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ORIGINAL INVESTIGATION

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Oto-facio-cervical (OFC) syndrome is a contiguous gene deletion syndrome involving *EYA1*: molecular analysis confirms allelism with BOR syndrome and further narrows the Duane syndrome critical region to 1 cM

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Abstract Branchio-oto-renal (BOR) syndrome is an autosomal dominant disorder involving hearing loss, branchial defects, ear pits and renal abnormalities. Oto-facio-cervical (OFC) syndrome is clinically similar to BOR syndrome, with clinical features in addition to those of BOR syndrome. Mutations in the *EYA1* gene (localised to 8q13.3) account for nearly 70% of BOR syndrome cases exhibiting at least three of the major features. Small intragenic deletions of the 3' region of the gene have also been reported in patients with BOR syndrome. We have developed a fluorescent quantitative multiplex polymerase chain reaction for three 3' exons (7, 9 and 13) of the *EYA1* gene. This dosage assay, combined with microsatellite marker analysis, has identified de novo deletions of the *EYA1* gene and surrounding region in two patients with complex phenotypes involving features of BOR syndrome. One patient with OFC syndrome carried a large deletion of the *EYA1* gene region, confirming that OFC syndrome is allelic with BOR syndrome. Microsatellite analysis has shown that comparison of the boundaries of this large deletion with other reported rearrangements of the region reduces the critical region for Duane syndrome (an eye movement disorder) to between markers D8S553 and D8S1797, a genetic distance of approximately 1 cM.

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Introduction

Branchio-oto-renal (BOR) syndrome is defined by the presence of at least three of the four following major features: hearing loss, branchial defects, ear pits and renal abnormalities (Melnick et al. 1976), although other minor features can be present. The disease is inherited in an autosomal dominant manner and, although penetrance is usually complete, there is considerable variability of phenotype within families (Chen et al. 1995; König et al. 1994; Heimler and Lieber 1986). Oto-facio-cervical (OFC) syndrome has features in common with BOR syndrome and clinicians have argued whether it is indeed a separate condition. OFC syndrome is also an autosomal dominant condition and presents with hearing loss, branchial fistulae, ear pits, facial abnormalities, hypoplasia of the cervical musculature (pronounced sloping shoulders), variable mental retardation and short stature (Allanson 1995; Dallapiccola and Mingarelli 1995). Thus, the two syndromes (BOR and OFC) share clinical features, although OFC syndrome is the more severe.

Mutations in the *EYA1* gene (localised to 8q13.3) can be identified in nearly 70% of BOR syndrome cases exhibiting at least three of the major features (Rickard et al. 2000), although families with some features of BOR syndrome that do not map to 8q13.3 have been reported (Kumar et al. 2000; Stratakis et al. 1998). Abdelhak et al. (1997) have used Southern blot analysis to identify several cases who have typical BOR syndrome and who carry small intragenic deletions of the conserved 3' region of the *EYA1* gene.

Rearrangements involving the BOR syndrome region, 8q13–21, have been identified by cytogenetics in three published cases (Table 1, Fig. 1; Haan et al. 1989; Vincent et al. 1994; Calabrese et al. 1998). The first, was identified in a large Dutch family affected with branchio-oto

Table 1 Comparison of the features of the patients from this study and three reported cases with deletions of the BOR region (*TRPS* tricho-rhino-phalangeal syndrome)

Clinical features	Patient A	Patient B	Case 1[a]	Case 2[b]	Case 3[c]
Hearing loss	+	+	+	+	–
Branchial defects	+	+	+	+	–
Ear pits	+	+	+	?	–
Renal abnormalities	+	+	–	+	–
Cholesteatoma	+	–	–	–	–
Developmental delay	+	+	+	+	+
Short stature	+	–	+	–	+
Sloping shoulders	+	–	?	+	?
Short neck	+	–	–	–	+
Hand/feet abnormalities	–	+	+	–	+
Hydrocephalus	–	–	–	+	–
Facial anomalies	–	–	+	–	+
Duane syndrome	–	–	–	+	+
TRPS	–	–	+	–	–

[a]Haan et al. 1989
[b]Vincent et al. 1994
[c]Calabrese et al. 1998

syndrome and tricho-rhino-phalangeal syndrome, involving the inherited rearrangement of a 470–600 kb deletion of 8q13.3 in association with the direct insertion of material from 8q13.3-q21.13 into 8q24.11 (Gu et al. 1996; Kalatzis et al. 1996; Haan et al. 1989). A second de novo case, involving a larger deletion of the region has been reported in a patient presenting with BOR syndrome, Duane syndrome, hydrocephalus, mild mental retardation and trapeze aplasia (Vincent et al. 1994). The third case involves a de novo deletion associated with an insertion of 8q13–21 into 6q25 in a patient with Duane syndrome, mental retardation and other dysmorphic features, but not BOR syndrome (Calabrese et al. 1998).

Duane syndrome is a congenital form of strabismus caused by abnormal development of the abducens nucleus and nerve and paradoxical innervation of the lateral rectus muscle. The most typical form is characterised by impaired ocular abduction and narrowing of the palpebral fissure and retraction of the globe on adduction (Duane 1905). A locus for Duane syndrome has recently been located on chromosome 2q31 by linkage, although it is thought to be a genetically heterogeneous disorder (Ott et al. 1999).

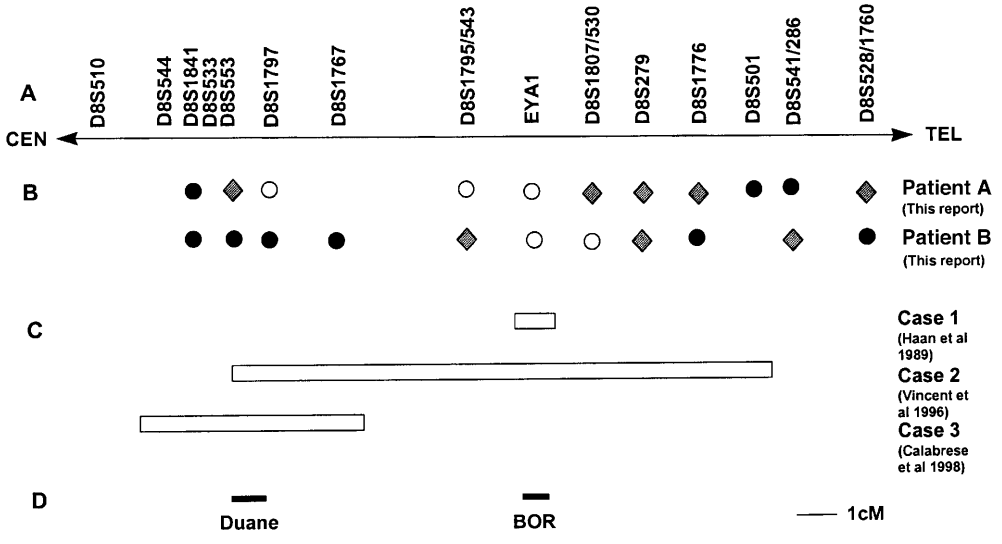
We have used dosage and microsatellite marker analysis to confirm a deletion of *EYA1* in two patients presenting with complex phenotypes that included features of BOR syndrome. Our results confirm that OFC is allelic with BOR syndrome and not genetically distinct. Comparison of the deletion boundaries of one of our cases with those of previously published rearrangements of the 8q13–21 region has narrowed the Duane syndrome critical region to less than 1 cM.

Fig. 1 The BOR syndrome region 8q12–21. **A** STS and gene map of the 8q12–21 region, derived from Marshfield integrated maps (http://research.marshfieldclinic.org). D8S501, D8S286 and D8S541 have been reordered with regard to the Marshfield map, as D8S501 was deleted in case 2, whereas D8S286 was not (Vincent et al. 1994). **B** Deletion results for patients A and B (some uninformative markers have been omitted for clarity; *filled circles* both parental alleles present, *open circles* loss of parental allele, *diamonds* uninformative marker). **C** Previously published deletions of the region (*Case 1* Haan et al. 1989, *Case 2* Vincent et al. 1994, *Case 3* Calabrese et al. 1998). **D** Newly defined Duane syndrome critical region

Materials and methods

Mutation detection of EYA1

Patient A was screened by single-strand conformation polymorphism (SSCP) analysis for all coding exons of *EYA1* (1'–16) by using published primers amplified under standard conditions (Abdelhak et al. 1997). The polymerase chain reaction (PCR) products from exons 1'–15 were denatured and run on SSCP gels overnight at 4°C. The gels were then silver-stained as described (Tyson et al. 1997). Because of its large size, exon 16 was digested with *Dde*I and run as above. In addition, patient A was sequenced for exons 1'–16 by using a BigDye Terminator Cycle Sequencing kit (PE



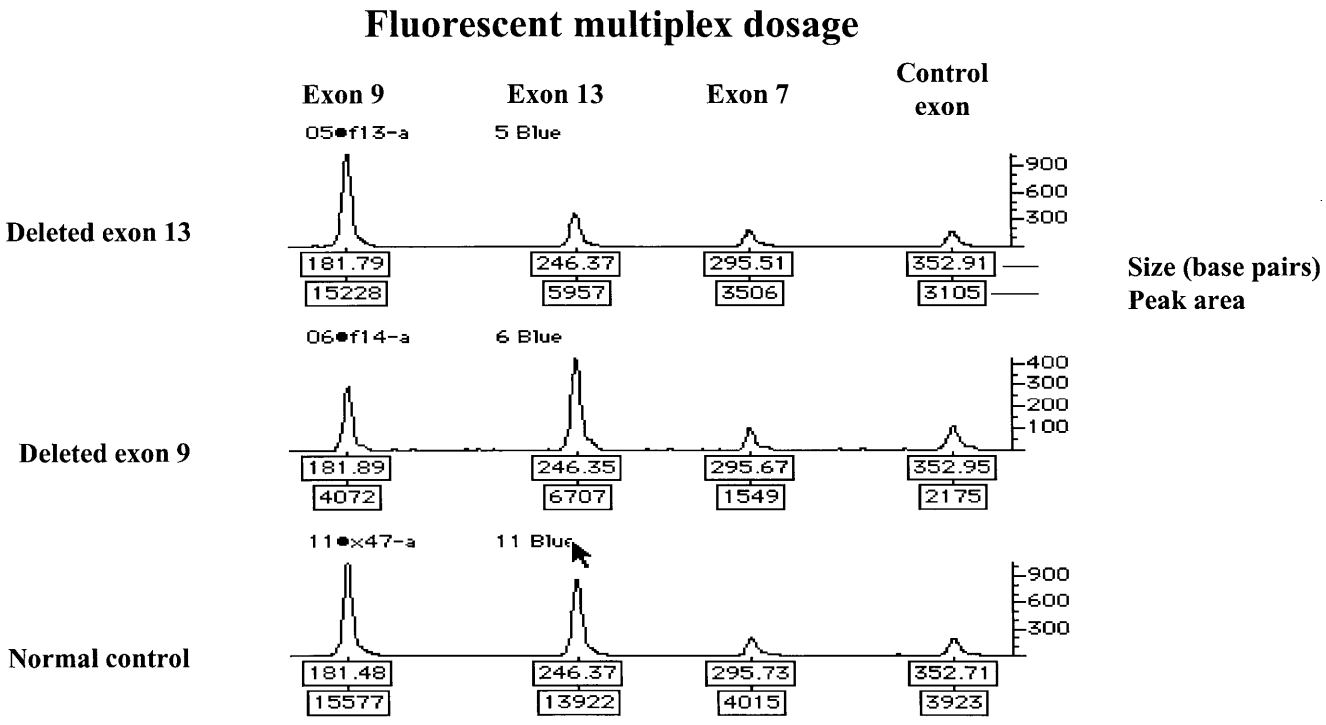


Fig. 2 Examples of electropheragram dosage data for exon 13 and 9 deletion controls and a normal control

Applied Biosystems) run on an ABI 377 DNA sequencer. Patient B was screened by SSCP for exons 4, 5 and 7–16, as our previous study had revealed these exons contained all the mutations detected to date (Rickard et al. 2000).

Fluorescent quantitative multiplex PCR

Fluorescent quantitative multiplex PCR was performed as described (Heath et al. 2000). Figure 2 shows some examples of electropheragram data generated by the dosage analysis. This approach involved two stages of amplification. The first round (P1) involved a PCR multiplex of unlabelled primers for exons 7, 9, and 13 and a control exon from another chromosome. All amplification primers were in the same reaction mix. Each primer consisted of

Table 2 Dosage quotients for patients A, B, an exon 13 deletion control and a normal control (*bold numbers* deleted exons). A DQ value of 0.5 or 2.0 indicates the deletion of an exon (depending on whether the peak area is the numerator or denominator in the equation). A DQ value of 1.0 indicates two copies of the exon are present

Exon	Mean of controls (peak area)	Sample (peak area)	Exon 7 (DQ)	Exon 9 (DQ)	Exon 13 (DQ)	Control exon (DQ)
Patient A						
Exon 7	2452	1333	1.00	0.95	0.79	**0.36**
Exon 9	10194	5836	1.05	1.00	0.83	**0.38**
Exon 13	8829	6063	1.26	1.20	1.00	**0.46**
Control exon	2289	3411	**2.74**	**2.60**	**2.17**	1.00
Patient B						
Exon 7	2452	624	1.00	0.93	0.93	**0.43**
Exon 9	10194	2798	1.08	1.00	1.00	**0.46**
Exon 13	8829	2427	1.08	1.00	1.00	**0.46**
Control exon	2289	1364	**2.34**	**2.17**	**2.17**	1.00
Exon 13 deletion control						
Exon 7	2452	3506	1.00	0.96	**2.12**	1.05
Exon 9	10194	15228	1.04	1.00	**2.21**	1.10
Exon 13	8829	5957	**0.47**	**0.45**	1.00	**0.50**
Control exon	2289	3105	0.95	0.91	**2.01**	1.00
Normal control						
Exon 7	2452	2512	1.00	0.94	0.99	1.14
Exon 9	10194	11144	1.07	1.00	1.06	1.21
Exon 13	8829	9097	1.01	0.94	1.00	1.14
Control exon	2289	2065	0.88	0.83	0.88	1.00

the unique published exonic primer sequence (Abdelhak et al. 1997) with a 5' universal tag (5'-TCCGTCTTAGCTGAGTGGCGTA-3' for the sense forward tag; 5'-AGGCAGAATCGACTCACCGCTA-3' for the antisense reverse tag). The unique sequence for the forward primer for the control exon was 5'-CAGCCTGGATGGCCAGAGAG-3' and the reverse primer sequence for the control exon was 5'-TATACCCTCAGTGCCCAACAGTGC-3'. P1 was performed at an annealing temperature of 55°C for 10 cycles. Product from P1 was transferred into a second reaction (P2) containing a labelled universal primer set, which was amplified for 20 cycles at an annealing temperature of 57°C; in P2, all synthesis was from the universal set, not the unique exonic sequence, resulting in a more uniform amplification product. This two stage approach gave more consistent results than the conventional one step fluorescent multiplex PCR. Three normal controls and a deletion control of exon 9 and another of exon 13 were included in each dosage experiment. No deletion control was available for exon 7. Gene dosage values were determined by comparing the peak areas of each sample (a value given directly by the ABI 377 sequencer) against one another and also against those from controls. The peak area data from the ABI electropheragrams were entered into an Excel spreadsheet (Table 2) and dosage quotients (DQs) calculated by using equations derived from multiplex PCR analysis developed for Duchenne muscular dystrophy (Yau et al. 1996). Essentially, DQ = (peak area of the test exon/peak area of the control exon)/(peak area of the mean of all controls for the same exon/peak area of the mean of the controls for the control exon) . If the dosage quotient values of the normal or deletion controls in a series varied significantly, the assay was repeated. The assay was repeated three times to ensure consistency of data.

Microsatellite marker analysis

DNA from the proband and parents of both individuals was amplified for published microsatellite markers D8S260, D8S553, D8S1841, D8S1797, D8S1767, D8S1795, D8S543, D8S1807, D8S530, D8S279, D8S1776, D8S501, D8S286, D8S1760, D8S541 and D8S528 (Généthon; Dib et al. 1996) by using fluorescently labelled forward primers run on an ABI 377 DNA sequencer.

Results

Clinical findings

Patient A has been described previously (Rajput et al. 1999). In brief, she presented with moderate hearing loss, bilateral pre-auricular pits, a single lacrimal pit, a cupped shaped ear and unilateral facial palsy. A congenital cholesteatoma was also identified in her right middle ear and was treated surgically. She was of short stature (3rd centile for height) and was investigated for failure to thrive as an infant. She had a short neck and pronounced sloping shoulders, with limited shoulder abduction. X-rays showed her to have lateral displaced scapulae. She had mild developmental delay with delayed milestones and her chromosomes were shown to be normal (Table 1). A renal ultrasound showed a small left kidney (size <5th centile for age) and a right kidney of normal size with a bifid pelvis. Both her parents were unaffected and intellectually normal.

Patient B is from a non-consanguineous Indian family and presented with severe to profound hearing loss, ear pits, a repaired branchial sinus and a dilated right kidney and upper ureter. He has learning difficulties. He had excision of bilateral post-axial polydactyly. In addition to these features, he presented with multiple café-au-lait patches associated with neurofibromatosis type 1 (NF1; Table 1). His chromosomes were shown to be normal. His sister also has skin stigmata of NF1, and a right-sided duplex kidney and related ureterocele. She has normal hearing but has learning difficulties. The mother of the children also has NF1 and was of short stature. She has a right-sided duplex renal system with iris Lisch nodules in her right eye. The father of the family showed no obvious clinical signs.

EYA1 mutation screening and fluorescent dosage analysis

No mutations were detected in either probands by SSCP analysis and no mutation was identified in patient A using sequence analysis. The DQs generated by fluorescent quantitative multiplex PCR analysis of patients A and B showed that both were deleted for exons7, 9 and 13 of the *EYA1* gene (Table 2). Further dosage analysis showed the parents of both cases and patient B's sister were not deleted for exons 7, 9 and 13 of the *EYA1* gene (data not shown), i.e. both patients possessed de novo deletions.

Microsatellite marker analysis

Analysis of markers within the region revealed that patient A lacked a paternal contribution at markers D8S1797 and D8S543. She had inherited both parental alleles at D8S1841, D8S501 and D8S541. Patient B lacked a maternal contribution at D8S1807 but showed both parental alleles at markers D8S1841, D8S553, D8S1797, D8S1767, D8S1776 and D8S528. These results, and the uninformative markers for both cases, are presented in Fig. 1.

Discussion

We have identified novel deletions involving the *EYA1* gene region in two unrelated cases presenting with features in addition to those of BOR syndrome. Patient A has been diagnosed with OFC syndrome, confirming this disorder to be allelic with BOR syndrome. This suggests that genes encompassed by the deletion, lying centromeric to *EYA1*, are responsible for the additional clinical features. Therefore, cases presenting with BOR syndrome (i.e. exhibiting at least three of the four major features) in the presence of additional features, particularly sloping shoulders, developmental delay and short stature may have deletions of the *EYA1* gene and surrounding region. The presence of developmental delay in patients A and B may be significant, as this has been noted as an additional feature, for example, in Saethre-Chotzen and Grieg syndrome, when a deletion of the disease-causing gene has been identified (Williams et al. 1997; Johnson et al. 1998).

The deletion identified in patient B is well defined, as the markers are more informative in this family. Although we have been unable to determine the boundaries of the deletion precisely, it is likely to be smaller than that of patient A and to involve less genetic material either side of *EYA1* (and therefore including fewer additional genes). Patient B exhibits additional features to BOR syndrome; however, these features are present in other family members who have been shown not to carry the deletion. NF1 and the renal abnormalities found in his sibling and mother are almost certainly segregating independently.

The boundaries of deletions in patients A and B differ at the centromeric end, the loss of genetic material in patient B being somewhat smaller. The centromeric boundary of the deletion in patient A has been identified as D8S1841, the chromosomal loss spanning D8S1797 to the *EYA1* gene. The breakpoint of her deletion must therefore lie between D8S1841 and D8S1797, a genetic distance of approximately 1 cM. The telomeric boundary is less well defined but must occur between the *EYA1* gene itself and marker D8S501.

In 1998, Calabrese et al. identified a case of Duane syndrome (Table 1, Fig. 1; case 3) with a complex rearrangement involving a deletion of the 8q13 region. Fluorescent in situ hybridisation analysis narrowed the centromeric breakpoint of the deletion to between D8S510 and D8S544, and the telomeric breakpoint to between D8S1767 and D8S543. Comparison with the case of a deletion spanning D8S1841 to D8S286, described by Vincent et al. (1994; Table 1, Fig. 1; case 2), reduced the Duane syndrome gene critical region to between D8S553 and D8S543. Patient A was not affected with Duane syndrome but deleted for marker D8S1797, further reducing the Duane syndrome critical region to between D8S553 and D8S1797.

Comparison with previously reported deletions of the region reduces the critical region for the gene causing Duane syndrome to approximately 1 cM (http://research.marshfieldclinic.org). Our refinement of the Duane syndrome critical region and the release of the first draft of the total sequence of the human genome, should hasten the identification of a Duane syndrome gene.

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