Familial Translocation t(1;9) Associated with Macromastia: Molecular Cloning of the Breakpoints

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Key Words

Breakpoints · Macromastia · Position effect · Reciprocal translocation · t(1;9)

Abstract

A familial reciprocal translocation associated with severe macromastia has been characterized by molecular cytogenetic and molecular analysis. Cloning of the translocation breakpoints revealed that no known gene has been disrupted by this translocation. Therefore, a position effect compromising the regulation of a still to be identified gene in the vicinity of the breakpoints can be assumed.

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Macromastia is a condition of abnormal enlargement of the breast tissue caused by gland hypertrophy, excessive fatty tissue, or a combination of both. It should be delineated from gynecomastia that refers to breast development in males. Four main forms can be distinguished: (1) pubertal or juvenile or virginal hypertrophy, (2) pregnancy-induced hypertrophy, (3) drug-related (for example peni-

cillamine) macromastia, and (4) macromastia caused by tumors such as fibroadenoma. Most cases of macromastia seem to be sporadic but familial occurrence has been reported in some instances. Sex-limited autosomal dominant inheritance has been suggested by Badejo (1984) in two unrelated families with unilateral giant breast in females (MIM 113670). Autosomal dominant juvenile hypertrophy of the breast associated with anonychia has been reported by Govrin-Yehudain et al. (2004). It is noteworthy that in this family macromastia was sex-limited whereas anonychia was present in both male and female carriers. Macromastia can also be associated with hypertrichosis, hirsutism, gum hyperplasia and hyperinsulinemia (Sood et al., 2000). We report on a reciprocal translocation t(1;9) associated with a sex-limited macromastia.

Patients

The pedigree of the family is given in figure 1. IV-1 presented because of three abortions in early pregnancy. Chromosome analysis revealed a translocation between the long arm of chromosome 1 and the long arm of chromosome 9: 46,XX,t(1;9)(q41;q31.3). The same translocation was detected in III-1, whereas normal karyotypes were found in IV-2, III-2, and II-1. II-3 and II-4 were stillbirths and II-5, affected by a hydrocephalus, died shortly after birth. Therefore, it is possible that unbalanced rearrangements may be the cause of these adverse courses. Because of a severe macromastia, reduction mammoplasty was performed in IV-1 at 14

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Fig. 1. Pedigree of the family. Filled circles: macromastia.

years of age. About 2,060 g breast tissue was removed at each side. Pathological examination revealed a marked fibrosis and additionally a fibroadenoma with a diameter of 3 cm on the right side. In III-1 breast reduction was performed at 16 years of age because of severe macromastia. It is likely that I-1 who was also affected by macromastia beginning at the time of puberty was a carrier of the same translocation. Hirsutism was also observed in IV-1, III-1 and I-1. In III-1 adrenal cause of hirsutism could be excluded.

Materials and Methods

Chromosome Analysis

Metaphase chromosomes from PHA stimulated peripheral blood lymphocytes of the available family members were analyzed by standard GTG-banding procedures.

FISH Analysis

FISH studies were performed using YAC and BAC clones selected from the MCN Reference Center at the Max Planck Institute for Molecular Genetics in Berlin (table 1). FISH analysis was performed according to standard protocols. The cells were counterstained with DAPI. Metaphases were captured with the Cyto-vision digital imaging system (Applied Imaging).

DNA Isolation and Southern Blot Analysis

DNA was extracted from peripheral blood by salting out procedure according to standard protocols. DNA was cleaved with restriction enzymes *Bgl*II, *Eco*RI, *Eco*RV, *Pst*I, *Pvu*II, *Sac*I, and *Taq*I. After separation on agarose gels DNA was transferred to Hybond N+ membranes (Amersham Biosciences, Freiburg, Germany) by alkaline transfer. Additionally, very high molecular weight DNA was isolated by embedding leukocytes in agarose

Table 1. Results of FISH analysis in order to narrow the translocation breakpoints

YAC/BAC clones	Location	Results
967b11	1q31–32	n. t.
852g06	1q32	n. t.
958e01	1q32–41	n. t.
851d09	1q32–41	n. t.
RP11 365d23	1q41	n. t.
RP11 459D6	1q41	n. t.
RP11 2007A17	1q41	n. t.
RP11 81E23	1q41	n. t.
RP11 25G08	1q41	brkp. spanning
RP11 664M17	1q41	t.
RP11 438g15	1q41	t.
RP11 22m7	1q41	t.
RP11 75h16	1q41	t.
RP11 361k17	1q41	t.
917h03	1q42	t.
830e05	1q42	t.
898c07	1q42	t.
828g06	1q42	t.
817c03	9q31	n. t.
RP11 473h13	9q31.3	n. t.
RP11 476F1	9q31.3	n. t.
RP11 115I1	9q31.3	n. t.
RP11 1079F2	9q31.3	brkp. spanning
RP11 1122O23	9q31.3	t.
RP11 386d8	9q31.3	t.
850d07	9q32–33	t.
908d10	9q32–33	t.
933c05	9q32–33	t.

brkp. = Breakpoint; n. t. = not translocated, i.e. proximal to breakpoint; t. = translocated, i.e. distal to breakpoint.

plugs and treatment with Pronase E. This DNA was cleaved with the rare cutter restriction enzymes *Sfi*I and *Sac*II and separated by pulsed field gel electrophoresis (PFGE) in the Gene Navigator System (Amersham Biosciences). Southern blots were consecutively probed with four multi-prime labelled PCR-products from the critical region. The four hybridisation probes were amplified from randomly chosen sequences of the breakpoint critical region: PCR 1 (nucleotides 63757–64155 from RP11-1079F2), PCR 2 (nucleotides 29308–29738 from RP11-1079F2), PCR 3 (nucleotides 21480–21806 from RP11-1079F2), and upEDG2 (nucleotides 3871–4114 from RP11-1079F2).

PCR Cloning of *t*(1;9) Breakpoints

Cloning of the breakpoint fusion products was performed by genome walking (BD GenomeWalker Kit, BD Biosciences). High molecular weight DNA from the patient was cleaved in three independent reactions using *Afe*I and *Dra*I, *Afe*I, *Dra*I and *Eco*RV, and *Pvu*II (New England Biolabs), respectively. The DNA was purified by phenol/chloroform extraction and precipitated by ethanol. Adaptor primers supplied in the kit were ligated and one

tenth of the ligation reaction was amplified in a nested PCR reaction (Expand Long Template PCR System, Roche). The PCR primers used were adaptor primer 1 provided in the kit and sequence-specific primer 1 (5'-TGATCTAGCTAGTGTATTCTGAGCG-3') for the first PCR, followed by adaptor primer 2 and sequence-specific primer 2 (5'-ACACAACTTTAAGCTCTTCAGGGTTAG-3'). Sequence-specific primers were generated from DNA sequence GenBank accession number NT_008470.431 and they were commercially synthesised (MWG Biotech). Afterwards, PCR aliquots of the reaction were run on a 1% agarose gel. The bands were excised from the gel, purified using a kit (QiaExII Gel Extraction Kit, Qiagen) and cloned into pCR2.1 plasmid vector (Invitrogen). Independent clones were sequenced (ALFexpress, Amersham Biosciences) and analysed using the BLAST program (NCBI). Cloning of the t(1;9) breakpoint on derivative chromosome 9 was verified by standard genomic PCR using primers generated from the sequence of the fusion product of chromosome 9 (5'-CCAATGAATGAGCAAAGAGG-3') and chromosome 1 (5'-TACACTTCAGACAATCAACAA-3'). Verification of the balanced translocation on derivative chromosome 1 was performed using primers 5'-TGAGAAGCAGGATA-3' from chromosome 9 and 5'-CTCAGTCACATGGCTCCAAC-3' from chromosome 1.

Expression Analysis

Semiquantitative reverse transcription PCR was performed on total RNA isolated from skin fibroblasts as described (Wieland et al., 2001). PCR amplification was performed using primers 5'-GCTGCCATCTCTCTACTTCCAT-3' and 5'-AAGATGGTGTGGTTGAGGGA-3' generating a 1.337-kb product from *EDG2* cDNA (GenBank accession NM_001401) and primers 5'-ACAGAGTACGTGGAGGTCCG-3' and 5'-TGGCATAGATC-TTCTCCGGT-3' generating a 153-bp product specific for *ESRRG* cDNA (GenBank accession NM_001438).

Results

Translocation t(1;9)(q41;q31.3) was detected in IV-1 and III-1, both affected by macromastia. In order to characterize the translocation breakpoints, sequential FISH analysis was performed with YAC- and BAC-clones with inserts derived from 1q31 to 1q42 and 9q31 to 9q33 (table 1). BAC RP11-1079F2 located in 9q31.3 and YAC RP11-25G08 located in 1q41 turned out to be two clones spanning the translocation (fig. 2).

Since clone RP11-1079F2 (start: 110877003, end: 111045349; <http://genome.ucsc.edu/>) overlaps the breakpoint while YAC RP11-1122O23 (start: 110955519, end: 11111928) is completely translocated, the critical region comprises about 78.5 kb. In order to further narrow the critical region Southern blot experiments were carried out.

Hybridisation of a *Sfi*I-blot with probes PCR1, PCR2 and PCR3 detected a junction fragment (B) which was not observed in controls. In contrast, hybridisation of a *Sac*II-

Fig. 2. FISH analysis with breakpoint spanning BACs: **A** Hybridisation with red labelled BAC RP11-25G08, located in 1q41, and green labelled YAC 852g06 as a control. In addition to the signal on the normal chromosome 1, RP11-25G08 showed two specific hybridisation signals, on both derivative chromosomes, and is therefore breakpoint spanning. Further, faint cross-hybridisation signals can be seen on the long arms of both chromosomes 10. **B** Hybridisation with breakpoint spanning BAC RP11-1079F2, located in 9q31.3. A faint hybridisation signal is found on the derivative chromosome 9 and a strong signal on the derivative chromosome 1. Additionally, cross-hybridisation signals can be seen on the short arms of both chromosomes 3.

blot with probe upEDG2 revealed another junction fragment (A) not seen in controls (fig. 3). While in conventional Southern blots probes PCR1, PCR2 and upEDG2 only detected the predicted fragments, probe PCR3 identified aberrant fragments that obviously represent junction fragments from the breakpoint region after restriction with *Bgl*II, *Pst*I, *Pvu*II, and *Sac*I. No junction-fragment

Fig. 3. Localisation of the hybridisation probes used for the breakpoint identification on derivative chromosome 9q31.3. *EDG2* and BAC RP11-1079F2 extend over this region. One junction fragment (JF-A) is detected by probe upEDG2 after restriction with *Sac*II, while PCR1, PCR2, and PCR3 only reveal the same fragments as in controls. After digestion with *Sfi*I probe a further junction-fragment (JF-B) is recognized by probes PCR1, PCR2, and PCR3. The breakpoint is expected in the region between probes upEDG2 and PCR3.

Fig. 4. Restriction map of the breakpoint-critical region on derivative chromosome 9q31.3. Junction fragments are recognized by probe PCR3 for *Bgl*I, *Pst*I, *Pvu*II, and *Sac*I, but not for *Eco*RI, *Eco*RV, and *Taq*I. Fragment sizes of normal fragments are given. The breakpoint-critical region consists of 828 bp flanked by restriction sites for *Pvu*II (5'-site) and *Taq*I (3'-site).

was seen after restriction with *Eco*RI, *Eco*RV, and *Taq*I supporting the hypothesis that the breakpoint must be located in the PCR3 5'-neighbouring region. Analysis of the restriction map confined the breakpoint critical region to a segment of 828 bp (fig. 4).

According to the Southern blot analysis the DNA sequence (GenBank accession number NT_008470.431) from chromosome 9 was retrieved from the US National Center for Biotechnology Information (NCBI) and analysed for restriction enzyme sites. Sequence-specific nested primers generated from the critical region on chromo-

some 9 produced two PCR products from the *Afe*I, *Dra*I and *Eco*RV digested patient DNA (fig. 5A). The PCR product of 1 kb corresponded in size to the normal fragment of chromosome 9, whereas the PCR product of 1.5 kb appeared to be a novel fragment. Sequence analysis of this PCR product revealed fusion of sequences from chromosome 9 and 1 (fig. 6). This allowed the precise location of the t(1;9) breakpoint on derivative chromosome 9. The identified breakpoint was unambiguously verified in the patient's DNA by PCR amplification of the sequence across the translocation breakpoints on chromo-

Fig. 5. Identification and cloning of the t(1;9) translocation breakpoints. **A** PCR based genome walking detected a breakpoint fragment (indicated by an arrowhead) in addition to the normal genomic fragment in *AfeI*, *DraI* and *EcoRV* digested patient DNA (lane 2). *AfeI*, *DraI* (lane 1) as well as *PvuII* (lane 3) cleavage yielded single fragments flanking the breakpoint on chromosome 9. **B** Breakpoint-specific t(1;9) PCR. Primers spanning the breakpoint on derivative chromosome 9 (upper panel) and derivative chromosome 1 (lower panel) were used in PCRs of patient DNA (lanes 2, 6). Single plasmids with the breakpoint junction sequence inserted from chromosome 9 (lanes 3, 4), a plasmid with the insert from the unarranged chromosome 9 (lane 5), DNA from a randomly chosen female (lane 7), and no template (lane 1) served as controls. DNA size markers are indicated at the left (lane M).

Fig. 6. Sequence across the t(1;9) breakpoint. The sequences spanning the breakpoint on chromosome 9 (upper panel) and on chromosome 1 (lower panel) are shown. GenBank Accession numbers of the corresponding genomic reference sequences from chromosome 9 (printed in upper case letters) and chromosome 1 (printed in lower case letters) are indicated. The nucleotides flanking the translocation breakpoints are highlighted and the position of the breakpoints on the respective genomic reference sequences are indicated beneath. PCR primers used for amplification across the translocation breakpoint are underlined. Analysis of the sequence spanning the chromosome 1 breakpoint revealed loss of 11 bp [taat-tactatt] from chromosome 1, insertion of 105 bp (indicated by italic letters) from an L1PA4 located on AC090014 mapping to chromosome 12, and loss of 38 bp [GGGATAGCAAAGACTTTGTAGGG-TCTGCATCATGATCT] from the chromosome 9 sequence.

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→ Chromosome 9: AC007157.6
CCAATGAATG AGCAAAGAGG TGATAGCTGA GATTATGCTC TTCTCATGGT GTACCATGCC

CAATGACTGA GCAAGGGCCT AATAAGTAGC TTCTCTAATG GGCCACCTTT TTCCCAAGAT

CCACGTcttt ttcactagca tgtaactcct aaagtacagt tttcaacttt tttcatctgt
102187/11977
tttttcatct gttttgtttc tagttcttta cacaatatga agcacatggc aagcactcta
taaagtgtta ttaagtgacg gactaactca ttgactggat gaatgagtga ctactgact

caccgaataa atggttgatt gtctgaagtg ta
Chromosome 1: AL451163.8 ←

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→ Chromosome 1: AL451163.8
ctcaqtcaca tggctccaac ctaatgacaa gtacaaagaa ggctgaaata cagactttat

gtgtacccag gaaaagaaaa ctacggtgaa catgcaacat tatctccact aaatattatt

→ AC090014.10
attaatgtcc tcttttaact tgaaattagt tttttccaat tctgtgcaga aagtcattgg
11965/40810
tagcttgatg gggatggcat tgaatctaca aattactttg ggcagaatgg ccattttcac
409014/

GTTGGCATCC CTTGCCCTAT CTGCTTCTC A
102226 Chromosome 9: AC007157.6 ←

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6

some 9 and 1 (fig. 5B). The breakpoint on derivative chromosome 9 was determined to be approximately 16 kb upstream of the *EDG2* gene. The breakpoint on derivative chromosome 1 appears to be far from any known or hypothetical gene of chromosome 1 (fig. 7).

In order to determine whether disruption of the upstream region of the *EDG2* or *ESRRG* gene results in alteration of gene expression we performed semiquantitative RT-PCR. No significant difference of *EDG2* and *ESRRG* expression was detected in skin fibroblasts derived from the patient's arm as compared to a control individual (data not shown).

Discussion

In both available female patients with macromastia the reciprocal translocation $t(1;9)(q41;q31.3)$ could be detected. It can be assumed that I-1, also affected by macromastia, carried this translocation. In this case the adverse course of II-3, II-4 and II-5 could be explained by unbalanced rearrangements. Because the female family members with normal breast development do not carry the translocation, it is very likely that macromastia is caused by this translocation. This form of macromastia seems to be restricted to the female sex because II-2 had no gynecomastia. In all three cases macromastia arose at the beginning of puberty suggesting an estrogen dependent effect.

In the past, molecular cloning of translocation or inversion breakpoints turned out to be a fruitful strategy for the identification of disease genes. Loss of function can be caused by gene disruption or position effects. Gain of function can emerge from the formation of fusion genes as it is well known in the pathogenesis of tumours. To our knowledge this translocation has not been observed in fibroadenomas or other benign tumours of the breast. We decided to characterize the breakpoints of this translocation in order to identify the molecular basis of macromastia in this family.

Using a combination of FISH, Southern blot analysis and PCR based genome walking the breakpoints of the derivative chromosomes 1 and 9 could be unambiguously determined. The breakpoint on chromosome 1 appears to be far from any known or hypothetical gene. The breakpoint on chromosome 9 is located approximately 16 kb

Fig. 7. Schematic presentation of the genomic region surrounding the balanced $t(1;9)$. A modified screen picture of the NCBI Map Viewer (www.ncbi.nlm.nih.gov/mapview) showing approximately 3-Mb segments of chromosome 9q31.3 and 1q41, respectively. Orientation of gene transcription is indicated by arrows at the top. The positions of the breakpoints (BP) are indicated by red arrows.

upstream of the *EDG2* gene. Again, this breakpoint does not disrupt a known gene. Therefore, a position effect is the most likely explanation. In a context of position effect caused by chromosomal rearrangements, transcriptional control can be disturbed by physical dissociation of a gene from its set of regulators or by alteration of chromatin structure. *EDG2* (Endothelial Differentiation Gene 2, Lysophosphatidic Acid Receptor 2, Ventricular Zone Gene 1) is a G protein-coupled receptor gene encoding a receptor for lysophosphatidic acid (LPA) (Hecht et al., 1996). An et al. (1997) detected two transcripts in most human tissues and the strongest expression was found in brain. LPAs are mediators generated by phospholipase cleavage of membrane phospholipids. LPAs can be detected in micromolar concentrations in serum and are involved in cell proliferation, platelet aggregation, smooth muscle contraction, chemotaxis, and tumour cell invasion. Targeted deletion of *Lpa1* in mice results in high lethality, impaired suckling behaviour in neonatal pups as well as reduced size and craniofacial dysmorphism in survivors. Therefore, it is unlikely that the translocation in our patients causes functional silencing of *EDG2*. Nevertheless, because overexpression of this gene cannot be ruled out, semiquantitative RT-PCR of *EDG2* was performed in fibroblasts, but no significant change could be detected as compared to a control individual. However, it must be considered that in this case fibroblasts may not be the appropriate tissue for expression studies. It is possible that a position effect is only effective in breast tissue, but unfortunately breast tissue of the patients is not available.

ESRRG (Estrogen Related Receptor Gamma or Estrogen Related Receptor 3) is located in 1q41, approximately 2 Mb of the breakpoint in chromosome 1. *ESRRG* is a member of the steroid/thyroid/retinoid receptor superfamily (Eudy et al., 1998). It is expressed in a variety of tissues including the mammary gland. Hong et al. (1999) demonstrated that the mouse homologue binds to an estrogen response element in the absence of any ligand. Therefore, it makes *ESRRG* as candidate gene rather unlikely. Unfortunately, this hypothesis could not be examined because *ESRRG* is not expressed in fibroblasts.

The identification of genes dysregulated by chromosomal rearrangements can be very difficult because the genomic regions containing *cis*-acting regulatory elements such as enhancers or repressors can stretch as much as 1 Mb in 5'- or 3'-direction (Kleinjan and van Heyningen, 2005). An approach for the detection of such long range regulatory elements may arise from the study of highly conserved regions between evolutionary diverged species.

The breakpoint characterisation of the derivative chromosome 1 revealed a complex rearrangement including loss of 11 nucleotides from chromosome 1, insertion of 105 nucleotides from chromosome 12 and loss of 38 nucleotides from chromosome 9. The inserted sequence from chromosome 12 is part of an LIPA4 sequence. Because LINE1 sequences are transposable elements, it is tempting to speculate that this sequence has promoted this translocation.

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