ELECTRONIC LETTER

Breakpoint analysis of a familial balanced translocation t(2;8)(q31;p21) associated with mesomelic dysplasia

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Mesomelic dysplasia (MD) is characterised by mildly short stature and shortening of the middle segments of the limbs. There are several subtypes of MD including dyschondrosteosis (Leri-Weill type), Langer type, Nivergelt type, Robinow type, Reinhardt type, Kozlowski-Reardon type, Werner type, and mesomelic dysplasia with synostoses.¹ Ventruto *et al*² reported an Italian family in which four members with a type of MD and vertebral abnormalities had a balanced translocation t(2;8)(q32;p13), which turned out to be t(2;8)(q31;p21) using our improved banding techniques, but three other phenotypically normal members were karyotypically normal. Thus, this type of MD seemed causally related or linked to the translocation. The female proband in the family had a short forearm with bowed and malformed radius, cubitus valgus with limited extension and supination, Madelung-type wrist deformity, atlantoaxial fusion, spina bifida occulta in the lumbosacral region, and the translocation. Kantaputra *et al*³ reported a large Thai family with another type of MD, Kantaputra type (MDK), similar to but with more severe manifestations than the MD in the Italian patients. MDK showed mildly short stature, shortening of the forearm/lower leg, carpal/tarsal synostosis, and dorsolateral foot deviation. Our previous linkage analysis of the Thai family using positional information from the translocation breakpoints in the Italian family showed that chromosome 2q24-q32, spanning about a 22.7 cM region, is implicated in MDK.⁴ The analysis also suggested that both conditions in the two families are identical or allelic. Furthermore, the *HOXD* cluster that is related to limb development has been mapped to 2q31.⁵ This led us to characterise the 2q31 breakpoint in the Italian family. Here we report the results of the breakpoint analysis.

MATERIALS AND METHODS

Fluorescence in situ hybridisation (FISH)

FISH analysis using P1 derived artificial chromosome (PAC)/cosmid DNA was performed on metaphase chromosomes of a patient with the 2;8 translocation from the Italian family reported by Ventruto *et al*² using standard methods.

Isolation of PACs and cosmids and construction of a cosmid contig

A RPCI-1, 3 PAC library was screened by PCR with primers each corresponding to *HOXD3*, 4, 8, 10, and 13, and *EVX2* (GenBank accession numbers, XM_056998, XM_042818, NM_019558, NM_002148, NM_000523, and XM_065465, respectively). Cosmid sublibraries were prepared from genomic DNA of the patient as described elsewhere.^{6,7} Cosmid DNA was extracted by PI-100 DNA isolation system (Kurabo, Osaka, Japan). A total of 260 cosmid clones were randomly chosen and used for construction of a contig covering the breakpoint. The contig was constructed by means of STS content mapping using PCR. New sequence tagged sites (STSs) were generated from end sequences of isolated clones.

Isolation of cosmid clone spanning the translocation breakpoint

A cosmid library of the patient using SuperCos1 vector was prepared according to the manufacturer's protocol (Stratagene, La Jolla, CA, USA). About a 4-genome-fold library was screened with two probes flanking the 2q31 breakpoint as described previously.⁷ These probes were prepared using the following two pairs of primers (5'-3'): 1f/1r, TAGAGGGA TGGCAAACCTCAG/GCTCTACCATTAGTTAGAGG; 2f/2r, CCCAT TACCAGAACTTCGTGA/CCATAATCAAATTGCTCCACA. The 1f/1r and 2f/2r later turned out to be 8.9 kb and 1.3 kb centromeric to the breakpoint, respectively.

DNA sequencing and computational analysis of sequences

PAC/cosmid DNA and PCR products were sequenced with ABI Prism Big Dye Terminator™ Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster, CA, USA) as previously described.⁷ Products were analysed on ABI 377 or 310 automated sequencers (PE Applied Biosystems).

New STSs were generated from clone end sequences after excluding repetitive sequences, using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). For analysis of entire cosmid/BAC sequences, RepeatMasker (<http://ftp.genome.washington.edu/cgi-bin/RepeatMasker>) was first used to eliminate repetitive sequences. PipMaker (<http://bio.cse.psu.edu/pipmaker/>) and Blast2 (<http://www.ncbi.nlm.nih.gov/gorf/b12.html>) analyses were performed to compare sequences flanking 2q31 and 8p21 breakpoints. HGMP-RC NIX Session (<http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix/>) was used to detect potential genes, exons, and promoters around each breakpoint.

RESULTS

Isolation of PAC clones and construction of cosmid contig spanning the 2q31 breakpoint

A total of six PAC clones were isolated by PCR with the five sets of primers corresponding to five *HOXD* genes. Among them, four PAC clones (349J14, 395P15, 440D1, and 342A21) showed FISH signals only on the normal chromosome 2 and der(2) chromosome. The other two clones (424A2 and 216K8) gave FISH signals on both der(2) and der(8) chromosomes in addition to the normal chromosome 2. Thus, these two PAC clones spanned the breakpoint.

A complete cosmid contig was constructed by PCR based STS content mapping (fig 1). Besides STSs for the *HOXD* genes, 10 new STSs (c11T3, c18T3, c55T3, c79T7, c55T7, c181T3, c188T3, c21T7, c181T7, and c188T7) were generated

Abbreviations: MD, mesomelic dysplasia; MDK, mesomelic dysplasia Kantaputra type; PAC, P1 derived artificial chromosome; STS, sequence tagged sites

Figure 1 A physical map of a contig covering the 2q31 breakpoint in the patient. From top to bottom, rows indicate positions of markers and *HOXD* genes; complete/draft sequences covering this region; a PAC clone (216K8); its derived cosmid clones; and genes. Thick lines show cosmid clones confirmed to span the breakpoint by FISH analysis. Horizontal arrows indicate the transcriptional direction of the genes and the vertical arrow shows the position of the breakpoint.

Figure 2 FISH analysis using a cosmid clone, c181, on the patient's chromosomes. The arrowhead indicates a normal chromosome 2 and the arrow the derivative chromosomes 2 and 8.

from end sequences of cosmid subclones. FISH analysis showed that five cosmid subclones (c55, c50, c58, c21, and c251) were mapped centromeric to the 2q31 breakpoint, c183 distal to it, and two clones (c181 and c188) at the breakpoint (fig 2). Thus, the 2q31 breakpoint was localised between the two STSs, c188T3 and c181T7.

Cloning of a cosmid containing the 2q31 junction fragment

A cosmid library was constructed from genomic DNA of the patient and screened by hybridisation using two probes generated by PCR with primers 1f/1r and 2f/2r. Among two clones isolated, a 2q31 breakpoint junction clone BR was identified. The BlastN searches showed that this clone had both the T3 end sequence hitting RP11-437N19 (Genbank accession number AC016739.5, mapped to chromosome 2) and the T7 end sequence corresponding to RP11-219J21 (GenBank accession number AC073069.2, mapped to chromosome 8). Detailed partial sequence analyses showed that the clone contained chimaeric sequences and turned out to be from the der(2) chromosome. PCR using a primer set (BR2F, AGAATGTGGCAATGTGGTGA at 2q31 and BR8R, CTGTTAG GACAGAGAGCTCC at 8p21) designed to amplify a junction fragment of the der(2) chromosome successfully yielded a product on the patient's DNA but not on control DNA (data not shown).

Figure 3 Comparison of sequences from the normal chromosomes 2 and 8, and from the breakpoints of the der(2) and der(8) chromosomes. In the der(8) chromosome, two nucleotides (CA) are deleted and 13 nucleotides of unknown origin are inserted. There is no nucleotide change in the der(2) chromosome.

Sequence comparison and characterisation of the 2q31 and the 8p21 breakpoints

PCR with a primer set (BR8F, AGATCACATATGCCAAAGGC at 8p21 and BR2R, ACACAGTGGGAACAATCG at 2q31) that was designed to amplify the der(8) breakpoint successfully created a product only on the patient's DNA. Sequence analysis of the product showed chimaeric sequences derived from RP11-437N19 (chromosome 2) and RP11-219J21 (chromosome 8). All sequences from normal chromosomes 2 and 8, and der(2) and der(8) chromosomes were aligned (fig 3). The der(8) sequence had a deletion of two nucleotides, CA, and an insertion of 13 nucleotides, TGGATACTCTTAA, with unknown origin, whereas the der(2) chromosome had no nucleotide changes (fig 3). The PipMaker and Blast2 comparisons of sequences that flanked the 2q31 and the 8p21 breakpoints did not identify any similar or repetitive sequences.

HGMP-RC NIX Session (<http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix/>) was used to detect potential genes, exons, and promoters around each breakpoint. Although there was no gene disrupted at the breakpoints, *MTX2* (Genbank accession number 16161620) and the *HOXD* gene cluster (*HOXD1*) (Genbank accession number 16157968) were identified 21 kb telomeric and 56 kb centromeric to the 2q31 breakpoint, respectively. Sequence analysis of *MTX2*, *HOXD9-13* of the patient did not show any nucleotide changes (data not shown). No gene was identified in a 430 kb region (230 kb centromeric and 200 kb telomeric) around the 8p21 breakpoint by the NIX session.

DISCUSSION

Since all four members affected with a type of MD in the Italian family² carried a balanced t(2;8)(q31;p21), and all three other unaffected members were karyotypically normal, the a priori hypothesis of our study was that a putative gene responsible for the skeletal disease in the family was affected by the translocation. We have characterised the 2q31 and the 8p21 breakpoint sequences in one of the patients and have shown that no known genes or gene-like sequences were disrupted at either breakpoint. Instead, we identified the *HOXD* gene cluster and *MTX2* which are 56 kb centromeric and 21 kb telomeric to the 2q31 breakpoint, respectively, and the 2q31 breakpoint was positively mapped within the linkage interval of the MDK family.⁴ We could not find any homology between sequences of chromosomes 2 and 8 flanking breakpoints,⁷ any repetitive sequences,⁸ nor any specific sequence structures possibly mediating constitutional translocations.⁹ *MTX2* protein is probably involved in mitochondrial protein import, but its precise function remains to be investigated. *Hoxd* genes, as well as *Hoxa* genes, are known to play important roles in the body plan in the vertebrate. These findings suggested several possibilities, (1) an enhancer of the *HOXD* genes cluster was disrupted by the chromosomal translocation, (2) misregulation of the entire *HOXD* cluster by an ectopic regulatory element normally located on 8p21, (3) a position effect on these genes close to the breakpoints altered their expression, (4) a coincidental rare instance of a gene with a mutation close to the breakpoint, but no evidence of aberrant sequences of *MTX2* and *HOXD9-13* in the patient, (5) the effect of chance, although this is unlikely because the probability would be as low as 1/1024.

Hoxd genes along the main body axis are regulated separately to their expression in the limbs and genitalia.¹⁰⁻¹² An enhancer responsible for the sequential activation of the *Hoxd* genes along the main body axis is located less than 28 kb upstream of the 5' end of the *Hoxd* cluster. Another enhancer, which appears to regulate the coordinated expression of the four most 5' *Hoxd* genes (*Hoxd10-Hoxd13*) and the adjacent *Evx2* gene in the developing autopod and genital tubercle, is also thought to lie well upstream of the 5' end of the *Hoxd* cluster.^{13,14} A spontaneous mouse mutant with mesomelic dysplasia, *Ulnaless* (*Ul*), has been mapped to the vicinity of the *Hoxd* cluster. The abnormalities in the *Ul* mouse are confined to the limbs and the penile bone. Moreover they are associated with reduced expression of the *Hoxd13* and *Hoxd12* in the developing autopod and genital tubercle, and ectopic expression of *Hoxd13* and *Hoxd12* in the developing zeugopod. They were therefore previously thought to result from a deletion or rearrangement of the digit enhancer. Abnormalities of MDK in the Thai family³ are also confined to the limbs, so they can be explained by a defect of digit enhancer.

The phenotype associated with the translocation in the family we described, however, includes abnormalities of the axial skeleton as well as the limbs. Moreover, the upper cervical as well as the lumbosacral vertebrae are affected, suggesting perturbation of 3' as well as 5' *HOXD* gene expression. These features cannot be explained only by an effect upon the digit enhancer. They may be the result of misregulation of the entire cluster by a regulatory element normally located on 8p21, although we do not have any clear evidence. Alternatively, they may be the result of removal/disruption of a hitherto unsuspected *cis* acting regulatory element for the entire cluster which is normally located downstream of the cluster's 3' end. Recently, it was shown that sequences responsible for regulating colinear *Hoxd* gene expression along the trunk are located within or close to the cluster itself, whereas sequences responsible for directing 5' *Hoxd* gene expression to the limbs and genitalia lie at least 100 kb upstream of the cluster's 5' end and/or at least 10 kb downstream of its 3' end.¹⁵ Thus it is possible that the 2q31 breakpoint which is 56 kb downstream of

Key points

- Breakpoints of a balanced chromosomal translocation, t(2;8)(q31;p21), observed in four patients with mesomelic dysplasia (MD) and vertebral abnormalities in an Italian family which was described by Ventruto *et al* in 1983, were cloned and analysed.
- One of the breakpoints, 2q31, had previously been implicated by our linkage analysis in mesomelic dysplasia, Kantaputra type (MDK). Dysplasia of the zeugopod in both the Italian and MDK patients suggested that the 2q31 region might harbour a gene(s) determining limb development.
- Although no genes or gene-like sequences were found at either breakpoint, the *HOXD* gene cluster is located 56 kb centromeric to the 2q31 breakpoint. Sequence analysis of both breakpoints showed an insertion of 13 nucleotides of unknown origin and a loss of two nucleotides from the derivative chromosome 8, but no sequence change in the derivative chromosome 2.
- Either disruption of an enhancer of the *HOXD* gene cluster by the chromosomal translocation or a position effect on those genes close to the breakpoints may explain the MD phenotype.

the 3' end of the *HOXD* cluster disrupts its unrecognised enhancer in humans.

Position effect may be an alternative explanation. It is recognised as a change of gene expression by a chromosomal aberration with an intact transcriptional unit, has been reported in several diseases,¹⁶ and can alter the expression of a gene even 900 kb away from a breakpoint.¹⁷ Thus, given that the distance from the breakpoint to the *HOXD* cluster is only 56 kb, it is possible that position effect is playing a role in altering gene expression at this cluster.

In conclusion, the 2q31 breakpoint of the MD family was confirmed to be located 56 kb from the 3' end of the *HOXD* cluster. Their phenotype may be explained either by a defect of *HOXD* enhancer or position effect on the *HOXD* cluster because of the chromosomal translocation.

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NOTE ADDED IN PROOF

Another paper on this subject¹⁸ was published after our work was accepted for publication. The two investigations were performed independently.

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REFERENCES

- International Working Group on Constitutional Diseases of Bone.** International nomenclature and classification of the osteochondrodysplasias (1997). *Am J Med Genet* 1998;**79**:376-82.
- Ventruto V**, Pisciotto R, Renda S, Festa B, Rinaldi MM, Stabile M, Cavaliere ML, Esposito M. Multiple skeletal familial abnormalities associated with balanced reciprocal translocation 2;8(q32;p13). *Am J Med Genet* 1983;**16**:589-94.
- Kantaputra PN**, Gorlin RJ, Langer LO Jr. Dominant mesomelic dysplasia, ankle, carpal, and tarsal synostosis type: a new autosomal dominant bone disorder. *Am J Med Genet* 1992;**44**:730-7.
- Fujimoto M**, Kantaputra PN, Ikegawa S, Fukushima Y, Sonta S, Matsuo M, Ishida T, Matsumoto T, Kondo S, Tomita H, Deng HX, D'Urso M, Rinaldi MM, Ventruto V, Takagi T, Nakamura Y, Niikawa N. The gene for mesomelic dysplasia Kantaputra type is mapped to chromosome 2q24-q32. *J Hum Genet* 1998;**43**:32-6.
- Limongi MZ**, Pelliccia F, Gaddini L, Rocchi A. Clustering of two fragile sites and seven homeobox genes in human chromosome region 2q31→q32.1. *Cytogenet Cell Genet* 2000;**90**:151-3.
- Tsukamoto K**, Tohma T, Ohta T, Yamakawa K, Fukushima Y, Nakamura Y, Niikawa N. Cloning and characterization of the inversion breakpoint at chromosome 2q35 in a patient with Waardenburg syndrome type I. *Hum Mol Genet* 1992;**1**:315-17.
- Matsumoto N**, David DE, Johnson EW, Konecki D, Burmester JK, Ledbetter DH, Weber JL. Breakpoint sequences of an 1;8 translocation in a family with Gilles de la Tourette syndrome. *Eur J Hum Genet* 2000;**8**:875-83.
- Kehrer-Sawatzki H**, Haussler J, Krone W, Bode H, Jenne DE, Mehnert KU, Tummers U, Assum G. The second case of a t(17;22) in a family with neurofibromatosis type 1: sequence analysis of the breakpoint regions. *Hum Genet* 1997;**99**:237-47.
- Kurahashi H**, Emanuel BS. Long AT-rich palindromes and the constitutional t(11;22) breakpoint. *Hum Mol Genet* 2001;**10**:2605-17.
- van der Hoeven F**, Zakany J, Duboule D. Gene transpositions in the HoxD complex reveal a hierarchy of regulatory controls. *Cell* 1996;**85**:1025-35.
- Kondo T**, Duboule D. Breaking colinearity in the mouse HoxD complex. *Cell* 1999;**97**:407-17.
- Herauld Y**, Beckers J, Gerard M, Duboule D. Hox gene expression in limbs: colinearity by opposite regulatory controls. *Dev Biol* 1999;**208**:157-65.
- Peichel CL**, Prabhakaran B, Vogt TF. The mouse Ulnaless mutation deregulates posterior HoxD gene expression and alters appendicular patterning. *Development* 1997;**124**:3481-92.
- Herauld Y**, Fraudeau N, Zakany J, Duboule D. Ulnaless (Ul), a regulatory mutation inducing both loss-of-function and gain-of-function of posterior Hoxd genes. *Development* 1997;**124**:3493-500.
- Spitz F**, Gonzalez F, Peichel C, Vogt TF, Duboule D, Zakany J. Large scale transgenic and cluster deletion analysis of the HoxD complex separate an ancestral regulatory module from evolutionary innovations. *Genes Dev* 2001;**15**:2209-14.
- Kleinjan DJ**, van Heyningen V. Position effect in human genetic disease. *Hum Mol Genet* 1998;**7**:1611-18.
- de Kok YJ**, Vossenaar ER, Cremers CW, Dahl N, Laporte J, Hu LJ, Lacombe D, Fischel-Ghodsian N, Friedman RA, Parnes LS, Thorpe P, Bitner-Glindzicz M, Pander HJ, Heilbronner H, Graveline J, den Dunnen JT, Brunner HG, Ropers HH, Cremers FP. Identification of a hot spot for microdeletions in patients with X-linked deafness type 3 (DFN3) 900 kb proximal to the DFN3 gene POU3F4. *Hum Mol Genet* 1996;**5**:1229-35.
- Spitz F**, Montavon T, Monso-Hinard C, Morris M, Ventruto ML, Antonarakis S, Ventruto V, Duboule D. A t(2;8) balanced translocation with breakpoints near the human HOXD complex causes mesomelic dysplasia and vertebral defects. *Genomics* 2002;**79**:493-8.