis, including craniosynostosis and distinct facial features. The translocation disrupts a region on chromosome 9 containing non-transcribed patches of high evolutionary sequence conservation and the *SOX6* gene on chromosome 11.

The breakpoint on chromosome 9 is located in a region without any annotated genes, but which is any how riddled by short sequences of 300-600 bp highly conserved in evolution (Figure 5). Despite several attempts we were not able to amplify these sequences from total RNA of different human and murine tissues (data not shown), indicating that these sequences most likely represent conserved non-genic sequences (CNGs) [11]. A large number of these CNGs have been identified in the human genome in the last years and some of them have been implicated with a gene regulatory function [11]. Recently, it has been demonstrated that translocations may cause disease by disrupting or separating this type of regulatory regions. Examples come from the *HOXD* complex, *RIEG/PITX2*, *TWIST* and *SOX9* genes where breakpoints affecting regulatory regions up to 900 kb up- or downstream of the transcription unit down-regulate gene expression [Review:11][Review:12][13]. Thus, in the present case we have to take into account that the phenotype may be caused by the disruption of sequences present in the breakpoint region of chromosome 9, which may influence the activity of flanking genes in cis. The region is flanked by the genes *TLR4* (toll-like receptor 4) (~0.7 Mb proximal to the breakpoint) and *DBCCR1* (deleted in bladder cancer 1) (~0.8 Mb distal to the breakpoint). While *DBCCR1* is ubiquitously expressed and encodes a protein generally involved in cell death and tumor development, *TLR4* is an interesting candidate gene, since it was recently shown that regulation of osteoclastogenesis by lipopolysaccharide is mediated via its interaction with TLR4 on both osteoclast- and osteoblast-lineage cells [14][15]. Consequently, Johnson et al. [16] demonstrated that *TLR4* mutant mice develop bones with higher mineral content.

The translocation on chromosome 11 disrupts the *SOX6* gene. *SOX6* is a member of the SOX gene family, encoding proteins of the HMG box superfamily of DNA-binding proteins, which are involved in diverse developmental processes. It has been demonstrated that SOX6, together with SOX5 (LSOX5) and SOX9, plays a
critical role in chondrogenesis [17][18][19][20][Review:21]. In addition SOX9 and LSOX5 were recently shown to be involved in neural crest development [22][23], suggesting a similar function for SOX6. The flat bones of the vertebrate skull vault develop from the cranial neural crest cells as well as paraxial mesoderm by intramembranous (and not endochondral ossification). Changes in suture formation may thus not be explained by an effect on chondrogenesis but rather on neural crest development, migration or differentiation. Nevertheless, Sox5-/-; Sox6-/- double null mouse embryos show severe generalized chondrodysplasia whilst the intramembranous ossification seems to be unaffected [18]. Consequently, no changes of the neurocranium were reported, which argues against a loss-of-function effect in the present case. In contrast, the translocation may have led to a shortened mRNA and a stable, truncated SOX6 protein in our patient. Interestingly, the translocation left exons 1-6 intact, which encode the N-terminal part of SOX6 including almost the complete domain important for dimerisation with other factors, possibly LSOX5 [Review:21]. Thus, a dominant-negative effect of a truncated protein via competitive binding is a possible scenario and could explain a mild phenotype, completely different to the knockout mice. Unfortunately SOX6 is not expressed in lymphoblastoid cell lines, and, as tissue samples from the patient were not available, it could not be tested whether such a truncated product is present. Screening of the SOX6 gene in 104 patients with various forms of craniosynostosis revealed a missense mutation in a single patient with an apparently isolated complex craniosynostosis involving the coronal and sagittal suture. Interestingly, an evolutionary conserved aspartate residue at position 68 was exchanged against asparagine as a result of the mutation. The affected amino acid is located in the N-terminal part of SOX6 and has so far not been assigned to any functional domain. Since the mutation was also found in the patient’s apparently non-affected mother it remains open, whether the mutation represents a rare polymorphism or is associated with a craniosynostosis form with reduced penetrance.

In a third scenario, regulatory regions from chromosome 9 translocated to the vicinity of the SOX6 gene might affect the expression of adjacent genes in chromosome region 11p15. A number of additional candidate genes come into play when considering an interval of ~1 Mb. The most intriguing one is CALCA (calcitonin/calcitonin related polypeptide) for which a regulatory role in bone formation as well as in prevention of bone resorption in hypercalcemic states has been assigned [24][25][26].

In summary, the present case is one of the very rare reports of a de novo balanced translocation found in a patient with syndromic craniosynostosis of yet unknown aetiology. The finding adds novel candidate genes to the list of factors underlying or at least influencing the development of craniosynostosis. Further analysis of the putative regulatory region, the possible function of SOX6, TLR4 and CALCA in cranial suture biology and mutation screening for this panel of candidate genes in additional patients will clarify if they play a role in craniosynostosis and in suture biology in general.

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“Written consent was obtained from the patient’s legal guardians for publication of clinical photographs.”
Figure legends

Figure 1 Patient. (A) Profile of patient illustrating flat midface, mildly protruding eyes, high forehead and low set, posteriorly rotated ears mimicking a Crouzon-like profile. (B-F) Cranial spiral CT scans at 3.5 months of age. Note fusion of lambdoid sutures and of occipital part of sagittal suture with external ridging. Written consent was obtained from the patient’s legal guardians for publication of these clinical photographs.

Figure 2 FISH mapping of translocation breakpoints. (A) Ideograms of chromosomes 9, 11, and their derivatives der(9) and der(11) in patient PM. (B) FISH analysis of the patient’s chromosomes with the breakpoint spanning clone RP11-451E16 which yields signals on the normal chromosome 9 and the derivative chromosomes 9 and 11 while clone RP11-71F16 (C) hybridized to normal chromosome 11 and the derivative chromosomes 9 and 11.

Figure 3 Breakpoint cloning. A) Southern blot analysis of DNA from the patient and a control digested with the indicated restriction enzymes. The blot was hybridised with probe 2, indicated in figure 3B (red box). In MspI digests the expected fragment (blue circle) was present in both patient and control DNA, whereas the rearranged fragment (red circle) was present only in DNA from the patient. B) Overview of MspI restriction sites (M) and probes used for Southern blot analysis (red boxes). Chromosome region 11p15 as deduced from genomic sequence of clone RP11-417O17 (top), chromosome region 9q32 as deduced from genomic sequence of clone RP11-451E16 (middle) and the putative situation on derivative chromosome 11 (bottom) are shown. The sizes of the depicted regions are indicated on the left side. Arrows indicate PCR primers used for amplification of the junction fragment. In addition, the normal and aberrant MspI fragments which hybridised with probe 2 on the Southern blot are shown. Exons 5 and 6 of the SOX6 gene are indicated as purple boxes. C) PCR with a forward primer from chromosome 11 (bold purple arrow in B) and primers flanking the 17 MspI sites on chromosome 9 as reverse primers (lanes 1-17). PCRs were carried out using patient and control DNAs (M = 1 Kb Plus DNA ladder (Gibco) size marker). The amplified junction fragment of 4 kb is indicated (asterisk). D) Chromosome 11, der(11) and chromosome 9 sequences at the der(11) breakpoint in the patient. Chromosome 9-derived sequences are shown in bold.

Figure 4 Multiple alignment of amino acid sequences from the N-terminal portion of SOX6 protein of different species. The accession numbers (in brackets) and the amino acid positions are indicated. The sequence XP_421000 from chicken represents a hypothetical protein, '+' - is less than mean value plus two standard deviations (SD), '*' - is more than mean value plus two SD.

Figure 5 Percent identity plot (PIP) comparing human with mouse and chicken sequence in a 200 kb interval around the breakpoint region. The breakpoint is indicated by a red bar. Part of the human sequence
(NT_008470) was compared with the available orthologous genomic sequences from mouse (NT_039260) and chicken (Contig93 (53-202)) using PipMaker. Regions (a-f) with a high degree of sequence conservation are indicated by purple bars. The putative CNGs have a length of 300-600 bp and a sequence identity to mouse and chicken of: 89.8% / 62.9% (a), 87.8% / 76% (b), 92% / 74.2% (c), 79.5% / 54.3%, 88% / 70.9% (e) and 85.5% / 84.4% (f).
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Figure 1
Figure 2
Figure 3
>patient (p.D68N)  PHS-EELPTLVSTIQQA AD WDSVLSSQQRME
>human (NM_033326) 51 PHS-EELPTLVSTIQQA D WDSVLSSQQRME 83
>mouse (NP_035575) 51 PHS-EELPTLVSTIQQA D WDSVLSSQQRME 80
>rat (XP_218980.2) 102 PHS-EELPTLVSTIQQA D WDSVLSSQQRMV 131
>gallus (XP_421000) 262 PHS-EELPTLVSTIQQA E WDGVIASAQHRME 291
>rainbow trout (I51083) 50 KGSMDELQP-LSSVPPES D WDSMVSAQQRME 79

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Figure 4
Figure 5