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A novel gene is disrupted at a 14q13 breakpoint of t(2;14) in a patient with mirror-image polydactyly of hands and feet

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Abstract Mirror-image polydactyly of hands and feet (MIP) is a very rare congenital anomaly characterized by mirror-image duplication of digits. To isolate the gene responsible for MIP, we performed translocation breakpoint cloning from an MIP patient with t(2;14)(p23.3;q13). We isolated a good candidate gene for MIP that was disrupted by the translocation of the patient. We had previously constructed a 1.2-megabase bacterial artificial chromosome (BAC)/P1-derived artificial chromosome (PAC) contig covering the 14q13 breakpoint of t(2;14)(p23.3;q13). From a 500-kb segment consisting of seven BAC/PAC clones in the contig, we isolated a novel gene (the mirror-image polydactyly 1 gene, designated as *MIPOL1*, GenBank Accession No. AY059470), in addition to the hepatocyte nuclear factor 3 alpha gene (*HNF3A,* GenBank Accession No. XM 007360). *MIPOL1* spans about 350 kb, comprises 15 exons, and encodes 442 amino acids. Northern blot analysis revealed that *MIPOL1* expression is definite but very weak in adult heart, liver, skeletal muscle, kidney, and pancreas, and in fetal kidney. In view of the genome sequence and the contig map constructed, the 14q13 breakpoint of the patient was identified as located in intron 11 of *MIPOL1*, indicating that the gene was disrupted by the translocation, and that the breakage resulted in MIPOL1 protein truncation. Whole-mount in situ hybridization in mouse resulted in mouse *Mipol1* signals all over E10.5–E13.5 mouse embryos. Two other unrelated patients with limb anomalies similar to MIP were subjected to mutation analysis of *MIPOL1*, but none had any mutations. We then isolated BAC clones from the other breakpoint, 2p23.3. A search for genes and expressed sequence tags in a more than 300-kb region around the 2p23.3 breakpoint found only the neuroblastoma-amplified protein gene (*NAG*, GenBank Accession No. NM 015909), which is located at least 50 kb centromeric to the breakpoint and is not likely to be related to MIP. *MIPOL1* is a good candidate gene for the MIP type of anomaly.

Key words Mirror-image polydactyly · Translocation t(2;14)(p23.3;q13) · Breakpoint cloning · 14q13 Breakpoint · Novel gene · *MIPOL1* · Coiled-coil domain

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Introduction

Mirror-image polydactyly of hands and feet (MIP) is a very rare congenital anomaly characterized by mirror-image duplication of digits. It is occasionally associated with dimelia of the ulnae and fibulae, tibial and/or fibular hypoplasia, nasal abnormality, and other malformations. Most cases are sporadic, but parent–child transmissions observed in familial cases suggest an autosomal dominant mode of inheritance (Laurin et al. 1964; Kogekar et al. 1993; Martin et al. 1993; Martinez-Frias et al. 1994; Hersh et al. 1995;

Hatchwell and Dennis 1996). We previously reported a Japanese boy with postaxial MIP without any other anomalies (Ohashi et al. 1995; Kim et al. 1997). Since his limb anomaly was associated with a *de novo* balanced chromosomal translocation, t(2;14)(p23.3;q13), we assumed that the translocation might have affected the function of a putative gene that might play a role in limb development (Matsumoto et al. 1997a;b). We also had previously constructed a 1.2-Mb BAC/PAC contig covering the 14q13 breakpoint of the patient (Matsumoto et al. 1997b). Here, we report on a novel gene that is disrupted at the 14q13 breakpoint.

Materials and methods

Isolation of transcribed sequences from the 14q13 breakpoint region

We attempted to isolate transcribed sequences from a 500-kb segment between markers AFM200ZH4 and P13T7 (Fig. 1a) within the previously constructed 1.2-Mb bacterial artificial chromosome (BAC)/p1-derived artificial chromosome (PAC) contig (Matsumoto et al. 1997b). To isolate CpG island-rich sequences, DNA from seven BAC/PAC clones (B319, B305, B368, P29, P7, P163, and P464) in the segment was double-digested with *Pst*I/*Sac*I, *Pst*I/*Bss*HII, or *Pst*I/*Eag*I, and the digests were subcloned into pBluescriptII SK(-) (Stratagene, La Jolla, CA USA). In parallel, DNA from all clones but B368 was sonicated, treated with Mung-bean nuclease (New England Biolabs, Beverly, MA, USA), and cloned into pBluescriptII SK(-). For exon trapping, after DNA was partially digested with *Sau*3AI, large fragments (>2kb) were extracted from agarose gel and cloned into pSPL3 according to the manufacturer's protocol (Gibco BRL, Carlsbad, CA, USA). DNA was subjected to sequencing with an autosequencer (model 377, PE Applied Biosystems, Foster City, CA, USA) by using a BigDye terminator sequencing kit (PE Applied Biosystems). The trapped sequences were analyzed with a BLAST search, and genomic sequences around the putative exons were subjected to the Grail II program. The BLAST search was performed against the expressed sequence tag (EST) and the genomic sequence databases.

Primers were designed for putative exons, and cDNA was amplified by 3′- and 5′-rapid amplification of cDNA ends (RACE) from human muscle and brain Marathon cDNA libraries (Clontech, Palo Alto, CA, USA). IMAGE cDNA clones matching the obtained sequence were subjected to sequence analysis. Exon–intron boundaries were confirmed by comparing the cDNA and genomic sequences.

Northern blot analysis

To confirm the expression of the newly isolated cDNA (hereafter designated *MIPOL1*), northern blot analysis was carried out. A 313-bp DNA segment was amplified by polymerase chain reaction (PCR) from the IMAGE clone using the primers Mipol1sense, 5′-TGACATGACATTACAGG AAT-3′; and Mipol1antis, 5′-TAGTAAACTCGAAGGG TC-3′, and used as a probe for exons 12 to 15 of *MIPOL1*. The probe was radiolabeled by a random primer labeling kit (Amersham, Buckinghamshire, UK) and hybridized at 60°C for 4h in the HybriExpress (Clontech), and then washed at 55°C in 0.2x SSC/0.1% sodium dodecyl sulfate (SDS). Autoradiography was performed at −80°C for 4 weeks. An oligonucleotide synthesized from the β-actin gene was used as a control probe.

Whole-mount in situ hybridization in the mouse

The mouse *Mipol1* cDNA was obtained by reverse transcriptase (RT)-PCR of total RNA from the mouse embryo. The RT-PCR product was cloned into a pT7T3α-19/*Sma*I vector and used as a probe for whole-mount in situ hybridization of E10.5–E13.5 mouse embryos. The probe contained *Mipol1* exons 14 and 15 that showed 85% homology

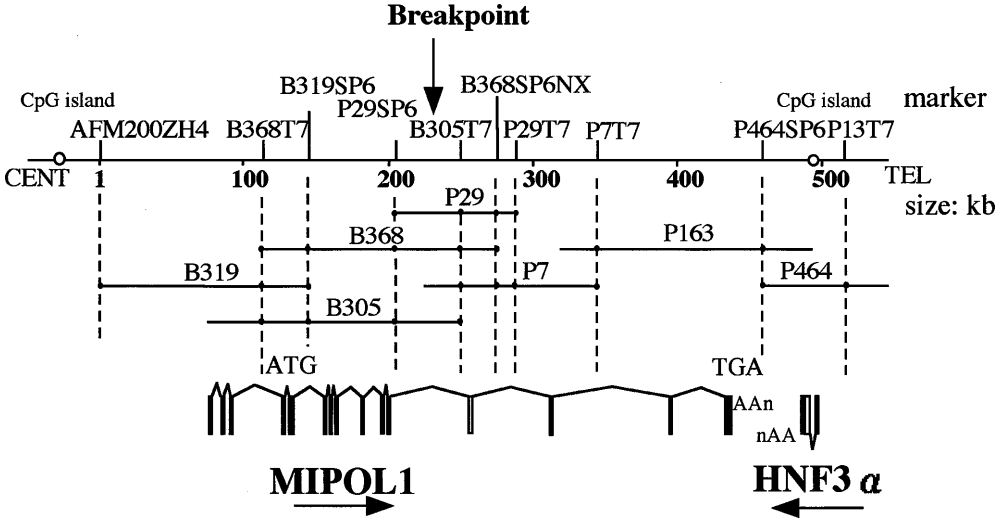

Fig. 1. Genomic organization of the mirror-image polydactyly 1 (*MIPOL1*) gene. The *MIPOL1* gene is about 350 kb long, consists of 15 exons, and is disrupted at exon 11 by the translocation. The *breakpoint* is indicated by the *vertical arrow* at the top of figure. Breakage has occurred between STS markers *P29SP6* and *B305T7*. The *MIPOL1* gene is transcribed from centromer to telomere, while the *HNF3A* gene is transcribed from telomere to centromere. *CENT*, centromere; *TEL*, Telomere

with those of the human *MIPOL1* gene. The in situ hybridization procedure was performed as described previously (Yoshiura et al. 1998).

Mutation analysis in patients with mirror-image polydactyly

Two unrelated, karyotypically normal patients, a Japanese boy with postaxial mirror-image polydactyly of the hands, and a Thai man with Laurin-Sandrow syndrome (Kantaputra 2001) were studied for the *MIPOL1* mutation as described previously (Kinoshita et al. 2000). Both disorders are clinically similar to MIP. PCR-direct sequencing was performed using 11 primer sets designed to amplify exons 5–15 of *MIPOL1* and their exon–intron boundaries. PCR (35 cycles) was done with a GeneAmp 9600 system (Perkin Elmer, Shelton, CT, USA) under the following conditions: denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s. After purification of the PCR product with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA), sequencing was carried out using the BigDye terminator cycle sequencing reaction kit (PE Applied Biosystems) and a Prism 377 DNA sequencer (PE Applied Biosystems).

Analysis of another breakpoint at 2p23.3

A fluorescent in situ hybridization (FISH) analysis was performed using BAC clones mapped to chromosome 2 as probes to search for the 2p23.3 breakpoint-covering clones, as described previously (Matsumoto et al. 1997b).

Results

Isolation of a novel gene, *MIPOL1*, and its protein showing weak homology to CARD14

With the CpG-island sequencing strategy, we detected only one transcribed sequence, which corresponded to the hepatocyte nuclear factor 3 alpha gene (*HNF3A*), within the 500-kb contig analyzed (Fig. 1). We also obtained some other sequences predicted to be exons with the random sequencing method. Two exons (exons 10 and 11) were isolated from the BAC B386 clone that showed split FISH signals on the patient's chromosome (Ohashi et al. 1995; Kim et al. 1997), and they matched two EST clones, zo50e01 and tg07a01. The sequences obtained by 3′-RACE from RNA of adult skeletal muscle and fetal brain completely matched those of the two ESTs, while the 5′-RACE gave two forms of PCR products, generated by alternative splicing. Alternative splicing occurred in the 5′-noncoding exons (exons 1–4), and both forms of cDNA contained a putative first methionine codon in exon 5. Therefore, the two transcripts may encode the same protein. Because this novel transcript did not completely match any known genes and it spanned the 14q breakpoint of the translocation of the patient, we concluded that it is a novel gene and designated it as *MIPOL1* (the mirror-image polydactyly gene 1 (GenBank Accession No. AY059470).

MIPOL1 contains a 1326-bp open reading frame spanning the region from the first ATG codon in exon 5 to the termination codon in exon 15. It is predicted to encode 442 amino acids that have two coiled-coil domains in the C-terminus region. The MIPOL1 protein is homologous in its second coiled-coil domain to a protein, CARD14 (caspase recruitment domain protein 14).

Expression of human *MIPOL1* and mouse *Mipol1*

Northern blot analysis of human *MIPOL1* in adult heart, liver, skeletal muscle, kidney, and pancreas, and in fetal kidney revealed a definite but very weak expression (Fig. 2), while gene expression was not detected in adult brain, placenta, or lung. At least three different transcripts (7 kb, 3 kb, and 2 kb) clearly appeared in adult heart. In adult skeletal muscle, the strongest signal was for the 2-kb transcript, which most likely corresponds to the sequence we identified, while we could not isolate the two other, longer transcripts. The whole-mount in situ hybridization of E10.5–E13.5 mouse embryos using mouse *Mipol1* as a probe did not give signals in specific areas or regions, including the limb bud, but showed diverse signals all over the embryo (data not shown).

Mutation analysis of *MIPOL1* in patients with mirror-image polydactyly

The PCR direct sequencing revealed no mutation in any exons or in the intron–exon boundary regions of *MIPOL1* in the two patients with disorders similar to MIP.

Genes flanking the 2p23.3 breakpoint

The FISH analysis revealed that a BAC clone (RP11-247H16) gave signals on both derivative chromosomes 2

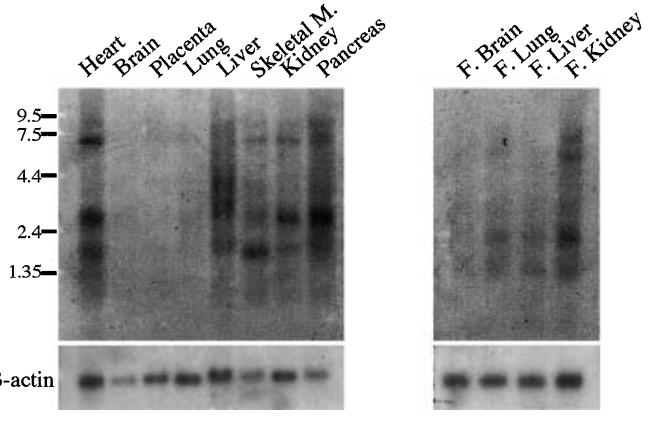

Fig. 2. Northern blot analyses of adult (*left*) and fetal (*right*) tissues. Three *bands* (7 kb, 3 kb, and 2 kb) are clearly visible in the *adult heart lane*, while the 3-kb signal is dominant in *liver*, *kidney*, and *pancreas*, and the 2-kb signal is dominant in *skeletal muscle*

and 14, in addition to the normal chromosome 2 (data not shown), indicating that the clone covers the 2p23.3 breakpoint. Because genomic sequences of this clone and overlapped clones are available (GenBank Accession Nos. AC008274, AC010988, and AC008282), we searched for genes and/or ESTs around the breakpoint. We found only one gene, the neuroblastoma-amplified protein gene (*NAG*, GenBank Accession No. NM 015909), which is located at least 50 kb centromeric to the breakpoint. The *NAG* gene spans a more than 300-kb genomic region and is transcribed from centromere to telomere. A search for a 200-kb genomic sequence telomeric to the breakpoint found no genes or protein-coding ESTs.

Discussion

We isolated a novel gene, *MIPOL1* (mirror-image polydactyly gene 1), which spanned the 14q13 breakpoint of t(2;14)(p23.3;q13) in the patient with MIP (Ohashi et al. 1995; Kim et al. 1997). *MIPOL1* in the patient was disrupted between exons 11 and 12 by this translocation, probably leading to a truncated MIPOL1 protein. Our extended gene search in the 1.2-Mb contig found no gene other than *MIPOL1* and *HNF3A*. We also verified the translocation breakpoint on chromosome 2 by FISH analysis. A BAC clone, RP11-247H16, showed split signals on derivative chromosome 2 and derivative chromosome 14. We performed a computational search around the 2p23.3 breakpoint, but no gene or EST other than *NAG* was identified. It is possible that there is a gene that was expressed very weakly. *NAG* is located at least 50 kb centromeric to the breakpoint and is unlikely to be related to MIP. Thus, it is most likely that *MIPOL1* is a strong candidate gene for MIP, although no mutations were detected in two karyotypically normal patients with a similar type of limb anomaly. It remains to be clarified whether other MIP patients have a mutation in *MIPOL1*, although it is difficult to locate patients with this rare disorder. Since the northern blot analysis disclosed the expression of two other, longer transcripts in human tissues, it also remains to be identified whether these have additional, hidden exons in the 5′ region, and whether the two patients with disorders similar to MIP have a mutation in these putative exons.

The function of *MIPOL1* and of its mouse orthologue, *Mipol1*, is unknown. No gene is known having high sequence homology to these genes. Although mouse *Mipol1* has not been cloned, its partial EST sequences available from the database show some 85% homology with those of human *MIPOL1*. Since human *MIPOL1* was expressed very weakly and rather ubiquitously in many tissues examined, except in brain, placenta, or lung, it may have no specific expression profile. A similar result was obtained by whole mount in situ hybridization of *Mipol1* in E10.5–E13.5 mouse embryos. The MIPOL1 protein has two coiled-coil domains in the C-terminus region. This suggests that MIPOL1 may interact with another protein. CARD14 (caspase recruitment domain protein 14) is a protein that has such a coiled-coil domain (Bertin et al. 2001) with significant homology to the MIPOL1 protein. It has been suggested that the CARD domain of CARD14 plays a role in the phosphorylation of Bcl10 and in the signaling of NF-κB, and its coiled-coil domain may work as the homo- or hetero-oligomerization domain (Bertin et al. 2001). It is thus likely that the second coiled-coil domain of the MIPOL1 protein may work as an oligomerization regulator by comparative protein–protein interaction.

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