Histone acetylation dependent allelic expression imbalance of BAPX1 in patients with the oculo-auriculo-vertebral spectrum

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The oculo-auriculo-vertebral spectrum (OAVS) (OMIM %164210) is a common developmental disorder characterized by hemifacial microsomia, epibulbar tumours, ear malformation and vertebral anomalies. Although rare familial cases suggest that OAVS has a genetic basis, no genetic defect has been identified so far. In a patient with OAVS and a chromosomal translocation t(4;8) we have found that the chromosome 4 breakpoint is 76.4 kb distal to the BAPX1 gene, which plays an essential role in craniofacial development. We did not detect any BAPX1 mutation in 105 patients, but observed a strong allelic expression imbalance (sAEI) in fibroblasts from five of 12 patients, but not in nine normal controls (Fisher’s exact test, \( P = 0.038 \)). sAEI was de novo in one patient and inherited in two other patients. Prolonged cell culture or treatment with the histone deacetylase inhibitor TrichostatinA led to reactivation of the downregulated allele. We propose that epigenetic dysregulation of BAPX1 plays an important role in OAVS.

INTRODUCTION

The oculo-auriculo-vertebral spectrum (OAVS) (OMIM %164210) is a common birth defect involving first and second branchial arch derivatives (estimated incidence, 1/5600) (1). It includes the Goldenhar syndrome and hemifacial microsomia and is characterized by hypoplasia of a number of craniofacial structures (e.g. the maxillary, temporal and zygomatic bones) as well as microtia or anotia. In addition to these anomalies, patients may present with preauricular tags, epibulbar tumours (dermoids) and vertebral anomalies (e.g. fused and hemivertebrae, spina bifida) (2,3). OAVS is highly variable and often affects only one side of the face (Fig. 1) (3). Although some familial cases following autosomal dominant or autosomal recessive inheritance have been described, the high frequency of sporadic cases and the occurrence of discordant monozygotic twins defy simple Mendelian inheritance (4,5). After genome-wide linkage analysis in one family, Kelberman et al. (6) identified a candidate region in 14q32. The authors considered Goosecoid (GSC) as a positional candidate gene for OAVS, but did not find any mutation within the coding region. Goosecoid functions as a transcriptional repressor and is expressed in the branchial arches during mouse embryogenesis (7). Mice with a homozygous disruption of the gsc gene exhibit multiple craniofacial abnormalities, notably hypoplasia of the mandible and branchial arch-derived bones and muscles, as well as underdevelopment of the external auditory meatus and inner ear defects (8,9). In zebrafish, gsc and another homeobox containing gene, bapx1, are known targets of hand2 expression. Furthermore, in hand2 mutant zebrafish, gsc expression is lost in the ventral first pharyngeal arch, whereas bapx1 expression expands ectopically into this domain, indicating that gsc represses bapx1 expression (10). In mice, Bapx1 and Gsc also act in the endothelin1–hand2 pathway, but deficiencies of either gene do not affect the expression of the other (11).

Bapx1 is a member of the NK2 class of homeobox genes (12,13). It is an early marker of chondrogenesis and is initially expressed in the posterior foregut at E8.0 in the mouse embryo. In the somites, Bapx1 expression is restricted to the sclerotomal part, giving rise to the axial skeleton. At stage E9.5, a further expression domain within the inferior portion of the first branchial arch appears, which later (E10.5) expands into developing structures of the craniofacial skeleton. At E16.5, Bapx1 is expressed in the axial skeleton in a rostrocaudal gradient and appears pronounced in the vertebral discs rather than in the intervertebral discs (14–16) and in a discrete domain within the mandibular component (Meckel’s cartilage) of the first branchial arch (11). Bapx1−/− mice exhibit lethal skeletal dysplasia. Malformations are more...
prominent in the cervical region and less severe in the caudal region. Cervical vertebrae show complete loss of their ventro-medial parts and ossification centres. In the chondrocranium, the basioccipital and the basisphenoid bones are hypoplastic, the exoccipital bone is dysmorphic and the supraoccipital bone is absent (14,17,18).

Numerous chromosomal rearrangements have been found in patients with OAVS, but apart from two cases involving 5p (19), no recurrent breakpoint has been identified (6,20,21). We have previously reported a patient with OAVS, multiple exostoses and a translocation 46,XX,t(4;8)(p15.3;q24.1) (22). Here, we describe that the translocation breakpoint on chromosome 4 is very close to the human BAPX1 gene. Although the gene is not disrupted by the translocation, there are several well-documented examples where chromosomal breakpoints outside developmentally relevant genes lead to disease, obviously by position effect (23). For example, SHH and SOX9, which in the mouse have been shown to be involved in the establishment of Bapx1 expression (24), are affected by chromosomal breakpoints up to 1000 kb 5' of their respective coding sequence (25,26). We have found abnormal BAPX1 expression in a significant number of patients with OAVS.

RESULTS

Molecular cloning of a translocation breakpoint on chromosome 4p15.3 and RAB28 mutation screening

The patient with OAVS, multiple exostoses and a translocation 46,XX,t(4;8)(p15.3;q24.1) has been described before (22,27). The translocation was inherited from the patient’s mother, who is a mosaic for this aberration. It was present in 11% of the mother’s fibroblasts, but not in her blood cells. The mother was not reported to have OAVS.

The chromosome 8 breakpoint disrupts the EXT1 gene in intron 5 (28) (unpublished data). In order to characterize the chromosome 4 translocation breakpoint, we have constructed a genomic λ-phage library from the patient’s DNA and isolated phage clones for both junction fragments with the help of probes for EXT1 exons 5 and 6. With a chromosome 4 probe derived from one junction fragment, we isolated one cosmid clone, two CEPH MEGA-YAC clones and four different RPCI-PAC clones containing normal chromosome 4 DNA. Sequencing revealed that the chromosome 4 breakpoint disrupts the RAB28 gene in intron 3. The translocation was associated with a 10 bp deletion on chromosome 4 and a 57 bp deletion on chromosome 8.

The ras-related RAB28 gene encodes a small GTPase that displays low sequence identity (31–33%) with other members of the Rab family. Human RAB28 is expressed in two isoforms (hRab28S, hRab28L) in a broad spectrum of tissues, whereas hRab28L is predominant in testis (29). We sequenced RAB28 in 75 patients with OAVS, but did not find any mutation (data not shown). Close to the chromosome 4 breakpoint (76.4 kb) is BAPX1, which is an excellent functional candidate gene for OAVS (Introduction).

BAPX1 mutation screening in patients with OAVS

To investigate whether BAPX1 plays a role in OAVS, we searched for mutations in the two exons, the exon/intron boundaries and the promoter region in 105 patients with OAVS. We identified nine sequence variants, seven within the coding region, one in the 5'-UTR and one in the predicted PAX6 transcription factor binding site of the promoter (30). Three polymorphisms with a minor allele frequency >0.03 were found in the promoter, the 5'-UTR and exon 1. Three of the remaining rare variants change the amino acids sequence, but only one (c.292 A>T, S98C) was not found in 300 normal controls. All sequence variants leading to an amino acid substitution (c.292 A>T, S98C; c.323 C>G, A108G; c.493 G>C, D165H) were also detected in unaffected relatives (results are summarized in Supplementary Material, Table S1).

BAPX1 expression analysis in patients with OAVS

To test whether the sequence variations might affect splicing or the stability of the transcript, we performed RT–PCR...
experiments. RNA was extracted from cultured primary fibroblasts, because BAPX1 is expressed neither in blood cells nor in cells from buccal smear (data not shown). We started with cultured fibroblasts taken from the left arm of patient P18 (Fig. 1A), who is heterozygous for the frequent polymorphism c.247 A > C as well as for the rare polymorphisms c.126 G > T and c.351 C > G. We obtained a PCR product of the expected size, which indicates a correctly processed mRNA (data not shown), but found a strongly skewed allelic expression ratio after sequencing of the RT–PCR product (37 days of culture, Fig. 2B). The silent allele was not the one carrying the rare sequence variants, but the frequent ones. After 53 (Fig. 2C) and 61 (Fig. 2D) days of culture, we observed reactivation of the silent allele. In a 12-day culture of a second skin biopsy taken from the same arm of this patient, the allele was absent again (Fig. 2E). Similar findings were made in fibroblasts taken from the left ear of patient P20 (Fig. 1B) during reconstructive surgery (data not shown).

**Quantitative analysis of the allelic expression**

For a quantitative analysis of the allelic expression, we carried out SNaPshot primer extension experiments on fresh fibroblast cultures obtained from 12 patients and nine normal controls who are heterozygous for an expressed polymorphism and who agreed to a skin biopsy. The samples analysed include the second skin biopsy of patient P18 mentioned above and a skin biopsy of the left arm of patient P20. Fibroblasts from the translocation patient were not available. In each case, 2 μg of total RNA from cultured primary fibroblasts (culture time 12–25 days) were reverse transcribed and an intron-spanning PCR with the primers BAPX1 RT–RCRf and BAPX1 RT–PCrR (Supplementary Material, Table S2) was carried out. An aliquot (60 ng) of purified PCR product was used for quantitative primer extension. For the analysis, the larger peak area was divided by the smaller peak area and the ratios were normalized with respect to the values obtained on genomic DNA (Fig. 3). The allelic expression ratios of five patients (P18, P20, P50, P90 and P110) lie outside of the 99.99% confidence interval (0.65–2.89) of the control values, assuming that the controls follow a normal distribution. We note that most of the normal controls show some degree of allelic expression imbalance (mean, 1.77), which may reflect genetic or epigenetic variation (31). The finding of strong allelic expression imbalance (sAEI) in five of 12 patients is statistically significant (Fisher’s exact test; \( P = 0.038 \)). There was no association between a particular sequence variant and its relative transcript level.

**Segregation pattern of sAEI in patients’ families**

To investigate the segregation pattern of sAEI, we studied fresh fibroblast cultures of the parents and sibs of patients P18, P20 and P50 (Fig. 1C). Parental samples of patients P90 and P110 were not available. In the family of P20, who is very mildly affected (3), only the patient has sAEI (Fig. 4A). sAEI was found in fibroblasts obtained from surgical intervention and in fibroblasts of the first skin biopsy, but not in the second skin biopsy from the same arm (data not shown), indicating that this patient is a mosaic for strongly skewed BAPX1 expression. Patient P18 inherited the underrepresented allele from her father, who also has sAEI (Fig. 4B). The father...
The loss of sAEI during prolonged cell culture (Fig. 2) suggested that sAEI is reversible and might have an epigenetic basis. To investigate whether DNA methylation or histone modification plays a role in sAEI, we treated fresh fibroblast cultures of patient P50 with the DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine (5-Aza-dC), the histone deacetylase inhibitor Trichostatin A (TSA) and a combination of both. As shown in Figure 5, treatment with 5-Aza-dC did not have any effect on the allelic expression ratio, whereas treatment with TSA or with TSA + 5-Aza-dC normalized the ratio to approximately 1.0. Similarly, in fibroblasts from patient P20, TSA normalized the allelic expression ratio.

**DISCUSSION**

An important question and a great challenge in human molecular genetics today is to decipher the mechanisms underlying complex and heterogeneous diseases that defy simple Mendelian inheritance. To date, no genetic defect has been found in OAVS, which is the second most common craniofacial birth defect. In fact, several authors have questioned that OAVS has a genetic basis and have suggested that OAVS is caused by blood vessel disruption in utero (32). The unilateral manifestation in nearly 50% of the patients (3) and the excess of discordant monozygotic twins (33–36) suggest that other explanations such as somatic mosaicism or epimutations should be taken into consideration.

In this work, we have shown that (i) allelic expression of BAPX1 in patients with OAVS can be heavily skewed, (ii) skewing can occur de novo or be inherited and (iii) skewing involves histone deacetylation. There is no evidence that BAPX1 is imprinted, which might explain some of the findings. Non-imprinted AEI is common among human genes (37–39), but so far it has only once been linked to human disease: as shown by Yan et al. (40), small changes in APC expression, which are most likely due to cis-acting regulatory genetic variation, can predispose to colon cancer. By studying CEPH families, Pastinen et al. (31) have obtained evidence for Mendelian transmission of AEI, imprinted transmission of AEI as well as discordant allelic expression in individuals carrying haplotypes identical-by-descent. The latter pattern is similar to the transmission pattern we observed in family P50. This pattern suggests that sAEI of BAPX1 may be secondary to a trans-acting factor, at least in this family.

We propose that sAEI of BAPX1 predisposes to OAVS. This hypothesis is based on the following two observations: (i) sAEI was only found in patients and their relatives. (ii) In patient P20, sAEI and the disease occurred de novo. This patient is mildly affected and appears to have somatic mosaicism for the skewed expression of BAPX1. The finding that the father of P50 and possibly the father of P18 are unaffected does not argue against a role of sAEI in OAVS, but probably reflects the multifactorial nature of OAVS. This may also be one explanation for the rareness of familial cases and the absence of OAVS features in the few patients with a cytogenetic aberration involving 4p15.3, although the two patients with Wolf–Hirschhorn syndrome and a deletion >14 Mbp including BAPX1 that have been described by Wieczorek et al. (41) have preauricular tags. It should be noted, however, that BAPX1 is not deleted in the majority of patients with the Wolf–Hirschhorn syndrome.

There are several reasons why sAEI of BAPX1 may be involved in OAVS. (i) Developmental pathways are highly dosage sensitive. It is likely that sAEI affects the total level of BAPX1 mRNA, although these changes are probably subtle. Owing to interindividual variation in the expression of BAPX1 and control genes, changes in the total level of BAPX1 mRNA are difficult to detect by real-time PCR (data not shown). (ii) BAPX1 is expressed in the first branchial arch, which shows impaired development in OAVS (2,3). In addition, BAPX1 knockout mice have defects of the vertebral column and of some craniofacial bones (14,17,18). (iii) In the zebrafish, second pharyngeal arch expression of bapx1 is

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**Figure 4.** Pedigrees of the three patients with strong AEI. In each family, the frequent polymorphism c.247 A > C and family specific variants were analysed. p, paternal haplotype; m, maternal haplotype; x, parental origin unknown; ?, inferred haplotypes; --, normal allelic ratio; > or <, sAEI; f.n.a., fibroblasts not available (sister of P18) or not analysed because of homozygosity (sister of P50); (?), phenotype unclear, because individual could not be examined.

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**Treatment of fibroblast cultures with 5-Aza-2'-deoxycytidine and Trichostatin A**

The loss of sAEI during prolonged cell culture (Fig. 2) suggested that sAEI is reversible and might have an epigenetic basis. To investigate whether DNA methylation or histone modification plays a role in sAEI, we treated fresh fibroblast cultures of patient P50 with the DNA methyltransferase inhibitor 5-Aza-2'-deoxycytidine (5-Aza-dC), the histone deacetylase inhibitor Trichostatin A (TSA) and a combination of both. As shown in Figure 5, treatment with 5-Aza-dC did not have any effect on the allelic expression ratio, whereas treatment with TSA or with TSA + 5-Aza-dC normalized the ratio to approximately 1.0. Similarly, in fibroblasts from patient P20, TSA normalized the allelic expression ratio.
repressed by gsc (42). In a familial case of OAVS, the genetic defect has been linked to GSC on human chromosome 14, although no mutation has been found in this gene (6). It is tempting to speculate that cis-acting regulatory variation at this locus, which indirectly affects BAPX1 expression, predisposes to OAVS in this family. (iv) As shown in the zebrafish, moz histone acetylation activity is required for the maintenance of hox1–4 expression domains in the first and second pharyngeal arches (10). Bapx1 and gsc are two early targets of moz and hox2 signalling in the second pharyngeal arch (10,42). Thus, histone acetylation is essential for the development of the first and second pharyngeal arch derivatives.

We conclude that epigenetic dysregulation is likely to play a role in OAVS. Such a mechanism can explain many of the genetic and phenotypic peculiarities of OAVS, for example, discordant monozygotic twins. It is possible that epigenetic dysregulation is also involved in other common, apparently sporadic birth defects in humans.

**MATERIALS AND METHODS**

**Patients**

All patients were examined by experienced clinicians (D.W. and G.G.-K.). Peripheral blood and skin fibroblasts were obtained after informed consent. The study was approved by the local Ethics Committee.

**BAPX1 mutation screening**

Genomic DNA was extracted from whole venous blood using the Flexigene DNA Blood Test Kit (Qiagen). PCR was performed in 25 µl, containing 400 ng genomic DNA, 10 × PCR buffer (Roche, Applied Biosystems), 400 nM primer (custom synthesized by Eurogentec), 160 µM dNTPs (containing 60% 7-Deaza-dGTP, Roche), 0.5 mM betaine and 2.5 U AmpliTaq (Roche, Applied Biosystems). The thermal cycling profiles were the following. BAPX1 exon 1.1: initial denaturation (96°C for 3 min); amplification (10 cycles 96°C for 30 s, 65°C for 30 s, 72°C for 1 min; 10 cycles 96°C for 30 s, 61°C for 30 s, 72°C for 1 min; 20 cycles 96°C for 30 s, 57°C for 30 s, 72°C for 1 min); final extension (72°C for 5 min). BAPX1 exon 2.1: initial denaturation (96°C for 3 min); amplification [five cycles 96°C for 30 s, 62°C for 30 s (−1°C per cycle), 72°C for 1 min; 30 cycles 96°C for 30 s, 57°C for 30 s, 72°C for 1 min]. BAPX1 exon 2.2: initial denaturation (96°C for 3 min); amplification (40 cycles 96°C for 30 s, 68°C for 30 s, 72°C for 2 min); final extension (72°C for 10 min). PAX6 binding site: initial denaturation (96°C for 5 min); amplification (35 cycles 96°C for 20 s, 60°C for 30 s, 72°C for 1 min); final extension (72°C for 5 min). The PCR products were cleaned up with the MultiScreen Filtration System (Millipore). The cycle sequencing reactions were carried out using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) following manufacturer’s instructions and were analysed in a Genetic Analyzer 3100 (Applied Biosystems). Primers used in all experiments are shown in Supplementary Material, Table S2.

**Cell culture experiments**

Human primary fibroblasts were obtained during reconstructive surgical interventions or by skin biopsies from the upper arm. In patients, the upper arm of the affected side was chosen. Cells were grown in AmnioMax-C100 Basal Medium (Gibco) plus AmnioMax-C100 Supplement (Gibco) in a humidified atmosphere containing 5% CO₂ at 37°C.

Cells were split 12–24 h prior to treatment with 5-Aza-dC (Sigma) and TSA (Sigma), respectively. Cells were then given one of the following treatments: (i) 50 µM 5-Aza-dC or an identical volume of phosphate-buffered saline (PBS) was used for 96 h, (ii) 1500 and 750 nM TSA or an identical volume of DMSO was used for 48 h, (iii) 50 µM 5-Aza-dC or an identical volume of PBS was used for 48 h followed by 3 and 1.5 µM TSA or an identical volume of DMSO for an additional 24 h. Medium for all treatments was changed every 24 h.

RNA was extracted using the RNA Blood Mini Kit (Qiagen) in accordance with manufacturer’s instructions.
Quantitative primer extension assay

Two micrograms of total RNA from cultured primary fibroblasts were reverse transcribed using the GeneAmp RNA PCR Kit (Roche, Applied Biosystems) in a three-step procedure (21°C for 10 min, 42°C for 30 min, 95°C for 5 min). PCR was carried out with the Advantage-2 PCR Kit (BD Biosciences) according to manufacturer’s instructions and the following thermal profile: initial denaturation (96°C for 5 min); amplification (37 cycles 96°C for 30 s, 61°C for 30 s, 68°C for 3 min). For the DNA controls, the PCR was carried out as described in ‘BAPX1 mutation screening’. The PCR products were purified with the MultiScreen Filtration System (Millipore), and 60 ng of the purified PCR product was used in the SNaPshot quantitative primer extension assay (Applied Biosystems) following manufacturer’s guide and using an annealing temperature of 54°C. The analysis was carried out in a Genetic Analyzer 3100 (Applied Biosystems). Primers used in all experiments are shown in Supplementary Material, Table S2.

Statistics

We have used the software package R 2.0.1 for all statistical analyses (http://r-project.org). Confidence intervals were computed under assumptions of normality. To increase robustness, a strict confidence level was used. Contingency tables were evaluated using Fisher’s exact test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank Melanie Trommler and Regina Kubica for excellent technical support; all the patients and their parents for participating in this study, especially the German parents’ support group ‘Netzwerk Goldenhar-Syndrom–Ohrmuscheldysplasie e.V.’, Drs C. Johnson and J.M. Graham Jr for referring the translocation patient to us. This work was supported by the Deutsche Forschungsgemeinschaft (WI 1440/6-3).

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