

LETTER TO JMG

Pierre Robin sequence may be caused by dysregulation of *SOX9* and *KCNJ2*

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Background: The Pierre Robin sequence (PRS), consisting of cleft palate, micrognathia and glossoptosis, can be seen as part of the phenotype in other Mendelian syndromes—for instance, campomelic dysplasia (CD) which is caused by *SOX9* mutations—but the aetiology of non-syndromic PRS has not yet been unravelled.

Objective: To gain more insight into the aetiology of PRS by studying patients with PRS using genetic and cytogenetic methods.

Methods: 10 unrelated patients with PRS were investigated by chromosome analyses and bacterial artificial chromosome arrays. A balanced translocation was found in one patient, and the breakpoints were mapped with fluorescence in situ hybridisation and Southern blot analysis. All patients were screened for *SOX9* and *KCNJ2* mutations, and in five of the patients expression analysis of *SOX9* and *KCNJ2* was carried out by quantitative real-time PCR.

Results: An abnormal balanced karyotype 46,XX,t(2;17)(q23.3;q24.3) was identified in one patient with PRS and the 17q breakpoint was mapped to 1.13 Mb upstream of the transcription factor *SOX9* and 800 kb downstream of the gene *KCNJ2*. Furthermore, a significantly reduced *SOX9* and *KCNJ2* mRNA expression was observed in patients with PRS.

Conclusion: Our findings suggest that non-syndromic PRS may be caused by both *SOX9* and *KCNJ2* dysregulation.

Cleft lip and/or palate (CL/P) is a common congenital malformation affecting approximately 2 per 1000 newborns worldwide. The aetiology of CL/P is largely unknown; however, recent studies focusing on syndromic forms of CL/P have identified specific genes that may also be involved in non-syndromic CL/P.¹ The Pierre Robin sequence (PRS (MIM 261800)) is a clinically well-defined subgroup of the CL/P population with an unknown aetiology. PRS is characterised by cleft palate, micrognathia and respiratory difficulties in the early neonatal period (caused by glossoptosis), and is often observed as a part of other Mendelian syndromes, such as Stickler's syndrome, velocardiofacial syndrome and Marshall's syndrome.² PRS is also seen as a part of campomelic dysplasia (CD (MIM 114290)), a rare skeletal dysplasia, consisting of bowing of the long bones (campomelia), malformation of the pelvis and spine, 11 pairs of ribs, hypoplastic scapulae, club feet, micrognathia, cleft palate and in some patients male-to-female sex reversal. When severely affected, the children die in the neonatal period owing to respiratory problems resulting from developmental defects in the respiratory system.³ CD is due to haploinsufficiency of the transcription factor *SOX9* on 17q24.3, caused by intragenic mutations, deletions involving the entire gene or chromosomal rearrangements up to about 950 kb upstream of the *SOX9*

coding region.^{4–6} The phenotypes of the breakpoint cases in CD, believed to be caused by disruption of regulatory regions, are generally less severe than the intragenic mutations.⁶

The description of patients with skeletal abnormalities, including PRS features caused by breakpoints 900 and 932 kb upstream of the *SOX9* coding region^{7,8} and the finding of a familial translocation t(2;17) cosegregating with PRS,⁹ suggests *SOX9* to be a candidate gene also for non-syndromic PRS. Animal studies support this hypothesis, as *SOX9* mutant mice present with cleft palate and hypoplasia of cartilage-derived skeletal structures¹⁰ and *SOX9* is expressed in the fetal mouse mandible.¹¹

To gain more insight into the aetiology of PRS, we performed genetic and cytogenetic analyses in 10 unrelated patients with non-syndromic PRS.

METHODS

The patients included in this study were identified through hospital records and selected if they had non-syndromic PRS (cleft palate, micrognathia and respiratory difficulties in the early neonatal period). One patient had an additional history of malignant hyperthermia and bilateral inguinal hernias. The patients were aged between 2 and 16 years.

Chromosome analysis was carried out with the G-banding technique using peripheral blood lymphocytes. Fine mapping of the breakpoints was performed using fluorescence in situ hybridisation (FISH), with bacterial artificial chromosome (BAC) and fosmid clones obtained from the Sanger Institute. Genomic DNA was isolated from blood according to standard protocols.

For Southern blot analysis the genomic DNA was digested with the restriction enzymes *EcoRI* and *MscI*. Two hybridisation probes (SB817 and SB520) were prepared by PCR using primer sets SB817U/L (5'-GTAAAGAAACGGTGCAGAAATA-3'/5'-CAATAGCAGAGCGCAGTAG-3') and SB520U/L (5'-TGGGACAGAAACATTACCTTG-3'/5'-TGATTGGAGGAGAAAACGAC-3'). The SB817 probe hybridised to the 5136 bp *MscI* and the 1712 bp *EcoRI* fragments, and SB520 detected the 7102 bp *MscI* and 3132 bp *EcoRI* fragments.

Array comparative genome hybridisation (CGH) was carried out using a sub-megabase resolution whole-genome tiling path BAC array consisting of the human genome high-resolution 32 k re-arrayed clone set (BACPAC Resources), the 1 Mb Sanger set (Wellcome Trust Sanger Institute, Cambridge, UK) and a set of 390 sub-telomeric clones (assembled by members of the COST B19 initiative: molecular cytogenetics of solid tumours).¹²

Abbreviations: BAC, bacterial artificial chromosome; CD, campomelic dysplasia; CGH, comparative genome hybridisation; CL/P cleft lip and/or palate; FISH, fluorescence in situ hybridisation; MIMdb, Mendelian Cytogenetics Network database; miRNA, microRNA; PRS, Pierre Robin sequence; QPCR, quantitative real-time PCR; SNP, single-nucleotide polymorphism

SOX9 and *KCNJ2* expression in cultured lymphoblastoid cell lines was analysed by quantitative real-time PCR (QPCR) using an Opticon DNA engine real-time PCR machine (MJ Research, Waltham, Massachusetts, USA) using an SYBR green kit (GE Healthcare, Waukesha, Wisconsin, USA). Relative gene expression was calculated using dilution curves and *UBC*, *YWHAZ*, *GAPDH*, *G6PD* and *B2M* mRNA as reference genes. Mean values of the housekeeping genes were calculated using the BestKeeper software tool,¹³ and the experiments were run in duplicate and repeated twice. Genomic PCR and sequencing were performed according to standard protocols (supplementary table showing primer sequences and PCR conditions, mRNA isolation and cDNA preparation is available at <http://jmg.bmj.com/supplemental>).

The study was approved by the local scientific ethics committee and written informed consent was obtained from the patients and from the parents of children who were aged <12 years.

RESULTS AND DISCUSSION

We performed chromosome analyses in 10 unrelated patients with features of PRS. All the cases had normal karyotypes except for one, where PRS cosegregated with a balanced translocation $t(2;17)(q23.3;q24.3)$ in a father and a daughter.

The translocation carrier (fig 1A, B) was a 15-year-old girl. The father also had PRS according to hospital records, but he declined to participate in further clinical and genetic studies. The patient had mild facial dysmorphism. In addition to micrognathia, she had a flat face, a broad nasal bridge, low-set ears and low-set hairline. She was born as the only child of a 33-year-old father and a 41-year-old mother with type 2 diabetes. Although the intellectual and psychomotor development was at the lower end of the normal spectrum, she attended a standard school. She had received speech therapy, and a pharyngeal flap operation had been performed to decrease hypernasal speech. No visual or hearing deficits were reported. Growth and sexual maturation were normal. The mother has a healthy daughter from an earlier relationship. No other family member has PRS, cleft palate or other malformations. Material from paternal grandparents was not available, but a paternal uncle and a maternal half-sister of the father had normal karyotypes.

Array CGH was performed in seven of the nine non-translocation patients with PRS, and it showed a 0.4 Mb microdeletion at 7q21.13 (chromosome position 88 473 693–88 909 911, UCSC Genome Browser, March 2006 assembly) in one of the patients. This result was confirmed by FISH. Parental DNA was not available to investigate the origin of this deletion. There is no known copy-number variation within this region (Database of Genomic Variants and unpublished observations by RU) and there is only one gene, *ZNF804B*, which encodes a C2H2-type zinc finger protein. Deletions at 7q21–q22 have been associated previously with PRS, but several other malformations such as ectrodactyly were part of the clinical phenotype.¹⁴ The other six patients did not show any significant changes with array CGH.

Translocation breakpoints of the $t(2;17)$ patient were mapped using FISH. The chromosome 2q23.3 breakpoint was within the BAC clone RP11-373H2 (AC113610) at chromosome position 150 777 641–150 874 211 (UCSC Genome Browser). This breakpoint is located in a gene-empty region, approximately 200 kb downstream of the *RND3* gene and 350 kb upstream of *FJL32955* encoding a hypothetical protein (LOC150596). *RND3* encodes a plasma membrane-bound GTPase involved in regulation of cellular response and cytoskeletal dynamics. *RND3* could be involved in the aetiology in the present case, but no phenotype is associated with

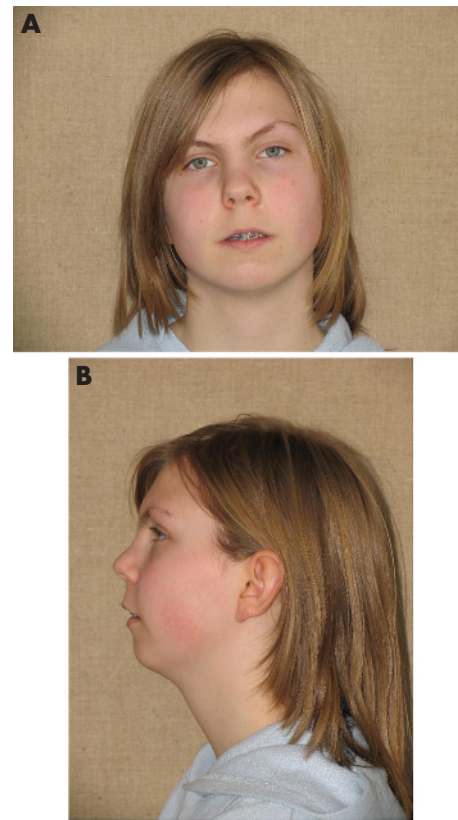


Figure 1 (A, B) Photographs of the patient with Pierre Robin sequence and the balanced translocation $t(2;17)(q23.3;q24.3)$. Informed consent was obtained for publication of this figure.

mutations or chromosomal rearrangements in the vicinity of *RND3*, and a position effect in the present case is speculative. Chromosome abnormalities involving 2q22 and 2q24 have been observed previously in patients with PRS. Jamshidi *et al*⁹ reported a familial translocation $t(2;17)(q24.1;q24.3)$ cosegregating with PRS, and mutations of *ZFH1B* at 2q22.3 cause Mowat–Wilson syndrome (MIM 235 730), in which submucous cleft palate is a part of the phenotype, although this syndrome does not otherwise resemble CD or PRS. However, these regions are located far from the present breakpoint and do not have an apparent effect on the phenotype of the patient.

We mapped the 17q24.3 breakpoint within an approximately 15 kb overlapping region of the BAC clone RP11-7D6 (fig 2A) and two fosmid clones (G248P89352E7 and G248P86963D5). The breakpoint was further mapped with Southern blot hybridisation using two probes, detecting an approximately 12 kb region within the overlapping region of the FISH clones (fig 2B, C). Using the hybridisation probe SB817 and *MscI* digestion, we detected the 5.1 kb normal fragment and a slightly larger band representing the junction fragment in the patient. This probe also detected the junction fragment using the restriction enzyme *EcoRI*. Hybridisation probe SB520 showed only the normal band pattern in the patient (data not shown). This suggests that the 17q breakpoint is within an approximately 5 kb region defined by two *MscI* recognition sites (fig 2C), 1.13 Mb upstream of the *SOX9* gene (chromosome position 66 485 382–66 491 517). The total gene-empty region upstream of *SOX9* is approximately 2 Mb where the proximal flanking gene is *KCNJ2*, which is located about 800 kb from the 17q breakpoint.

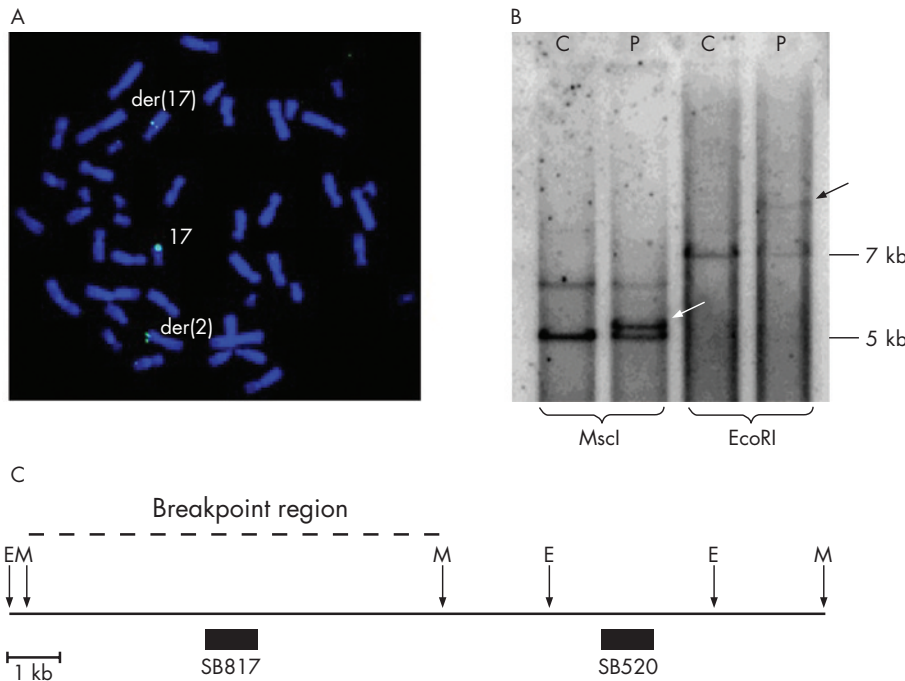


Figure 2 The breakpoint region at 17q. (A) FISH analysis showing the breakpoint-spanning bacterial artificial chromosome (BAC) clone RP11-7D6. (B) Southern blot hybridisation using probe SB817 and the restriction enzymes *MscI* and *EcoRI*. With *MscI* digests, the 5 kb normal fragment and a larger band representing the junction fragment (white arrow) are detected in the patient. With *EcoRI* digests, besides the 7 kb normal fragment, the patient has a larger fragment (black arrow). (C) The physical map of the breakpoint region. The 12 kb interval is within the 15 kb overlapping region of the spanning BAC (fig 2A) and the fosmid clones. The breakpoint region (dashed line) is mapped between the two *MscI* restriction sites (chromosome position 66 485 382–66 491 517). The vertical arrows show the *MscI* (M) and *EcoRI* (E) restriction sites. The filled boxes represent the two hybridisation probes SB817 and SB520. C, control; P, patient.

We sequenced the coding region of *SOX9* in all the 10 patients with PRS and detected several nucleotide polymorphisms, but no pathogenic mutations. We also screened the 3' untranslated region of *SOX9* for sequence variations, as *in silico* predictions of microRNA (miRNA) target sites suggest that *SOX9* expression is regulated by miRNAs (miRBase Targets V2.0). miRNAs are short non-coding RNAs involved in downregulation of genes at the translational level by binding to specific target sites in the 3' untranslated region of the mRNA.¹⁵ No sequence variations in the predicted miRNA target sites were identified. Furthermore, we searched for evolutionarily conserved sequences, and hence potentially regulating regions (in human, chimpanzee, mouse, rat, dog, chicken, zebrafish and fugu, UCSC Genome Browser) and identified a conserved region at chromosome position 66 520 880–66 521 660. This region overlaps with the candidate regulatory element “*SOX9cre1*” (chromosome position 66 520 446–66 521 622, UCSC Genome Browser) proposed by Velagaleti *et al*⁸ (fig 3). Screening this conserved region in the 10 patients with PRS revealed two unreported consecutive single-nucleotide polymorphisms (SNPs) at 66 520 950–66 520 951 (A/T and G/A) in two patients. These SNPs were also detected in two of 18 patients with isolated cleft palate and in 20 of 95

normal controls. We therefore concluded that the two consecutive SNPs were not associated with PRS.

Haploinsufficiency of *SOX9* as a cause of CD has been shown by deletions involving the entire gene in two unrelated patients with CD,^{4,5} and we considered whether expression levels of *SOX9* were affected in patients with PRS. By QPCR, we detected a significantly reduced *SOX9* expression ($p = 0.04$, two-tailed Mann–Whitney U test) in cultured lymphoblastoid cell lines from five non-translocation patients with PRS compared with 11 controls (table 1). Unexpectedly, the expression of *SOX9* in the translocation patient was higher than in the group of non-translocation patients with PRS, although still at the lower end of the normal range. A possible explanation for this is that some upstream regulators (<1 Mb from *SOX9* in the present case) are still active in the translocation patient. Likewise, Wirth *et al*¹⁶ found the same expression levels in both *SOX9* alleles in a lymphoblastoid cell line from a t(13;17) translocation patient, where the 17q breakpoint was located >130 kb from *SOX9*.

The expression data should be interpreted with caution, as expression in the patients may not reflect expression during embryonic development. Moreover, expression studies were

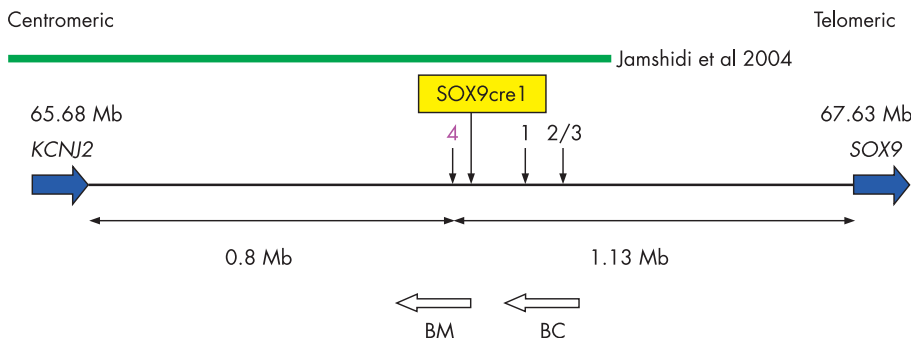


Figure 3 Schematic overview of the 2 Mb gene-empty region between *KCNJ2* and *SOX9*, showing known breakpoints >900 kb upstream of *SOX9*. Patients 1 and 2 were reported by Hill-Harfe *et al*⁷ and patient 3 by Velagaleti *et al*⁸. The patients have breakpoints in the following approximate chromosome positions: patient 1 at 66.7 Mb, and patients 2 and 3 at 66.73 Mb. Patient 4 is the translocation patient presented here with a breakpoint at 66.49 Mb. The green bar represents the breakpoint interval in the three-generation family with PRS reported by Jamshidi *et al*.⁹ Velagaleti *et al*⁸ suggested a potential regulatory region, *SOX9cre1*, at 66.52 Mb. BM is the spliced human EST BM678241 and BC is the human mRNA BC039327.

carried out in lymphoblastoid cell lines, not in chondrocytes, which would have been preferred, but were unavailable.

The breakpoint located most upstream within the *SOX9* regulatory landscape reported so far, about 932 kb from the coding region, was associated with hypoplastic scapulae and 11 pairs of ribs and Robin sequence (family F).⁷ The 17q breakpoint identified in this study was located 1.13 Mb upstream of *SOX9*, which is 200 kb further upstream than the breakpoint in family F, and the absence of missing ribs in the present patient points to a milder phenotype. Thus, the present case constitutes the mildest phenotype and the most distantly located breakpoint upstream of the *SOX9* gene reported to date (fig 3 shows a schematic overview of breakpoints located >900 kb upstream of *SOX9*). Whether the mild phenotypes may be categorised as CD or represent a distinct clinical entity is a subject of debate.^{17,18} Considering the growing reports of distantly located chromosomal rearrangements cosegregating with milder types of skeletal dysplasia, it is possible that CD, acampomelic CD, mild acampomelic CD and PRS may represent a continuum of phenotypes caused by dysregulation of *SOX9*.

The gene most proximal to the 17q breakpoint is *KCNJ2* (fig 3), which encodes a potassium channel. In humans, *KCNJ2* mutations have been detected in Andersen's syndrome (MIM 170390), which presents with potassium-sensitive periodic paralysis, variable cardiac arrhythmias and dysmorphic features overlapping with CD to some extent, including low-set ears, micrognathia, cleft palate and scoliosis. Moreover, mutations in *KCNJ2* cause cleft palate in mice.¹⁹ To our knowledge, CD with periodic paralysis and cardiac arrhythmias has not been reported, but PRS occurring with cardiac arrhythmias has been described previously²⁰ and a higher prevalence of congenital cardiac anomalies has been found in patients with PRS (13.6%) compared with the general population with CL/P (6.7%).²¹ We sequenced the coding region of *KCNJ2* in the 10 patients with PRS, including the translocation patient, without detecting any mutations. Furthermore, we investigated the *KCNJ2* expression using QPCR analyses and detected a significant reduction in the

KCNJ2 expression in the non-translocation patients with PRS compared with 11 controls. The expression levels of *KCNJ2* were very low in both controls and patients; consequently, two different *KCNJ2* primer sets were tested, and these were in agreement (table 2).

Our expression data therefore suggest that *KCNJ2* may play a role in the PRS phenotype, together with *SOX9*. It has recently been reported that chromosomal aberrations may change the expression of several neighbouring genes,²² thus supporting a role for both *KCNJ2* and *SOX9* in the aetiology of PRS.

Our sequencing results indicate that mutations in the *SOX9* and *KCNJ2* coding regions, in *SOX9* miRNA target sites and in an evolutionarily conserved upstream region of *SOX9* are not a frequent cause of PRS. Identifying and sequencing other candidate *cis*-regulatory regions is highly relevant but complicated, time and cost consuming, as regulatory elements may reside even within neighbouring genes.²³ Since translocations upstream of *SOX9* are causing phenotypes consistent with CD, *cis*-regulatory elements have been sought in the conserved neighbouring regions of *SOX9* and many have been suggested (reviewed by Hill-Harfe *et al*). A candidate regulatory element *SOX9cre1*, about 1.1 kb, was suggested using *in silico* analysis and analysis of chromosomal rearrangements (fig 3).⁸ The breakpoint in the present case is located even more upstream than the *SOX9cre1*, thus one or more of the *cis*-regulatory regions must be located either in our breakpoint region or even more upstream, or both. Another explanation of the effect of the translocation could be that altered organisation of the chromatin structure causes changes in gene regulation (eg, euchromatic to heterochromatic conformation).²³

In the region upstream of *SOX9*, several non-coding transcripts, but no protein-coding transcripts, have been identified.^{6,7} The 17q breakpoint is located in the intronic region of a 226 kb spliced human EST (BM678241), which has been detected in fetal and adult eyes.²⁴ BM678241 is located

Table 1 *SOX9* expression in lymphoblasts (quantitative real-time PCR)

Sample	Gender	Expression values of <i>SOX9</i>
PRS 1	XY	5.4
PRS 2	XY	5.4
PRS 3	XX	1.7
PRS 4	XY	5.3
PRS 5	XX	7.1
PRS mean (range)		5.0 (1.7–7.1)
PRS 6 t(2;17)	XX	13.7
C 1	XY	10.6
C 2	XX	34.6
C 3	XY	10.4
C 4	XY	15.0
C 5	XX	22.4
C 6	XY	40.2
C 7	XX	22.5
C 8	XX	1.0
C 9	XY	21.4
C 10	XX	4.0
C 11	XY	31.9
C mean (range)		19.5 (1.0–40.2)
p Value		0.04

PRS, Pierre Robin sequence; PRS 1–6, six patients with PRS, PRS 6 is the translocation patient; C 1–11, controls.

*The expression value of *SOX9* was normalised to the mean values of the housekeeping genes *UBC*, *YWHAZ*, *B2M*, *G6PD* and *GAPDH*. Mean values of the housekeeping genes were calculated using the BestKeeper software tool.¹³

The experiments were run in duplicate and repeated twice. Values of PRS 1–5 to C 1–11 were compared and p values were calculated using the two-tailed Mann-Whitney U test.

Table 2 *KCNJ2* expression in lymphoblasts (QPCR)

Sample	Gender	Expression values of <i>KCNJ2</i> A	Expression values of <i>KCNJ2</i> B
PRS 1	XY	2.5	1.2
PRS 2	XY	3.0	1.0
PRS 3	XX	2.0	1.6
PRS 4	XY	2.7	3.0
PRS 5	XX	1.0	2.1
PRS mean (range)		2.2 (1.0–3.0)	1.8 (1.0–3.0)
PRS 6 t(2;17)	XX	3.7	2.5
C 1	XY	8.2	2.1
C 2	XX	70.3	23.7
C 3	XY	2.4	1.2
C 4	XY	10.8	4.3
C 5	XX	31.2	16.3
C 6	XY	17.1	5.6
C 7	XX	35.2	20.0
C 8	XX	6.7	1.1
C 9	XY	27.6	30.4
C 10	XX	7.6	2.3
C 11	XY	70.3	29.1
C mean (range)		26.1 (2.4–70.3)	12.3 (1.1–30.4)
p Value		0.007	0.05

PRS, Pierre Robin sequence; PRS 1–6, six patients with PRS, PRS 6 is the translocation patient; C 1–11, controls.

As the expression of *KCNJ2* was low, QPCR was performed with two different primer sets A and B (supplementary table). The expression value of *KCNJ2* was normalised to the mean values of the housekeeping genes *UBC*, *YWHAZ*, *B2M*, *G6PD* and *GAPDH*. Mean values of the housekeeping genes were calculated using the BestKeeper software tool.¹³

The experiments were run in duplicate and repeated twice. Values of PRS 1–5 and C 1–11 were compared and p values were calculated using the two-tailed Mann-Whitney U test.

Key points

- The aetiology of isolated Pierre Robin sequence (PRS) is unknown.
- Expression analysis and cytogenetic analysis were performed in patients with isolated PRS.
- The data point to a role for *SOX9* and *KCNJ2* dysregulation in isolated PRS.

proximal to BC039327, a human mRNA exclusively expressed in testis and disrupted by the 17q breakpoint in family F (fig 3). It was suggested that BC039327 was a functional RNA with an unknown role, since there was no testis phenotype in family F.⁷ Likewise, the role of BM678241 in relation to our breakpoint is unclear, as our translocation carrier does not have a phenotype involving the eyes.

Considering gene regulation, it is also necessary to include genes involved in the *SOX9* and *KCNJ2* signalling pathways. *SOX9* is one of several factors involved in chondrogenesis,²⁵ and mutations and sequence variations in genes encoding collagen subunits (*COL2A1*, *COL11A1* and *COL11A2*) were found in patients with non-syndromic PRS,²⁶ supporting the fact that PRS may result from dysregulation of *SOX9* signalling pathways in general.

In summary, our data suggest that dysregulation of the genes *SOX9* and *KCNJ2* may be involved in PRS, evidenced by a familial translocation with a breakpoint located in the gene-empty region between *SOX9* and *KCNJ2*, and by reduced expression of *SOX9* and *KCNJ2* in non-translocated patients with PRS. To determine the extent of the involvement of *SOX9* and *KCNJ2* in PRS, skeletal x ray surveys and sequencing of *SOX9* and *KCNJ2* candidate *cis*-regulatory regions and signalling partners in a larger group of patients are needed.

ELECTRONIC DATABASE INFORMATION

The URLs for data presented herein are as follows:

- Database of Genomic Variants, <http://www.projects.tcag.ca/variation/>
- Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>
- BACPAC Resources, <http://bacpac.chori.org>
- UCSC Genome Bioinformatics site, March 2006 assembly, <http://genome.ucsc.edu/>
- The Mendelian Cytogenetics Network Database (MCNdb), <http://www.mcndb.org/>
- NCBI dbSNP Build 125, <http://www.ncbi.nlm.nih.gov/SNP/>
- miRBase Targets Version 2.0, <http://microrna.sanger.ac.uk/targets/v2/>
- Sanger Institute, <http://sanger.ac.uk/>

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Supplementary table showing primer sequences and PCR conditions, mRNA isolation and cDNA preparation is available at <http://jmg.bmj.com/supplemental>

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Competing interests: None.

Informed consent was obtained for publication of fig 1.

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