The breakpoint identified in a balanced *de novo* translocation t(7;9)(p14.1;q31.3) disrupts the A-kinase (PRKA) anchor protein 2 gene (AKAP2) on chromosome 9 in a patient with Kallmann syndrome and bone anomalies

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Abstract. We report the molecular characterization of a patient with Kallmann syndrome and bone anomalies bearing a balanced *de novo* translocation t(7;9)(p14.1;q31.3) which completely disrupts the A-kinase anchor protein 2 gene (AKAP2) on chromosome 9. In order to investigate the role of AKAP2 in the pathogenesis of the disease, we analyzed the expression of Akap2 in mouse embryos. The expression pattern was consistent with the phenotype observed and mAkap2 was actually found in the olfactory bulb and in the cartilagineous structures of the embryo. Since AKAP2 is supposed to bind and compartmentalize the PKA, we also analyzed the distribution and quantity of PKA in limphoblastoid cell lines of the patient compared with a control; these experiments did not demonstrate any differences between the cell lines. Furthermore a collection of 98 DNA samples from sporadic Kallmann patients was screened for mutations in this gene. The analysis revealed two different sequence variations observed in two patients but not in 200 control chromosomes: since they have been detected also in the unaffected mother of one of the two patients we can assume that they are rare polymorphisms, although we cannot exclude that they represent mutations with incomplete penetrance. Our findings suggest that the complex phenotype with Kallmann syndrome and bone anomalies observed in our patient could be the result of the interruption of the AKAP2 gene. However, a position effect mediated by the translocation could not be excluded. The screening of AKAP2 in other Kallmann patients will be necessary to elucidate its role in the pathogenesis of the disease.

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Introduction

Kallmann syndrome (KS) (MIM308700) is a genetic condition, characterized by the existence of hypogonadotropic hypogonadism associated with anosmia or hyposmia (1).

The major symptoms of KS can be explained as a defect of normal olfactory-system development: anosmia and hyposmia are due to the absence of the olfactory lobes, and hypogonadism is a consequence of the deficiency of hypothalamic gonadotropin-releasing hormone (GnRH). Sometimes these main features are associated with abnormalities in the development of the kidney (unilateral renal aplasia or hypoplasia), mirror movements of the hands (synkinesia), pes cavus, high arched palate, uncoordinated eye movements (nystagmus) and cerebellar ataxia.

KS is a genetically heterogeneous disease and cases of autosomal dominant or recessive and X-linked inheritance have been reported.

Only two genes have been associated with KS. Mutations in the KAL-1 gene are responsible for an X-linked type mapped on the short arm of the X chromosome by Franco and collaborators (2). Recently, Dode showed that mutations in the FGFR1 gene cause an autosomal type of KS (3).

As a consequence of patients' potential infertility, large pedigrees are not available and, therefore, it is difficult to apply genetic analysis based on linkage to the identification

of autosomal KS genes. For this reason much attention is given to balanced translocations as indicative of candidate regions.

The present work describes the cytogenetic and molecular characterization of a patient with a complex phenotype including KS and bone anomalies, presenting a balanced *de novo* translocation between chromosome 7 and chromosome 9. The patient was negative for mutations in KAL-1, which is mutated in 15% of KS patients, and in KAL-2 (FGFR1), a recently described autosomic gene mutated in KS. Since no mutations or deletions have been found in the patient tested for mutations in the KAL-1 and KAL-2 gene, the translocation was characterized by the molecular point of view, looking for genes that were interrupted at the breakpoints and likely responsible for the complex phenotype observed.

Materials and methods

Cytogenetic and FISH analysis. Chromosome analysis on peripheral blood lymphocytes and EBV-transformed lymphoblastoid cell line from the proband and his parents was performed according to standard cytogenetic techniques.

The rearrangement breakpoints were refined at the BAC level by FISH analysis using the RPCI-11 library. BAC clones were obtained from the YAC Screening Center at DIBIT, Milan.

BAC DNA was extracted using the PhasePrep BAC DNA kit (Sigma) and directly labeled with SpectrumGreen and SpectrumOrange by nick translation (Vysis, Inc.). Slides were counterstained with Vectashield-DAPI antifade medium (Vector Laboratories, USA). Signals were visualized under a Nikon E1000 microscope equipped with a cooled charged-coupled device (CCD) camera and Genikon image analysis software.

Mapping the breakpoints. A selection of genomic clones expected to be located in the cytogenetic bands involved in the translocation were used as probes for FISH on patient's chromosomes. BAC clones were selected from the human RPCI-11 library according to the UCSC human genome assembly (http://genome.ucsc.edu/; May 2004 freeze).

Array-CGH analysis. Array-CGH was performed by using the Spectral Genomics 1 Mb chip (www.spectralgenomics.com). The chip provides a 1 Mb resolution with >2,600 BAC clones (genome-wide) spotted on the array. Along with the arrays we used SpectralWare software that allows the analysis of the acquired images giving a graphical view of the results. Patient and control DNAs were digested with EcoRI (NEB) and products were run on agarose gel 1% for quality assessment. The digested DNA was purified and precipitated by standard methods. We used the Dye-Reversal strategy (Dye-Swap) on two different microarrays for Cyanine3 (Cy3) and Cyanine5 (Cy5) dye-labelled control and patient DNAs (BioPrime DNA labeling System, Invitrogen). DNAs were co-hybridized on one microarray and at the same time labelled in an opposite way and co-hybridized on a second microarray. For hybridization and washing, in brief, 25 μl of reaction mix with the patient DNA was co-precipitated with an equal volume of labeled control DNA with 50 μg human COT1 and resuspended in 45 μl of hybridization buffer. After denaturing at 72˚C for 5 sec and incubation at 37˚C for 30 sec, the DNA was placed over a microarray, covered by a 24x60-mm coverslip and incubated in an hybridization chamber (Corning) at 42˚C for 14-16 h. After hybridization the microarray was washed in PBS/0.05% Tween-20, then in 50% formamide at 45˚C and again in PBS at RT and finally washed in bidistilled water and promptly dried. The dye emission capture was performed by a dual-laser scanner (Axon 4000B) and analyzed by the SpectralWare program. Each array was simultaneously analyzed at two different wave lengths (635 and 532 nm). Controls are provided within the array by Spectral Genomics to normalize the signal data.

PAC digestion. Digestion of the PACs was accomplished through the use of EcoRI and AatII enzyme. Buffers and enzyme were purchased by the New England BioLabs. The standard digestion of the DNA was performed by using 5 U of enzyme for each microgram of DNA, at the recommended temperature (37˚C).

Fragments amplification by PCR. Exons in a size range of 200-400 bp were amplified. Exon number 8 was divided into 13 overlapping subfragments of approximately 200 bp. Primers of 20 bp in length were designed. All PCR was carried out through the use of the following conditions: 30 sec at 95˚C, 30 sec at 60˚C, 30 sec at 72˚C, for 30 cycles. An initial denaturation of 10 min at 95˚C and a final extension of 5 min at 72˚C degree was also used.

Screening mutations by SSCP analysis. The gel composition for the SSCP was 8% of acrylamide, 5% of glycerol and 1X TBE buffer. Gels were ran using BioRad PAGE apparatus, overnight at an approximate voltage of 170 constant volts. Staining of the gel was carried out through the silver-staining procedure.

Screening mutations by direct sequencing. All the sequences were run on a 3100 Applied Biosystem Analyzer sequencer. A standard protocol for sequence reaction made use of 100 ng of DNA from PCR and 800 ng of plasmid DNA. For PCR products and plasmid DNA, 5 and 10 pmoli of primers respectively were used. The cycles were 96˚C for 10 sec, 60˚C for 3 min for 25 cycles with an initial denaturation of 96˚C for 10 sec.

RNA extraction. The RNA deriving from patient's lymphoblasts was isolated using an ULTRASPEC RNA isolation system (BIOTECX). In a second step it was transcribed by using MMLV reverse transcriptase (Clontech Laboratories).

Confocal microscopy. Cells were fixed and permeabilized by the Triton/paraformaldehyde method as described by Higgins *et al* (1988). Non-specific protein binding to cells was blocked by 30-min incubation with 5% (v/v) fetal calf serum diluted in PBS. Cells were then treated with 1 μg/ml antibody directed towards the catalytic subunit or the regulatory subunit of PKA (anti-PKAα cat or anti-PKAIIα reg, Santa Cruz Biotechnology). Anti rabbit Ig (H+L) Alexa Fluor 488 conjugate (Molecular Probes), 0.5 μg/ml, was used as a secondary antibody. Cells were mounted on coverslips with Vectashield antifade reagent

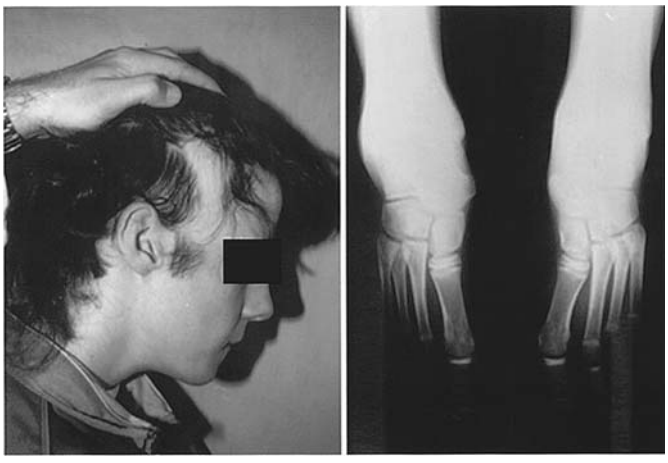

Figure 1. The propositus showing alopecia areata and the absence of the fifth toe bilaterally.

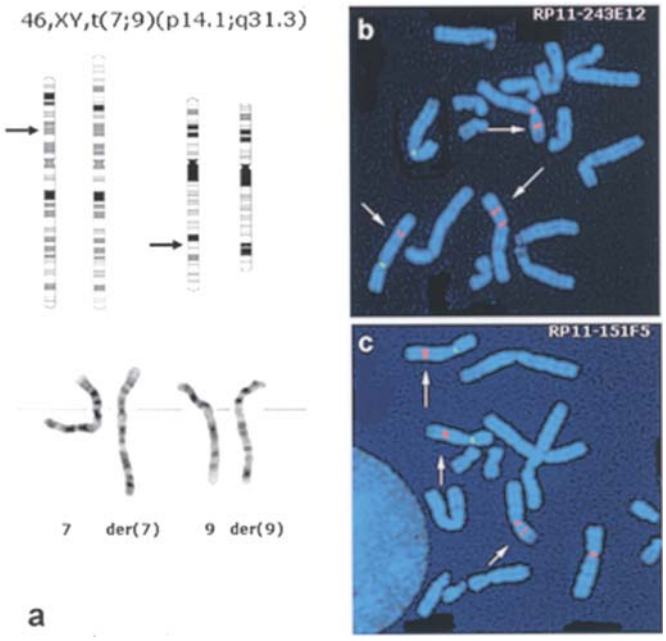

Figure 2. (a) Partial karyotype and schematic representation of the *de novo* balanced translocation 46,XY,(7;9)(p14.1;q31.3). (b) FISH on proband metaphase spreads with RP11-243E12: red signals (arrows) are present on normal chromosome 7 and on derivative chromosomes der(7) and der(9). (c) FISH on proband metaphase spreads with BAC RP11-151F5: red signals (arrows) are present on normal chromosome 9 and on derivative chromosomes der(9) and der(7). Centromeric probe CEP7 (Vysis) (red) and CEP9 (Vysis) (green) indicate respectively chromosomes 7 and 9.

(Vector Laboratories) and images of the samples were collected by confocal microscopy using a Bio-Rad MRC1024 instrument on a Nikon Diaphot 200, using a planapochromat x60 objective with normal aperture 1.4. The excitation/emission wavelengths were 488/522 nm.

Western blotting. Total cell samples, corresponding to 10^5 cells, were subjected to SDS-PAGE (10% polyacrylamide gel) and the separated proteins were transferred to nitrocellulose membranes (BioRad) by electroblotting. Membranes were incubated for 1 h with 0.1 μg/ml anti-PKAα cat antibody or 0.1 μg/ml anti-actin antibody (goat polyclonal IgG, Santa Cruz Biotechnology). Immunoreactivities were revealed with 0.05 μg/ml peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), developed with an enhanced chemi-luminescence detection system (Amersham Biosciences).

In situ hybridization. In order to obtain a probe for *in situ* hybridization, a fragment of the mouse Akap2 cDNA (NM_009649) corresponding to nucleotides 1891-2514 was amplified by RT-PCR using E15.5-E17.5 mouse olfactory bulb cDNA as a template, and subcloned into pBluescript. The probe was linearized with appropriate restriction enzymes to transcribe either sense or antisense 35S-labelled riboprobes. *In situ* hybridization studies were performed as previously described (4).

Results

Case report. The propositus, a 16.7-year old male, is the second child of healthy non-consanguineous parents. Pregnancy and delivery were uneventful, and his birth weight was 2.7 kg. His major complaint was delayed puberty and short stature. Further evaluation revealed a mild short stature (159.5 cm), hypogonadotropic hypogonadism and anosmia, associated with the following features (Fig. 1): malformed ears, prominent frontal bossing, high arched palate, prominent jaw, micro-gnathia with dental malocclusion, long phyltrum, hypotelorism with a left convergent strabismus and a marked epichantus. He had kyphosis, flat arches of feet with absence of the fifth toe bilaterally, hyperextensible joints, fusion of the scaphoid with the astragalus bone on the right foot and hip hypoplasia. He showed an alopecia aerata at the nucal and parietal region. The olfaction test revealed the presence of anosmia. He also had chryptorchidism, fimosis, and a testicular volume of 2 ml.

Magnetic Resonance Imaging (MRI) showed an asymmetry of the lamina cribrosa and the absence of the right olfactory bulb and sulcus.

Endocrinological investigations confirmed the diagnosis of hypogonadotropic hypogonadism (undetectable basal gonadotropine levels and inadequate response of LH and FSH to GnRH stimulation test). The circadian rhythms of LH and FSH also confirmed the prepuberal status, showing undetectable gonadotropine concentration, both during the day and night. Further laboratory investigations excluded other hypothalamic-pituitary hormone axis deficiencies.

Cytogenetic studies. Chromosome and FISH analysis showed, in the proband, a balanced reciprocal translocation: 46, XY, t(7;9)(p14.1;q31.3) (Fig. 2). Karyotypes of his parents were normal.

Using multiple BAC probes for FISH analysis, we found that the BAC RP11-243E12 (AC018634) spanned the break-point on chromosome 7 giving signals on both the derivative 7 and 9 chromosomes and on the normal chromosome 7. Using the same approach, when BAC clone RP11-151F5 (AL158823) was used, signals were seen on the normal chromosome 9 and on der(9) and der(7).

Moreover, using comparative genomic hybridization (CGH-Array) analysis of the patient DNA, we were also able to exclude the presence of further deletions and duplications in the genome with a 1 Mb resolution (data not shown).

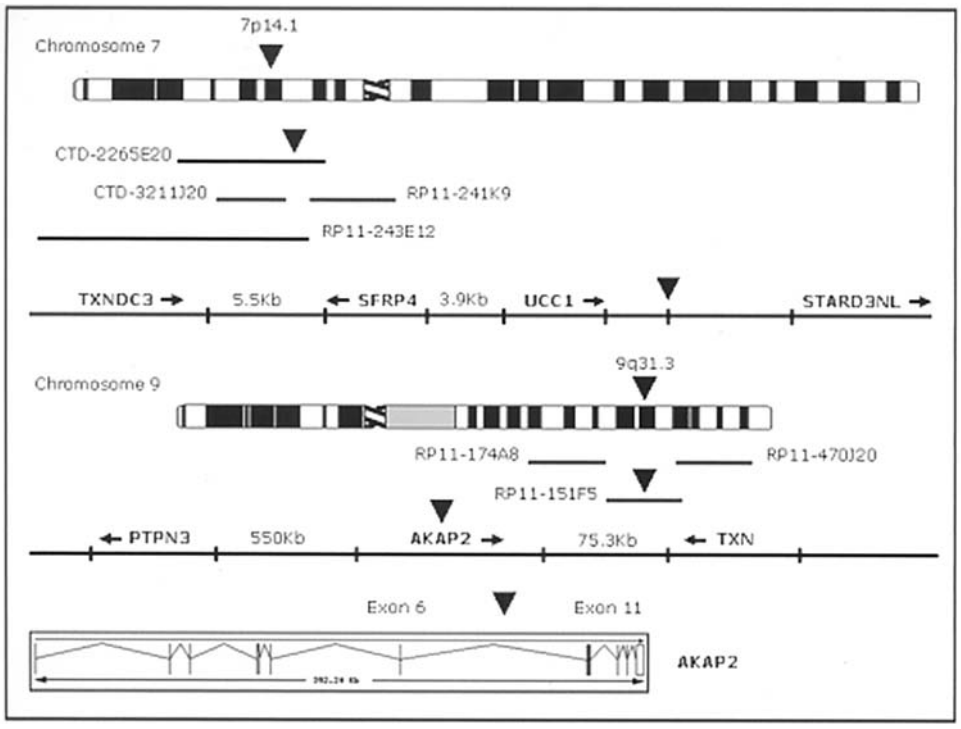

Figure 3. Schematic representation of the breakpoints on chromosome 9 and 7. BAC clones covering the breakpoints and the genes in the critical region are represented.

The UCSC genome browser made possible the identification of a few candidate genes in these clones. On BAC RP11-243E12, mapped on chromosome 7, three genes were identified: TXNDC3 (MIM 607421; Thioredoxin domain-containing 3), SFRP4 (MIM 606570; Secreted frizzled-related protein 4), and UCC1 (MERP1), which encodes a type-II transmembrane protein and is similar to two families of cell adhesion molecules, the protocadherins and ependymins.

To further refine the location of the breakpoint within the identified BAC RP11-243E12 we constructed a contig from the UCSC public human genome database. We tested by FISH the clones CTD-2265E20, CTD-3211J20 and RP11-241K9. The clones CTD-2265E20 and RP11-241K9 spanned the breakpoint, while the CTD-3211J20 didn't span the breakpoint (Fig. 3). This analysis allowed us to narrow the chromosome 7 breakpoint to an approximate 12-kb region within BAC CTD-2265E20. Using a subcloning approach we used these PACs in order to identify the minimal region spanning the breakpoint. Although we did not find any genes that were directly disrupted by the chromosome 7 breakpoint, we did identify the UCC1 (MERP1) gene approximately 22 kb distal to the 7p14.1 breakpoint.

BAC RP11-151F5 spanning the chromosome 9 breakpoint contains exons 7, 8, 9, and 10 of the AKAP2 gene (MIM 604582; A-Kinase Anchoring Protein 2); exons 1 and 2 are on BAC RP11-406O23 (AL158829) and exons 3, 4, 5, and 6 are on BAC RP11-174A8 (AL353806) both proximal to the breakpoint while, distally, exon 11 was comprised on BAC RP11-470J20 (AL353598). These observations made it clear that the translocation was interrupting the AKAP2 gene.

The AKAP2 gene was interrupted by the translocation. In order to obtain a molecular demonstration of the gene interruption, Southern blotting was carried out by making a comparison between the patient and a control individual. Field Inverted Gel Electrophoresis (FIGE) was used to separate the large fragments resulting from the digestion. Clone 700700, including the central part of the AKAP2 gene, from a Japanese cDNA collection was used as a probe. Different patterns of fragment lengths between patient and control were observed, demonstrating that the translocation was interrupting the AKAP2 gene in the patient (data not shown).

We proved that the expression of the AKAP2 gene was monoallelic taking advantage of a polymorphism of an AAGCTG repetition found by sequencing exon 8. Exon 8 is telomeric to the translocation and the primers were designed at the same side of the breakpoint. The sequence reported in the database has a double repetition (AB023137 base 2711) whereas the DNA of the patient and other normal controls has a triple repetition. We synthesized cDNA from RNA extracted from patient's lymphoblasts. The patient who was heterozygous for this polymorphism at the genomic level, showed a single allele in the cDNA.

Mutation analysis of candidate genes on chromosome 7 and 9. As mentioned above, analysis of available genome sequences in the public databases and the subcloning approach roughly showed that the 3' end of the gene UCC1 (MERP1) is located 22 kb far from the breakpoint. To investigate the role of this gene in the pathogenesis of KS, a collection of 98 DNA samples from KS patients with supposed autosomal transmission, was screened. Mutation screening was carried out on these patients by SSCP and sequence analysis for the UCC1 (MERP1) gene. No mutations were observed.

To investigate the role of the AKAP2 gene, the same collection of KS patients was used to carry out the mutation

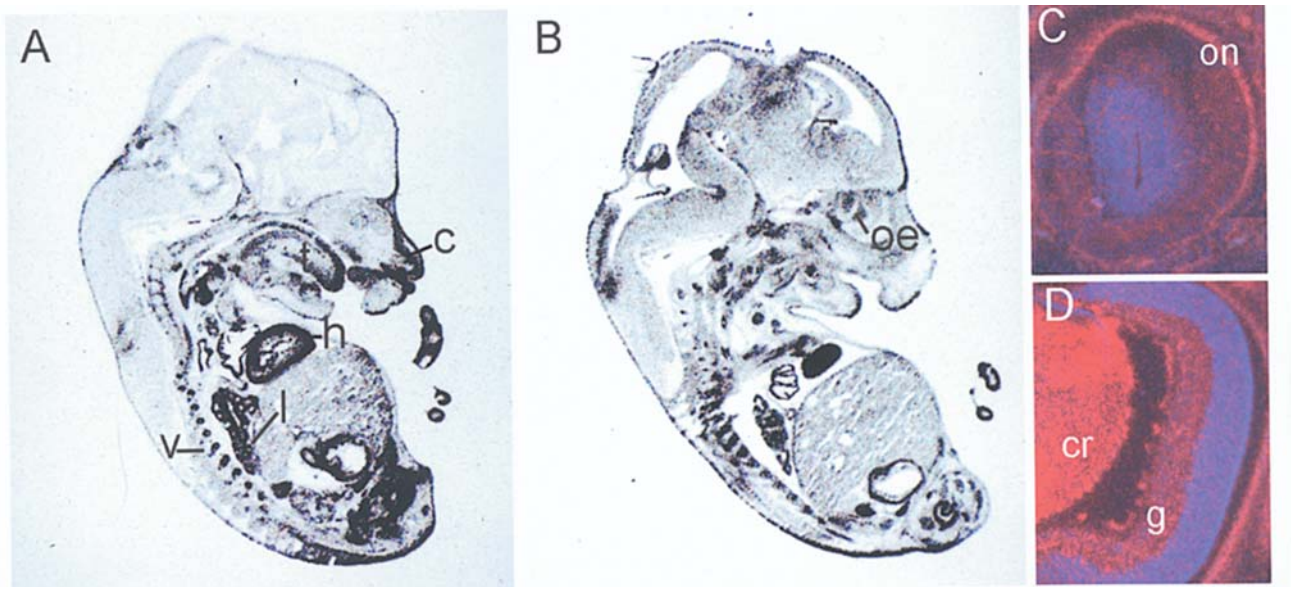

Figure 4. *In situ* hybridization studies of mouse Akap2 during development. (A and B) Autoradiography of sagittal sections of E13.5 mouse embryos shows high levels of expression in the heart (h), lung (l), facial cartilage (c), tongue (t), vertebral precursors (v), and olfactory epithelium (oe). Coronal sections across the olfactory bulb (C), and the eye (D) of a E16.5 mouse embryo, subjected to *in situ* hybridization. In the olfactory bulb, specific mAkap2 signal is restricted to the olfactory nerve layer (on). High levels of expression are found in the lens (cr), and in the ganglion cell layer of the neural retina (g).

screening. The exon-intron structure was established by comparing the cDNA with the genomic sequence in the public databases: 11 exons were identified in the range of 150-350 nucleotides except for the 2400-bp long exon 8. For the mutation screening, SSCP and sequence analysis were used. We divided exon 8 in 13 overlapping fragments of approximately 200 nucleotides. We also studied the exon/intron structure of Paralemmin-2, a second isoform which can be differentially expressed as a natural fusion protein with the A-kinase anchor protein AKAP2/AKAP-KL. During the mutation screening several polymorphisms were detected (data not shown). Most relevant, in two patients we identified two substitutions in two different exons: in one patient, an A to C transversion in exon 8 gave rise to the P631T aminoacidic change; the other patient showed an A to G transition in exon 9 that gave rise to the T1012A aminoacidic change. The conservation of these residues among species was studied. One of them, the T1012A, was well conserved also in the mus musculus ortholog, Akap-kl (PolyPhen, http://www.bork.embl-heidelberg.de/PolyPhen/). The two patients were also screened for mutations in the whole KAL-1 gene which did not reveal any mutations. In order to investigate whether these two substitutions could represent rare polymorphisms, 100 DNA samples from healthy control individuals were sequenced. The result of the observation was that none of them carried the same substitutions.

In order to establish if these mutations are inherited or *de novo*, the DNA from the parents of one of these patients, carrying the T1012A mutation, was also obtained and sequenced. The mother showed the T1012A mutation and, surprisingly, she also presented the P631T mutation on the other allele, suggesting that they represent a rare polymorphism.

Functional studies. In order to understand the possible role of AKAP2 in the pathogenesis of autosomal KS some functional studies were performed. The localization of the PKA in lymphoblast cultures was studied in our patient compared with lymphoblasts from control individuals. The PKA protein was also studied by Western blotting and subjected to quantitative analysis to compare cells of the patient carrying the translocation with those of normal controls. These experiments did not reveal any differences between patient and control cells.

Akap2 is expressed in the olfactory system. We next sought to determine whether AKAP2's expression pattern is consistent with the proposed involvement in KS, and with the clinical phenotype of the patient bearing the t(7;9) translocation. To this purpose, we cloned a 600-bp fragment of the murine orthologous cDNA by RT-PCR and used it as a probe for radioactive *in situ* hybridization experiments on mouse embryonic tissue sections. Our probe detects all different mAkap2 isoforms as previously described (4). We analyzed the mAkap2 expression pattern at 13.5 days of embryonic development, and found that the gene is highly expressed in the heart, lung, facial cartilage, cartilage precursors of the vertebral bodies, and olfactory epithelium (Fig. 4A and B). High expression levels in the lung were previously reported (5). We further investigated the mAkap2 expression pattern in the olfactory system from E13.5 to E19.5. We found that mAKAP2 is always expressed in the olfactory epithelium, and is also transcribed in the olfactory bulb. Expression in the olfactory bulb is characteristically confined to the olfactory nerve layer (Fig. 4C). High levels of expression were also found in the lens and the ganglion cell layer of the retina at E16.5 (Fig. 4D). These studies show that mouse Akap2 is expressed in the olfactory system, and support the hypothesis that the gene may play a role in the development of these structures. Furthermore, we found high levels of expression in cartilagineous structures of the embryo, consistent with the clinical phenotype of the propositus of this study.

Discussion

The analysis of balanced translocations is an important tool towards the identification of disease genes (6-8). We had the opportunity to study a patient with a *de novo* balanced translocation, showing a complex phenotype characterized by KS and bone anomalies.

This association was of particular interest because it suggests a new autosomal gene involved in KS. Until a few years ago only the X-linked, KAL-1 gene was identified in this genetically heterogeneous syndrome (9). More recently, an autosomal dominant form of KS, was associated with mutations in FGFR1, which is the first gene responsible for an autosomal form (KAL-2) (3). Gain of function mutations in FGFR1 were already associated with a form of craniosynostosis and the result of the mutations was supposed to enhance the affinity for certain FGF ligands. On the contrary, KS results from loss-of-function mutations in FGFR1 (3,10). Moreover some of the mutations associated with FGFR1 could cause the pathology through a dominant negative effect (3). Notably, some KS families that were described in the study by Dode and collaborators, showed that non-penetrance of the disease in some mutation carriers can simulate a recessive transmission of KS.

After accurate characterization of the critical region involved in the translocation on chromosome 7, no evidence for involvement of any genes in this region was obtained. A gene was found at roughly 22 kb from the breakpoint. This gene belongs to Ependymins, a family of secreted glycoproteins that, in many teleost fish, form the major component of the cerebrospinal fluid. The mammalian ependymin-related proteins (MERPs) are a family of proteins with a wide different expression pattern, which have also been involved in synaptic plasticity (11). The gene located in the critical region on chromosome 7 encodes the upregulated in colorectal cancer gene 1 protein [UCC1; synonym: mammalian ependymin related protein 1 (MERP1)]. This gene shows significant sequence similarity to the highly divergent piscine glycoproteins termed ependymins which are synthesized by leptomeningeal fibroblasts and secreted into the cerebrospinal fluid (12). On the basis of the information from the literature, this gene was considered a good candidate. Although in a collection of 98 sporadic KS patients the search for mutations in this gene was unsuccessful, we cannot exclude a position effect on its expression, due to the translocation.

On chromosome 9 the characterization of the translocation revealed that the AKAP2 gene was interrupted in the patient. This gene is a member of the A-kinase anchoring protein (AKAP) family, whose role is to anchor and bind protein kinase A (PKA). The specificity of PKA function is in part achieved through its compartmentalization into different subcellular locations through an interaction with AKAPs. Little information about this gene and its protein is available, only the murine forms are characterized and the homologous gene is Akap-kl.

We studied the localization of mAkap2 in mouse embryos. Our finding demonstrated that mAkap2 is expressed in the olfactory epithelium and in the olfactory bulb. Furthermore, a high level of expression in cartilagineous structures of the embryo was found. These studies show that Akap2 may play a role in the development of these structures.

Even if the role of the gene in the pathogenesis of KS is not demonstrated, some interesting evidence is found in recent literature. In particular, to our knowledge, no deletions involving the AKAP2 gene have been described. Furthermore, the role of intracellular PKA signaling in the axonal path-finding of olfactory sensory neurons in transparent zebrafish embryos was examined by Yoshida and collaborators (13), who suggested that the regulation of PKA signaling plays a key role in long distance axonal pathfinding.

We studied the localization and quantity of PKA in the lymphoblastoid cell lines of the patient compared with a control. Unfortunately these experiments didn't reveal any differences between patient and control.

In order to clarify the pathogenetic role of AKAP2 a collection of 98 sporadic KS patients were screened for mutations. We detected two mutations in AKAP2 in two different patients which were not found in 100 healthy controls nor in the other 96 patients of the case collection. This observation raises some interesting considerations. It is not surprising that only two mutations were found in this group of patients, since KS is a genetically heterogeneous disease. Therefore, several genes could be involved in the pathogenesis, such as those involved in the development of the olfactory bulb and migration of GnRH neurons. However, the simultaneous presence of these two different exonic substitutions in trans in the healthy mother of one of the two patients, suggests that the two substitutions are rare polymorphisms. Nevertheless, at least for one of these two variants, we cannot totally exclude their potential role, in the pathogenesis of KS trought incomplete penetrance.

In conclusion, we suggest that the complex phenotype with KS and bone anomalies observed in our patient might be the result of the interruption of the AKAP2 gene. However the evidence collected so far does not allow us to estabilish an involvement of the gene in autosomal KS. Nevertheless we cannot exclude that the two mutations in AKAP2 observed in two out of 98 KS patients collected might be the real cause for an autosomal form with incomplete penetrance. Additional studies should include mutational screening of AKAP2 in other KS patients to elucidate its role in the pathogenesis of the disease. Functional studies to assess the function of AKAP2 would also be useful.

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