LETTER TO JMG

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

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Craniosynostosis is a congenital developmental disorder involving premature fusion of cranial sutures, which results 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 and 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. Mutations in the genes for *FGFR2* and *FGFR3* were excluded. Standard chromosome analysis revealed a de novo balanced translocation t(9;11)(q33;p15). The breakpoint on chromosome 11p15 disrupts the *SOX6* 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 evolutionarily highly conserved non-genic sequences and may thus lead to dysregulation of flanking genes on chromosome 9 or 11 involved in skull vault development. 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.

Craniosynostosis is the premature fusion of one or more cranial sutures and is a relatively common birth defect with a prevalence of 1 per 2100–3200 births.¹ The aetiology of craniosynostosis is heterogeneous 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, brachycephaly or turriccephaly, mid-face hypoplasia, hypertelorism with exorbitism, and, depending on the timing and type of affected sutures, neurological complications.

Current knowledge on the pathomechanisms of craniosynostosis and 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 have been found in several conditions, including Pfeiffer, Crouzon, Jackson-Weiss, Apert, and Muenke syndromes.^{2–3} Mutations in the *MSX2* gene, encoding a homeobox transcription factor, were identified in a family with Boston-type craniosynostosis, and loss of function mutations in the transcription factor gene *TWIST* were identified in a subset of patients with Saethre-Chotzen syndrome.^{2–3} However, 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 approximately 80% of all craniosynostoses, and involve both environmental and genetic risk factors (reviewed by Cohen¹). 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 on chromosome 9 with a large number of highly conserved non-genic sequences (CNGs). 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.

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 510 mm (50th centile), weight 2670 g (<10th centile), and occipitofrontal circumference (OFC) 330 mm (just above 10th centile). The child 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 the hands or feet such as syndactyly, brachydactyly, or broad first rays. The suspicion of bilateral lambdoid and posterior sagittal craniosynostosis was supported radiographically (fig 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 from peripheral lymphocytes

Abbreviations: CNG, conserved non-genic sequence; FISH, fluorescence in situ hybridisation; OFC, occipitofrontal circumference

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 the lambdoid sutures and of the occipital part of the sagittal suture with external ridging. Written consent was obtained from the patient's legal guardians for publication of these clinical photographs.

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 the normal range; at 5.5 years of age, length was 1.07 m (10th centile and within parental target centile range), weight 17 kg (25th centile), and OFC 510 mm (25th–50th centile). There was mild developmental speech delay (on average five word sentences, attending normal kindergarten with 1 additional hour per week for speech therapy). Repeated hearing examinations 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 *FGFR2* and *FGFR3* genes. No mutation was found which could account for the phenotype (data not shown). In addition, we excluded the P252R mutation in the *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 yeast artificial chromosome (YAC) clones from the regions of interest. Fine mapping of breakpoints was performed with smaller bacterial

and plasmid (BAC and PAC) clones. DNA samples were prepared according to standard protocols and were labelled 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 analysed with a Zeiss epifluorescence microscope.

Molecular investigations

Genomic DNA was extracted from peripheral blood lymphocytes. For mutation screening, all 18 coding exons of the *FGFR2* and *FGFR3* genes were amplified by PCR using intronic primers from *FGFR2* (sequences available on request) and *FGFR3*.⁵ Direct cycle sequencing was carried out by using a commercial kit and automatic sequencers (Big Dye Terminator sequencing kit on an ABI377 and an ABI3730 automatic sequencer; all Perkin Elmer) 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, of 417 bp (probe 1; forward 5'-tttactgaggaatggacagta-3', reverse 5'-ggccgttttgatacacctt-3'), 402 bp (probe 2; forward 5'-gagaataggtagctatccatg-3', reverse 5'-cagtgcctcaccgcatcaag-3'), and 422 bp (probe 3; forward 5'-gaagatagtgcaggttgctat-3', reverse 5'-aacttctactgtgtgtgtgc-3') in size, from the *SOX6* gene (fig 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) (fig 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 (fig 2B,C and data not shown).

To refine the breakpoints further we performed Southern blot analysis of DNA from the patient and a control. Hybridisation with probe 2 from BAC clone RP11-417O17 revealed a 4.3 kb *Msp*I fragment not present in control DNA (fig 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 *Msp*I site in the sequence of the chromosome 9 BAC clone RP11-451E16 on the 3' side (fig 3B). The combination with primer M14 generated a PCR product of 4 kb only in the patient's DNA and 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

(fig 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 5' part of the gene intact. Exons 2–6 encode 259 of the 808 amino acids in 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 has a large number of short sequences of 300–600 bp in length, which are highly conserved in evolution (up to 90%) down to chicken (fig 5). Reverse transcription PCR experiments with RNA from different murine tissues, including complete mouse embryos at stages E15.5, E17.5, and E18.5, and skulls from (P0) newborn mice, did not reveal that any of these elements is transcribed (results not shown). Therefore, the sequences most probably represent CNGs. As *SOX6* has a major role 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

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) hybridised 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 B (red box). In the *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 the *MspI* restriction sites (M) and probes used for Southern blot analysis (red boxes). Top, chromosome region 11p15 as deduced from the genomic sequence of clone RP11-417017; middle, chromosome region 9q32 as deduced from genomic sequence of clone RP11-451E16; and bottom, the putative situation on derivative chromosome 11. 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 that 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 is a 1 kb Plus DNA ladder size marker; Invitrogen). The amplified junction fragment of 4 kb is indicated (*). (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**.

sutures. The mutation resulted in an amino acid exchange from aspartate to asparagine (p.D68N). The mutation affected a highly conserved amino acid in the N-terminal half of *SOX6* (fig 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.

DISCUSSION

Characterisation of breakpoints in patients with apparently balanced chromosome rearrangements has proven a valuable tool in the identification of disease genes.^{6,7} However, 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 showed a very similar craniofacial phenotype to our patient, with brachycephaly, a flat midface, a high forehead, downslanting palpebral fissures,

and low set ears. The deletion most probably 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 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 has a large number of short sequences of 300–600 bp highly conserved in evolution (fig 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 probably represent CNGs.¹¹ Several of these CNGs have been identified in the human genome in past years and some have been implicated with a gene regulatory

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 2SD, * is more than mean value plus 2SD.

Figure 5 Percentage identity plot comparing human with mouse and chicken sequences 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: (A) 89.8%/62.9%, (B) 87.8%/76%, (C) 92%/74.2%, (D) 79.5%/54.3%, (E) 88%/70.9%, and (F) 85.5%/84.4%.

function.¹¹ Recently, it has been demonstrated that translocations may cause disease by disrupting or separating this type of regulatory regions. Examples are the *HOXD* complex and *RIEG/PITX2*, *TWIST*, and *SOX9* genes, in which breakpoints affecting regulatory regions up to 900 kb upstream or downstream of the transcription unit downregulate gene expression (reviewed by Dermitzakis *et al*¹¹, Kleinjan *et al*¹², Velagaleti *et al*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* (~0.7 Mb proximal to the breakpoint) and *DBCCR1* (~0.8 Mb distal to the breakpoint). While *DBCCR1* is ubiquitously expressed and encodes a protein generally involved in cell death and tumour development, *TLR4* is an

interesting candidate gene, as 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} Johnson *et al*¹⁶ demonstrated that *TLR4* mutant mice consequently 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^{17–20} (reviewed by Lefebvre²¹). 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 and paraxial mesoderm by intramembranous (and not endochondral ossification). Changes in suture formation may thus be explained not by an effect on chondrogenesis but rather on neural crest development, migration, or differentiation. Nevertheless, *Sox5*^{-/-};*Sox6*^{-/-} double null mouse embryos show severe generalised 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, in our patient, the translocation may have led to a shortened mRNA and a stable, truncated SOX6 protein. 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 (reviewed by Lefebvre²¹). 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 sutures. Interestingly, an evolutionarily conserved aspartate residue at position 68 was changed to 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. As the mutation was also found in the patient's apparently non-affected mother, it remains debatable 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. Several additional candidate genes come into play when considering an interval of ~1 Mb. The most intriguing is *CALCA* for which a regulatory role in bone formation and prevention of bone resorption in hypercalcaemic states has been assigned.^{24–26}

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 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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Clinical Evidence also needs to recruit a number of new peer reviewers specifically with an interest in the clinical areas stated above, and also others related to general practice. Peer reviewers are healthcare professionals or epidemiologists with experience in evidence-based medicine. As a peer reviewer you would be asked for your views on the clinical relevance, validity, and accessibility of specific topics within the journal, and their usefulness to the intended audience (international generalists and healthcare professionals, possibly with limited statistical knowledge). Topics are usually 1500-3000 words in length and we would ask you to review between 2-5 topics per year. The peer review process takes place throughout the year, and out turnaround time for each review is ideally 10-14 days.

If you are interested in becoming a peer reviewer for *Clinical Evidence*, please complete the peer review questionnaire at www.clinicalevidence.com/ceweb/contribute/peerreviewer.jsp