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Translocation and Deletion around *SOX9* in a Patient with Acampomelic Campomelic Dysplasia and Sex Reversal

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Key Words
Acampomelic campomelic dysplasia · Deletion · Sex reversal · *SOX9* · Translocation

Abstract
Campomelic dysplasia (MIM 114290) is a severe malformation syndrome frequently accompanied by male-to-female sex reversal. Causative are mutations within the *SOX9* gene on 17q24.3 as well as chromosomal aberrations (translocations, inversions or deletions) in the vicinity of *SOX9*. Here, we report on a patient with muscular hypotonia, craniofacial dysmorphism, cleft palate, brachydactyly, malformations of thoracic spine, and gonadal dysgenesis with female external genitalia and müllerian duct derivatives in the presence of a male karyotype. X-ray examination and clinical examinations revealed no signs of campomelia. The combination of molecular cytogenetic analysis and array CGH revealed an unbalanced translocation between one chromosome 7 and one chromosome 17 [46,XY,t(7;17)(q33;q24).ish t(7;17)(wcp7+,wcp17+;wcp7+wcp17+)] with a deletion of approximately 4.2 Mb located about 0.5 Mb upstream of *SOX9*. STS analysis confirmed the deletion of chromosome 17, which has occurred de novo on the paternal chromosome. The proximal breakpoint on chromosome 17 is localized outside the known breakpoint cluster regions. The deletion on chromosome 17q24 removes several genes. Among these genes *PRKAR1A* is deleted. Inactivating mutations of *PRKAR1A* cause Carney complex. To our knowledge, this is the first report of a patient with acampomelic campomelic dysplasia, carrying both a deletion and a translocation.

Campomelic dysplasia (CD; MIM 114290) is a rare and severe skeletal malformation syndrome. Characteristic features include congenital bowing of long bones (i.e. campomelia), hypoplastic scapulae, deformed pelvis and spine, and a reduced number of ribs. Craniofacial defects such as cleft palate, micrognathia, flat face, and hypertelorism are also common. Most patients do not survive the neonatal period due to severe respiratory distress. In about two thirds of 46,XY CD patients, partial or complete male-to-female sex reversal is observed [Houston et al., 1983; Mansour et al., 1995]. An atypical form with absence of campomelia, referred to as acampomelic CD (ACD), is found in about 10% of patients and is more frequent among those individuals surviving the neonatal period [Mansour et al., 2002].

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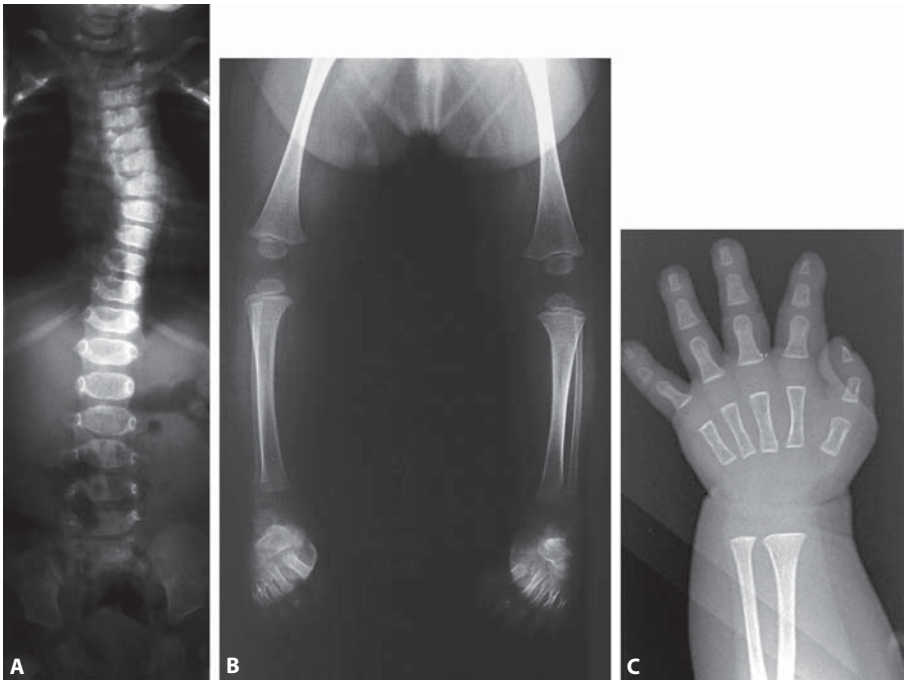

Fig. 1. X-rays of the patient at the age 20 months. **A** The chest showed severe scoliosis. **B** Lower extremities of the patient exhibited no signs of campomelia. **C** Shortening of the thumb is striking.

In the majority of patients, de novo heterozygous loss-of-function mutations in the coding region of the transcription factor gene *SOX9* on 17q24 are detected. Chromosomal aberrations (translocations, inversions and deletions) are rare causes of CD/ACD. In only few cases, breakpoints have been determined precisely [Hill-Harfe et al., 2005; Leipoldt et al., 2007]. Leipoldt et al. [2007] defined 2 breakpoint cluster regions with a proximal breakpoint cluster between 50 and 375 kb and a distal breakpoint cluster between 789 and 932 kb upstream of *SOX9*. Recently, Lecointre et al. [2009] reported on a deletion of 960 kb upstream of *SOX9* causing a familial ACD. Here, we report on an ACD patient with male-to-female sex reversal and translocation [t(7;17)] as well as a ~4-Mb deletion located 492 kb upstream of *SOX9* in chromosome 17.

Materials and Methods

Case Report

The patient is the second child of nonconsanguineous parents (a 32-year-old father and a 28-year-old mother) and was born in the 39th week of pregnancy with a birth weight of 2,940 g and a length of 49 cm. After birth craniofacial dysmorphism including epicanthus, broad nasal bridge, low-set dysmorphic ears, and cleft palate as well as muscle hypotonia and short hands were obvious. By X-ray examination no signs for campomelia were seen, but vertebral malformations, including fusion of cervical and thoracal vertebrae, only 11 pairs of ribs, severe scoliosis, and a double kidney on the left side were detected. Chromosome analysis revealed a male karyotype despite female external genitalia, uterus and inguinal gonads.

Now, at 5 years of age, the patient has a severe progressive scoliosis (fig. 1) and lordosis. There is a mild muscle hypotonia, and she is able to walk. She speaks clearly and answers precisely to questions. Sensorineural hearing deficit requires the use of hearing aids.

Cytogenetic Analysis

Chromosome preparations of the patient were made from cultured B-lymphocytes (EBV-transformed lymphoblastoid cell culture), according to standard procedures. Karyotype analysis was done on GTG-banded chromosomes at a banding level of 400 (ISCN). Chromosome preparations of the parents were made from PHA-stimulated peripheral blood lymphocytes and analyzed by standard GTG banding procedures at a banding level of 450 (ISCN). FISH studies were performed with whole chromosome painting probes for chromosome 7 and 17 according to the supplier's protocol (Q BIOgene, Heidelberg, Germany; Total Chromosome DNA probe Chromosome 7 Green, Chromosome 17 Red).

DNA Isolation and STR Analysis

High-molecular-weight DNA was isolated from peripheral blood by salting out procedure and from cultured lymphoblastoid cell lines by phenol-chloroform extraction, both according to standard protocols. For STR (Short Tandem Repeats) analysis primers for following loci were used: D17S807, D17S1870, D17S1350, D17S1304, D17S1351, and D17S1352. PCR was performed in a total volume of 20 μl reaction buffer containing ~200

ng of genomic DNA, 0.4 μM of each primer, 0.2 mM dATP, dGTP, and dGTP, 10 μM dCTP, 0.2 μCi α[^{32}P]dCTP, 1.5 mM $MgCl_2$, and 0.5 U *Taq* polymerase (Invitrogen, Karlsruhe, Germany). Products were separated on 6% denaturing polyacrylamide gels and visualized by autoradiography.

Array CGH

Array CGH was performed as described previously [Erdogan et al., 2006]. In brief, 2 μg of amplified patient and reference DNA were labeled by random priming (Bioprime Array CGH, Invitrogen, Carlsbad, Calif.) with Cy3 and Cy5 (Amersham Biosciences, Piscataway, N.J.), respectively, and hybridized onto a tiling path BAC array, consisting of the human 32k BAC Re-Array Set (BACPAC Resources Center; http://bacpac.chori.org/pHumanMinSet.htm). For the analysis and visualization of array CGH data, the software package CGH-PRO [Chen et al., 2005] was employed. Raw data were normalized by 'Subgrid LOWESS'. For the assessment of copy number gains and losses, we used conservative log2 ratio thresholds of 0.3 and –0.3, respectively. Deviant signal intensity ratios involving 3 or more neighboring BAC clones were considered to be potentially pathogenic, unless they were covered by more than one known DNA copy number variant, as listed in the Database of Genomic Variants (http://projects.tcag.ca/variation/) or covered by >50% of their length at least once in our reference set of more than 700 samples.

Southern Blot Analysis

DNA was cleaved with restriction enzymes (New England Biolabs, Frankfurt/M., Germany), separated on 0.7% agarose gels and transferred to Hybond XL membranes (GE Healthcare, Freiburg, Germany) by alkaline transfer. Southern blots were consecutively probed with multi-prime labeled PCR-products from the critical region. The hybridization probes were amplified from randomly chosen sequences of the breakpoint critical region from N0074N19. Primers for probe up SOX9–8 were: upSOX9_8_F: TTGATGCATACACATCTGGGA and upSOX9_8_F: TTACACTCCTGGAGTATGTCA.

Identification of the Breakpoints

Genomic walking was carried out using the BD Genome Walker™ Universal Kit (BD Biosciences Clontech, Palo Alto, Calif.). In short, genomic DNA of the patient was cleaved in 4 independent reactions with blunt-end cutting restriction enzymes *Eco*RV, *Hpa*I, *Pvu*II, and *Stu*I. After phenol-chloroform extraction and ethanol precipitation, the adaptor supplied in the kit was ligated to both ends of the genomic DNA fragments. Subsequent amplification of these walking libraries was carried out in 2 nested rounds using the High Fidelity Expand Long Template PCR System (Roche, Mannheim, Germany). For the first PCR, for determination of the chromosome 17 telomeric breakpoint, adaptor-specific primer 1 (AP1) from the kit and a self-designed sequence-specific primer (SOX9_BP_GSP1: 5′-AAGTCTACCAGTTTACTGCTCTGTAACAAG-3′) were used. A nested amplification round was carried out using primers AP2 (supplied in the kit) and SOX9_BP_GSP2 (5′-GTTCTCTAAGCCCTTTTAGCTCTATTCCAC-3′). After each round, PCR-products were analyzed on 1% agarose gels. Appropriate bands were cut out from the agarose gel, purified and either directly sequenced or after being cloned into pGEM-T Easy vectors (Promega, Mannheim, Germany). Sequencing reactions were carried out using the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Freiburg, Germany) and analyzed on a MegaBace 500 sequencer (GE Healthcare). The resulting sequences were subjected to FASTA searches (www.ebi.ac.uk.fasta33). The proximal breakpoint of chromosome 17 was obtained by amplifying the walking libraries with primers AP1 and Chr.7_1_R (5′-TGGGACCTTGGTTTAGACCACAGAGTG-3′) and AP2/BP_Chr7_2_R (5′-CTGAGGTCAGGCAGGATGCCTGATGTA-3′), respectively. All breakpoints were verified by sequencing PCR products across the translocation breakpoints.

Results

The patient's phenotype indicated the diagnosis of ACD. Conventional cytogenetic analysis and whole chromosome painting revealed a karyotype 46,XY,t(7;17)(q33;q24).ish t(7;17)(wcp7+,wcp17+;wcp7+wcp17+). By sequencing *SOX9* no alteration could be detected. Array CGH analysis identified an approximately 4.2-Mb-spanning deletion located 5′ to and ~500 kb apart from *SOX9*. N0737P07 (RP11–737P07) and N0074N19 (RP11–74N19) were identified as proximal and distal breakpoint clones, respectively. This deletion could be confirmed by STR analysis (fig. 2). For the critical region, only a maternal allele could be amplified from the patient's DNA; the paternal allele was missing. The absence of the paternal allele indicates a de novo occurrence of the deletion on the paternal chromosome. Furthermore, chromosome analysis of the parents revealed normal karyotypes.

The precise telomeric breakpoint region from chromosome 17q24 was narrowed down by quantitative Southern blot hybridization experiments. Therefore, Southern blots from equal amounts of DNA of the patient and one control person, cleaved with various restriction enzymes, were produced. For the generation of hybridization probes sequences, 9 almost equally distributed sections of N0074N19 were selected for PCR amplification (= probe upSOX9–1 to upSOX9–9). Probes localized telomeric to the breakpoint hybridized in normal dosage to the patient's DNA, whereas probes localized centromeric to the breakpoint hybridized in half of the normal intensity. Hybridization with probe upSOX9–8 revealed junction fragments for 4 restriction enzymes (*Bgl*I, *Eco*RI, *Sac*I and *Tth*111I) in the patient's DNA in comparison to the control suggesting close vicinity to the chromosome 17 telomeric breakpoint. According to a restriction map of the breakpoint region derived from a database sequence (AC118653), the predicted breakpoint region could be confined to an interval of approximately 800 bp. For the identification of the breakpoint at the nu-

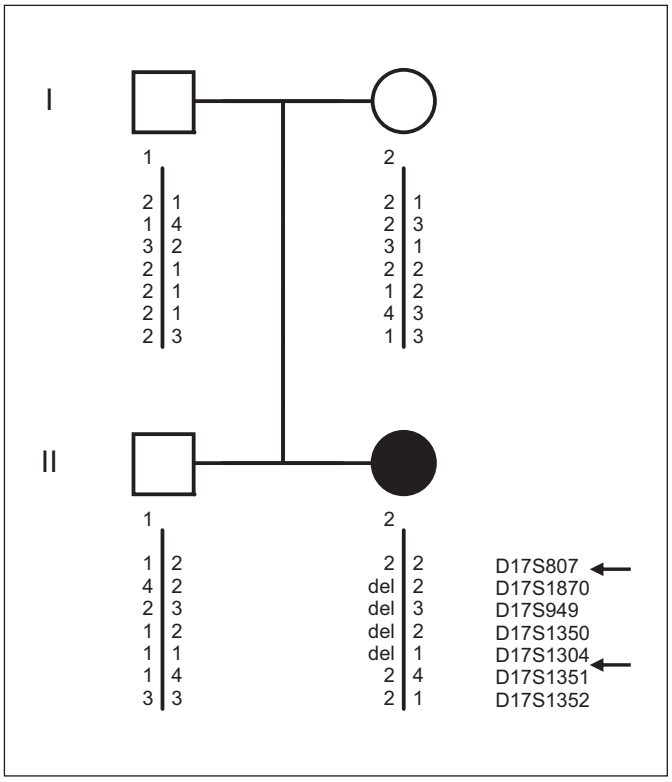

Fig. 2. Results of STR analysis confirmed a deletion in the patient, which has occurred on the paternal chromosome. Presence of a reciprocal translocation t(7;17) is not considered in this figure.

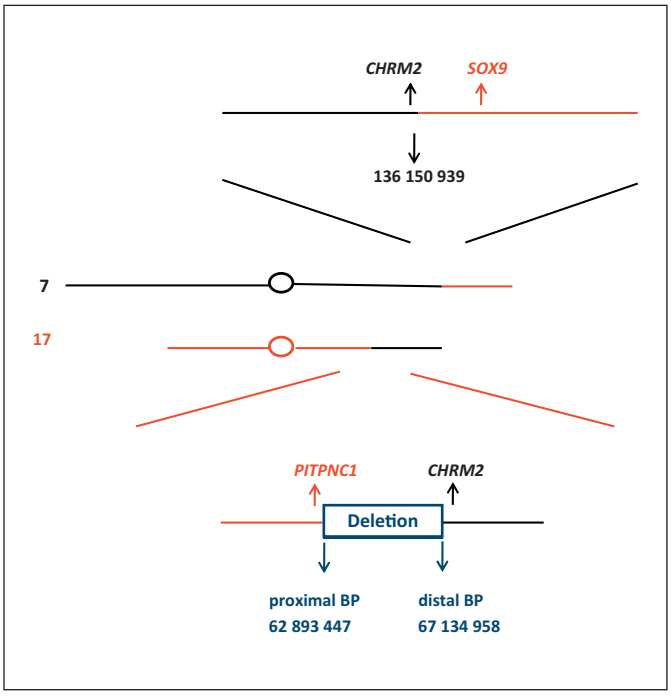

Fig. 3. Localization of translocation/deletion breakpoints in the derivative chromosomes 7 (black) and 17 (red). *PITPNC1* on chromosome 17 is partly deleted by the 4.2-Mb deletion, *CHRM2* is interrupted by the translocation. Numbers refer to positions of breakpoints according to HG17. The genes involved in the deletion are given in table 1.

cleotide level, a chromosome walking library of the region of interest was established (see Methods). From this library, a breakpoint-spanning fragment was amplified and sequenced. Sequence analysis revealed identity to chromosomes 17q24 and 7q33. Starting with a PCR-primer derived from the chromosome 7q33 sequence, a fragment covering the 17q24 centromeric breakpoint could be amplified from our walking library. After sequencing the corresponding cloned fragment, the breakpoints could be assigned exactly. The 7q33 breakpoint corresponds to BAC clone AC020581 at position 136,344,223 (http://genome.ucsc.edu/) within the *CHRM2* gene. The centromeric breakpoint on chromosome 17 is localized after position 62,893,447 in the region of *PIPNC1*, while the telomeric breakpoint is localized before nucleotide 67,134,958 (http://genome.ucsc.edu/). Thus, the deletion comprises of 4,241,511 Mb and is located 493,799 bp centromeric to *SOX9* according to HG17 (fig. 3). According to the databases, several genes map to the deletion interval (table 1). The sequences spanning the breakpoints are given in figure 4.

Discussion

In the patient with ACD, a deletion of 4.2 Mb, located approximately 0.5 Mb upstream from *SOX9,* and a de novo translocation (7;17) have been detected. It can be assumed that the deletion arose in the context of this translocation. Each aberration alone – the translocation and the deletion – may be sufficient to explain the phenotypic characteristics of the patient. She has survived the neonatal period as it has been described for several ACD patients with aberrations outside of the *SOX9*-coding region [Pfeifer et al., 1999]. Besides point mutations within *SOX9*, translocations, inversions and deletions centromeric to *SOX9* cause CD or ACD.

So far, in the literature only few deletions within the *SOX9* region have been reported. In the first patient, the breakpoint has not been determined exactly, but it is reported that the deletion is localized in 17q23.3–24.3 and comprises the entire *SOX9* gene as well as the regions upstream and at least 10 kbp downstream [Olney et al., 1999]. Pop et al. [2004] reported 2 deletions: the first deletion oc-

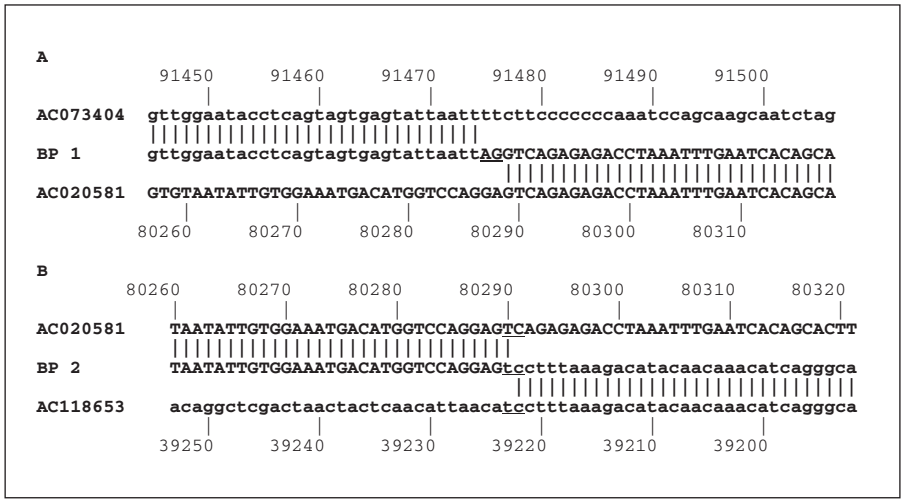

Fig. 4. A Sequences spanning breakpoint 1 (= BP1; proximal 17q/distal 7q) and **B** sequences spanning breakpoint 2 (= BP2; proximal 7q/distal 17q). Sequences of chromosome 7 are given in upper case while those of chromosome 17 are given in lower case. Numbers refer to positions within the corresponding BAC. The double-underlined dinucleotide AG at the junctions between chromosome 17 and chromosome 7 in BP1 could not be assigned to one of the BACs and must therefore represent an insertion. The underlined TC dinucleotide at BP2 is present in the corresponding sequences of chromosome 7 and chromosome 17 and cannot unequivocally be attributed to one or the other sequence.

Table 1. Genes included in the deletion of chromosome 17 (according to HG17)

Gene	Localization	Disease
PITPNC1	62804386–63120107	
NOL11	63144521–63170772	
BPTF	63252242–63410956	
C17orf58	63417680–63420164	
KPNA2	63462310–63473431	
ENS00000154251	63633345–63635979	
Q6ZU00	63677569–63677958	
Q66K37	63706572–63708031	
AMZ2	63755310–63776634	
SLC16A6	63775933–63799002	
ARSG	63814772–63930467	
WIPI1	63929018–63965210	
PRKAR1A	64019705–64040503	Carney complex type 1 (MIM 160980) intracardiac myxoma (MIM 225960) primary pigmented nodular adrenocortical disease 1 (MIM 610489)
FAM20A	64044607–64109125	
ABCA8	64375028–64463128	
ABCA9	64482369–64568731	
ABCA6	64586442–64649610	
Q9P162	64656019–64656177	
ABCA10	64655772–64752582	
ABCA5	64754387–64834885	
MAP2K6	64922433–65050046	
KCNJ16	65583021–65643339	
KCNJ2	65677271–65687755	Andersen cardiodysrhythmic periodic paralysis (MIM 170390) short QT syndrome 3 (MIM 609622)
ENSG0000214155	67363766–67365559	
Q8IVH9	67548060–67548212	

curred in a male CD patient and comprises more than 4 Mb. Also this deletion removes the entire *SOX9* gene. Interestingly, it also had occurred on the paternal chromosome. The second deletion is located 380–1,869 kb centromeric to *SOX9* and was found in an ACD patient with 46,XY sex reversal. Lecointre et al. [2009] reported on a familial ACD caused by a 960-kb deletion encompassing a fragment from 517 kb to 1.477 Mb upstream of *SOX9* which removes several highly conserved sequences.

Analysis of deletions upstream of *SOX9* is of great impact for the identification of regulatory sequences. Bagheri-Fam et al. [2006] assayed the regulatory potential of 7 conserved sequence elements (E1 to E7) located between 290 kb 5′ and 95 kb 3′ to human *SOX9* in a transgenic mouse model. Among them E1 being located 28 kb 5′ to *Sox9* controls the expression in the node, notochord, gut, bronchial epithelium as well as in the pancreas, while E3 located 251 kb 5′ to *Sox9* enhances the expression in the neural crest cells of the inner ear. Although our patient is affected by a hearing deficit, the deletion detected does not comprise these regulatory sequences. Also the homologous sequence to the recently identified testis-specific enhancer of *Sox9* [Sekido and Lovell-Badge, 2008] is not deleted. Therefore, other regulatory elements may be missing, or a position effect may influence the function of known regulatory sequences. For example, the developmental enhancer of *SOX9* located 1.44 Mb upstream of *SOX9* is involved in pathogenesis of Pierre-Robin sequence [Benko et al., 2009]. Therefore, the cleft palate, being present in our patient and being one of the characteristics of PRS, may be explained by the inclusion of this developmental enhancer into her deletion.

Database analysis revealed that several protein-coding sequences map into the deleted region (Ensembl release 49; table 1). One of these genes is *PRKAR1A,* encoding the cAMP-dependent protein kinase regulatory subunit type Iα [Kirschner et al., 2000]. Inactivating mutations of this gene are associated with Carney complex type 1 (CNC1, MIM 160980) as well as isolated primary pigmented nodular adrenocortical disease (PPDNAD, MIM 610489) and intracardiac myxoma. CNC, a multiple neoplasia syndrome, is charaterized by endocrine tumors, spotty skin pigmentation, cardiac and other myxomas, psammomatous and pigmented schwannomas, large-cell calcifying Sertoli cell tumors (LCCSCTs), and mammary ductal adenomas as well as other rather rare lesions. CNC and PPNAD are inherited in an autosomal-dominant manner. So far, neither Carney complex nor isolated PPDNAD or intracardia myxoma were diagnosed in the patient.

Both chromosome 17 breakpoints in the patient map outside the translocation breakpoint clusters defined by Leipoldt et al. [2007] which are located between 50 and 375 kb (proximal breakpoint cluster) and between 789 and 932 kb (distal breakpoint cluster) centromeric to *SOX9*.

The proximal deletion breakpoint on chromosome 17 in the patient is located within the *PITPNC1* gene, a member of the phosphatidylinositol transfer protein family. The corresponding cytoplasmic protein transfers phosphatidylinositol from one membrane compartment to another. Two transcript variants encoding distinct isoforms have been identified for this gene.

The breakpoint on chromosome 7q33 is located within the *CHRM2* (muscarinic cholinergic receptor 2) gene. The muscarinic cholinergic receptors belong to a large family of G protein-coupled receptors. The functional diversity of these receptors is defined by the binding of acetylcholine to these receptors and includes cellular responses such as adenylate cyclase inhibition, phosphoinositide degeneration and potassium channel mediation. Muscarinic receptors influence many effects of acetylcholine in the central and peripheral nervous system. The muscarinic cholinergic receptor 2 is involved in mediation of bradycardia and a decrease in cardiac contractility. Multiple alternatively spliced transcript variants have been described for this gene.

As *CHRM2* and *PITPCN1* are both transcribed in the same direction (+ strand), the translocation (7;17) could mediate the formation of a fusion gene with an alternative function or control.

For the management of this patient, it is important to keep in mind that adverse consequences of these deleted or disrupted genes can occur during further development.

Acknowledgements

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