Clinical and Molecular Characterisation of Children with Pierre Robin Sequence and Additional Anomalies

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Abstract

Pierre Robin Sequence (PRS) is usually classified into syndromic and nonsyndromic groups, with a further subclassification of the nonsyndromic group into isolated PRS and PRS with additional anomalies (PRS-Plus). The aim of this research is to provide an accurate phenotypic characterisation of nonsyndromic PRS, specifically the PRS-Plus subgroup. We sought to examine the frequency of sequence variants in previously defined conserved noncoding elements (CNEs) in the putative enhancer region upstream of SOX9, the regulation of which has been associated with PRS phenotypes. We identified 141 children with nonsyndromic PRS at the Royal Children's Hospital, Melbourne from 1985 to 2012 using 2 databases. Clinical and demographic data were extracted by file review and children categorized as 'isolated PRS' or 'PRS-Plus'. A subset of children with PRS-Plus was selected for detailed phenotyping and DNA sequencing of the upstream SOX9 CNEs. We found 83 children with isolated PRS and 58 with PRS-Plus. The most common PRS-Plus malformations involved the musculoskeletal and ocular systems. The most common coexisting craniofacial malformation was choanal stenosis/atresia. We identified 10 children with a family history of PRS or cleft palate. We found a single nucleotide substitution in a putative GATA1-binding site in one patient, but it was inherited from his phenotypically unaffected mother. PRS-Plus represents a broad phenotypic spectrum with uncertain pathogenesis. Dysmorphology assessment by a clinical geneticist is recommended. SOX9 CNE sequence variants are rare in our cohort and are unlikely to play a significant role in the pathogenesis of PRS-Plus.

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The clinical features of Pierre Robin Sequence (PRS; OMIM 261800) include micrognathia, glossoptosis, and upper airway obstruction [Robin, 1923]. Cleft palate, a variable feature, has often been incorporated into the definition. PRS is most commonly described in terms of 2 broad categories: syndromic and nonsyndromic. Syndromic PRS refers to children with the traditional features as a component of a known syndrome, of which there are more than 50 [summarized in Tan et al., 2013]. Nonsyndromic PRS can be further subcategorized into

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children with: only the core components (isolated PRS) or additional malformations, but not in a pattern recognized as a known syndrome or genetic condition (which we hereafter term PRS-Plus). PRS is clearly a clinically heterogeneous condition, and the full phenotypic spectrum of the nonsyndromic PRS group requires detailed characterisation.

Several theories have been proposed to explain the pathogenesis of PRS, but the most prevailing involves the notion of primary mandibular hypoplasia. This theory states that during embryonic development, an intrinsic or extrinsic factor leads to micrognathia, which in turn causes failure of the tongue to drop from between the palatal shelves resulting in cleft palate [Cohen, 1999].

The identification of microdeletions, translocation breakpoints, and sequence variants disrupting putative regulatory elements in the chromosome 17q24 noncoding regions far up- and downstream of *SOX9* (OMIM 608160) in children with isolated PRS provided the first definitive insight into the pathogenesis of PRS [Benko et al., 2009]. Disruption of *SOX9* by intragenic mutations and more proximal chromosomal translocation breakpoints cause campomelic dysplasia (OMIM 114290), characterized by skeletal dysplasia, genital anomalies including sex reversal, and craniofacial involvement with PRS as a component feature. Additional support for the dysregulation of *SOX9* in the pathogenesis of PRS has been provided by the identification of chromosomal anomalies in children with PRS-Plus, notably involving the skeletal system [Fukami et al., 2012; Gordon et al., 2014; Smyk et al., 2015].

The optimal criteria for diagnosis and management of children with PRS remain contentious. There is no consensus about a definition of PRS and optimal management of the airway and feeding difficulties. Without a comprehensive understanding of the full phenotypic spectrum of syndromic and nonsyndromic PRS, accurate diagnoses and tailored management strategies are challenging and difficult to evaluate systematically. The aim of this research is to provide a detailed phenotypic description of a cohort of children with PRS managed at one tertiary children's hospital, with a specific focus on the PRS-Plus group. A secondary objective of this study was to identify sequence variants in previously described [Gordon et al., 2014] conserved noncoding elements (CNEs) in the 'PRS region' ~1.2 Mb upstream of *SOX9* in children with PRS and musculoskeletal anomalies and/or a family history of cleft or PRS as we hypothesized that this group would be most likely to have an underlying genetic basis.

Methods

Data Collection and Phenotypic Classification
Our cohort of children with nonsyndromic PRS was identified from the Royal Children's Hospital (RCH) in Melbourne, the major tertiary referral centre for PRS in the state of Victoria, Australia. The RCH Cleft Registry and the Victorian Clinical Genetics Services (VCGS) database were cross-referenced to identify all children with PRS born between January 1985 and December 2012. Clinical and demographic data were extracted by detailed review of the RCH medical record and VCGS genetic file. Any children with syndromic PRS (established by clinical or molecular means) were excluded from the study.

We defined isolated PRS as referring to children with only the traditional features of PRS, and PRS-Plus was defined as children with the core features plus one or more congenital anomaly. Trivial or clinically insignificant cardiac lesions (e.g., spontaneously closing patent ductus arteriosus) and common medical conditions such as asthma, eczema, gastroesophageal reflux, and conductive hearing loss secondary to middle ear effusion were not considered significant enough to constitute a diagnosis of PRS-Plus.

Subgroup Selection
We selected patients with a family history of PRS or cleft from the nonsyndromic PRS cohort (isolated PRS and PRS-Plus) for clinical review and molecular characterisation. Based on the clinical and molecular link with campomelic dysplasia, we also selected a subgroup of PRS-Plus children with musculoskeletal anomalies for the same analysis.

Clinical Assessment and Molecular Characterisation
Dysmorphology and anthropometric data were collected by clinical assessment using a standardized form (J.X.X.) with independent verification (T.Y.T.). DNA from lymphocytes in saliva was extracted by standard manufacturer protocol (Oragene, Ottawa, Canada). Standard Sanger sequencing of 14 CNEs described previously [Gordon et al., 2009] was performed. Mutation Surveyor software version 3.2 (by Soft Genetics) was used to align sequencing data with the reference genome on the UCSC (University of California, Santa Cruz, Calif., USA) Genome Browser (http://genome.ucsc.edu/), version number GRCh37/hg19 and SNP database version number dbSNP141. CNE variants were examined in the UCSC Genome Browser and primary databases including Transfac (http://www.gene-regulation.com/pub/databases.html) and 1000 Genomes (1000genomes.org) [Matys et al., 2006; 1000 Genomes Project Consortium et al., 2015].

Results

We identified 174 children with PRS in the RCH Cleft Registry and VCGS database. After 33 children were excluded for having a diagnosed syndrome or pathogenic chromosomal abnormality (table 1), 141 children remained as the nonsyndromic PRS cohort. All children in our cohort had cleft palate as they were ascertained through the cleft registry. Note that the 2 related children
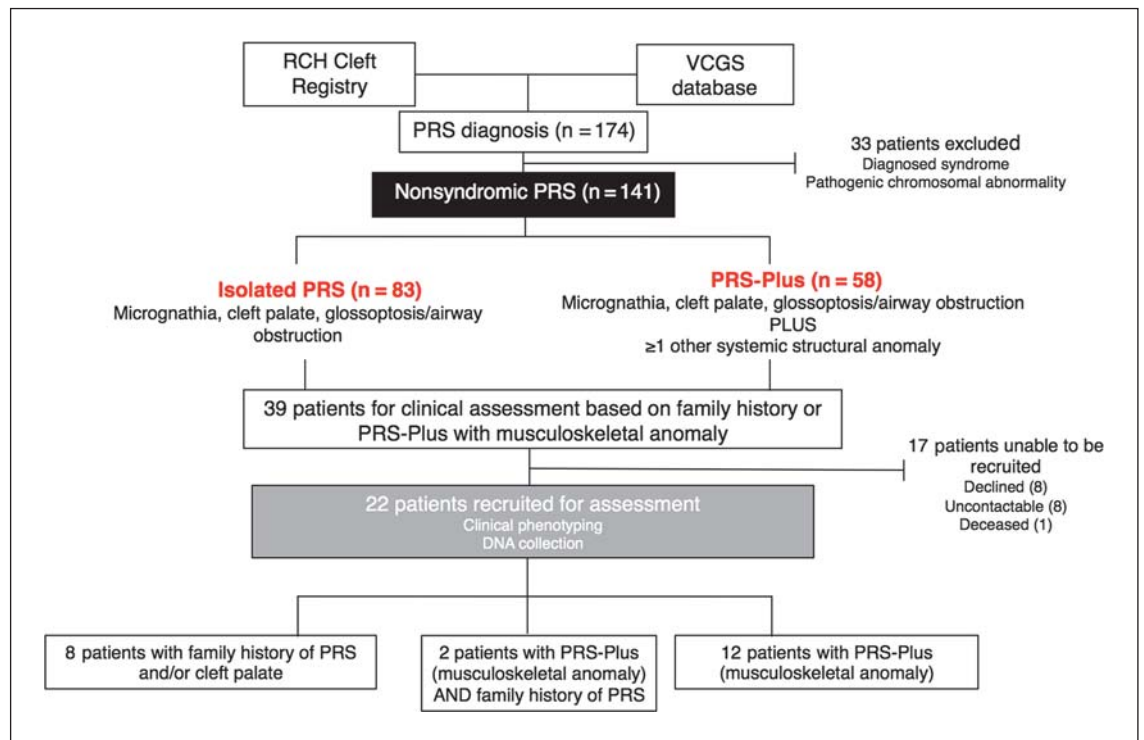
Fig. 1. Methods. This flowchart details the process of the medical record review of the PRS cohort followed by subgroup selection for detailed phenotyping and molecular analysis.

Table 1. Diagnoses of syndromic PRS excluded from study

Reason for exclusion	Patients (n = 33)
Stickler syndrome	11
22q11 deletion syndrome	2
22q12 deletion syndrome	2
Translocation 2;17	2
Marshall syndrome	1
Foetal alcohol syndrome	1
Treacher-Collins	1
Deletion 5q	1
Duplication 7q	1
Duplication 4q35	1
Deletion 19q13.43	1
Duplication 8p23.3	1
Van der Woude syndrome	1
Rapp-Hodgkin syndrome	1
Fragile X syndrome	1
Smith Magenis syndrome	1
Campomelic dysplasia	1
Mandibulofacial dysostosis	1
Translocation 9;11	1
Osteopathia striata with cranial sclerosis	1

with a balanced t(2;17) translocation have been previously reported [Jamshidi et al., 2004]. Of the 141 children with nonsyndromic PRS, 83 were classified as having isolated PRS and 58 with PRS-Plus by medical record review (fig. 1). Demographic information of the nonsyndromic PRS (isolated PRS and PRS-Plus) cohort is detailed in table 2.

Phenotypes of Nonsyndromic PRS

Of the 58 children with PRS-Plus, 36 (62%) had additional craniofacial dysmorphology noted from medical record review (online suppl. table 1; for all suppl. material, see www.karger.com/doi/10.1159/000449115). The most common facial dysmorphic features were hypertelorism (6), low-set ears (5), and preauricular skin tags (4). Musculoskeletal and ocular anomalies were the most frequently observed abnormalities, with 26/58 (44.8%) and 23/58 (39.6%) children affected, respectively. Of the 26 children with musculoskeletal anomalies, the most common conditions were congenital talipes equinovarus (n = 4), congenital dislocated hips (n = 4), metatarsus varus (n = 3), pectus excavatum (n = 2), and scoliosis (n = 2). Strabismus was the most common ocular anomaly (n = 9).

Thirty-four of the 58 children with PRS-Plus (59%) had malformations affecting only one body system beyond the craniofacial region (online suppl. table 2). Two or more body systems beyond the craniofacial region were affected in 24 children with PRS-Plus (41%). The central nervous system, whilst uncommonly affected in single-system children, was the third most common body system involved in children with multisystem anomalies. These included epilepsy (n = 3), hypoplasia of the corpus callosum (n = 2), sensorineural hearing loss (n = 2), and cerebral palsy (n = 2). All anomalies found in each of the 24 multisystem-affected children are listed in online supplementary table 3.

The most common craniofacial anomaly besides PRS in our cohort was choanal stenosis/atresia, with 8 children having this condition; 3 as the only additional malformation beyond PRS, and 5 had other associated anomalies.

Family History of Cleft

Of the 136 children in our nonsyndromic PRS cohort with documented family history, 22 (16.2%) had a relative with orofacial cleft; of these, 13 (59.1%) occurred in a first-degree relative, and 9 (40.9%) occurred in a second-degree or more distant relative. These were diagnosed with PRS (n = 9; 40.9%) or cleft palate only (n = 6; 27.3%). There was one pair of siblings with isolated PRS, and their father had cleft palate only.

Detailed Phenotyping of the Selected Subgroup

On the basis of family history of PRS or cleft, or PRS-Plus with musculoskeletal involvement, 39 children were selected for comprehensive phenotyping, but 17 of these children were unable to be recruited for various reasons (fig. 1). Of the remaining 22 with a musculoskeletal anomaly (12), family history of cleft/PRS (8), or both (2), we identified additional dysmorphic features in 7 (31.8%) children, but did not make any syndrome diagnosis, nor did their PRS classification change. Approximately 60% of this selected subset (13/22) had already seen a clinical geneticist as part of standard care.

DNA Sequencing of the Noncoding Elements of SOX9

Sanger sequencing of 14 CNEs [Gordon et al., 2009] in the upstream region of *SOX9* was undertaken in 22 children with PRS and either a musculoskeletal anomaly (12) or a family history of cleft/PRS (8), or both (2). In total, 4 children (4/22; 18.2%) were found to have a *SOX9* CNE variant observed in dbSNP141 at an allele frequency of less than 1% (online suppl. table 4). These 4 children com-

Table 2. Demographic characteristics of the entire nonsyndromic PRS cohort (n = 141), with subclassification into isolated PRS (n = 83) and PRS-Plus (n = 58) groups

	Total cohort	Isolated PRS	PRS-Plus
Current age, years			
1–5	33 (23.4)	21 (25.3)	12 (20.7)
6–10	33 (23.4)	25 (30.9)	8 (13.8)
11–15	41 (29.1)	20 (24.1)	21 (36.2)
16–20	22 (15.6)	11 (13.3)	11 (19.0)
21+	12 (8.5)	6 (7.2)	6 (10 3)
Gender			
Male	62 (44.0)	30 (36.1)	32 (55.1)
Female	79 (56.0)	53 (63.9)	26 (44.8)
Mortality			
Alive	139 (98.6)	82 (98.8)	57 (98.3)
Deceased	2 (1.4)	1 (1.2)	1 (1.7)

Percentages are given in parentheses.

prised 3 with PRS-Plus and musculoskeletal anomalies, while 1 had isolated PRS with a family history of cleft palate (online suppl. table 5). In the 3 children with PRS-Plus and musculoskeletal anomalies, heterozygous variants were identified within CNEs 1, 3, and 4. Of interest was the variant occurring in CNE1 within a predicted *GATA1* transcription factor binding site. This was found in a patient (PRS136) with PRS, talipes valgus, and mild pectus excavatum. The single heterozygous nucleotide substitution (T to C) was identified in CNE1 in chr17:68658181 (USCS browser hg19 build) located 1.459 Mb from the start codon of *SOX9*. The change was present in 1 of 2,298 samples reported in the dbSNP141 database, but not in 2,504 samples in 1000 Genomes. Parental analysis confirmed that the variant was inherited from a phenotypically unaffected mother.

Discussion

Phenotypic Spectrum of Children with Nonsyndromic PRS

The nomenclature, and more importantly, categorisation of children with PRS and additional anomalies without a defined syndrome have traditionally been inconsistent. Some studies refer to this group as PRS with associated anomalies [Holder-Espinasse et al., 2001; Bütow et al., 2009; Caouette-Laberge et al., 2012; Thouvenin et al., 2013], whilst others prefer Unique-PRS [Smith and Senders, 2006] (online suppl. table 6). These children are

sometimes grouped with syndromic PRS [Shprintzen, 1988, 1992; Marques et al., 1998; Cruz et al., 1999; van den Elzen et al., 2001; Li et al., 2002; Printzlau and Andersen, 2004; Evans et al., 2006; de Buys Roessingh et al., 2007; Izumi et al., 2012; Patel et al., 2012; Gomez-Ospina and Bernstein, 2016] but also grouped with nonsyndromic children in other studies [Sheffield et al., 1987; Bütow et al., 2009; Al-Samkari et al., 2010]. These differences in classification of the PRS-Plus group make interstudy comparisons challenging. Our study sought to develop a more detailed phenotypic analysis of those in the PRS-Plus group, representing 41% of our cohort. These children appear at this time not to be able to be diagnosed with any known syndrome, even after assessment by a clinical geneticist. Since 2010, it has been standard practice at our centre to refer all neonates with PRS for clinical genetics assessment, and an ophthalmology assessment, especially in children with myopia, to exclude Stickler syndrome.

Positional limb deformities are one of the most commonly observed additional anomalies in children with PRS [Smith, 1961; Hanson and Smith, 1975; Williams et al., 1981; Caouette-Laberge et al., 1994; Bütow et al., 2009]. Extrinsic forces such as multiple pregnancy and oligohydramnios have been hypothesized to have an impact on the in utero positioning of the jaw leading to micrognathia [Poswillo, 1966; DeMyer and Baird, 1969; Knottnerus et al., 2001; Aggarwal and Kumar, 2003]. Positional limb deformities such as congenital talipes equinovarus and metatarsus varus may also occur as a result of compressive forces in utero, such as polyhydramnios, oligohydramnios, or twin pregnancy. All the PRS-Plus children with positional limb deformities in this cohort (n = 7) were singleton pregnancies. Two pregnancies were complicated by polyhydramnios, one pregnancy was complicated by intrauterine growth restriction, and another by premature rupture of membranes at 20 weeks' gestation with resultant oligohydramnios. Of these 4 children with concomitant pregnancy complications, 3 were categorized as PRS-Plus with multisystem anomalies. Our observation that the positional limb deformity occurred with other systemic anomalies suggests an aetiology that is more complex than amniotic fluid disturbance in these children.

Noncoding Elements of SOX9

Our focus on the CNEs upstream of *SOX9* was largely driven by previous work implicating their involvement in the pathogenesis of the nonsyndromic PRS phenotype [Benko et al., 2009; Fukami et al., 2012; Amarillo et al., 2013; Gordon et al., 2014; Smyk et al., 2015]. Given the clinical and molecular link with campomelic dysplasia (OMIM 114290), we hypothesized that children with PRS and additional skeletal anomalies might have CNE sequence variants that alter *SOX9* expression and impact on skeletal development. We also reasoned that those with a family history of PRS or cleft would be more likely to have a genetic basis than those without.

Genetic analysis in this study was restricted to identifying sequence variants in CNEs that might act as putative regulatory elements of *SOX9*. The majority (13/22; 59.1%) of the children in the subgroup analysis had seen a clinical geneticist and had been investigated with either a chromosome microarray or conventional karyotyping with FISH for 22q11 deletion. Only one child had a microarray abnormality (maternally inherited 153-kb duplication of chromosome 5q35.5) that was not considered causative for his phenotype.

In one patient with PRS-Plus (talipes valgus and pectus excavatum in addition to PRS), we identified a variant in a predicted GATA1 transcription factor binding site, located within CNE1 upstream of *SOX9* (genome location chr17:68658181). The same variant had been identified in 1 out of 2,298 samples reported in the dbSNP141 database. There are no publically available phenotypic data for this single database individual. *GATA1* (OMIM 305371) is located in the X chromosome and codes for a protein which plays an important role in erythroid development [Simon et al., 1992], but no known regulatory interaction with SOX9. Germline mutations in *GATA1* cause various hematological diseases [Nichols et al., 2000; Mehaffey et al., 2001; Yu et al., 2002; Hollanda et al., 2006; Sankaran et al., 2012], but are not known to be associated with PRS. Our finding that this variant was inherited from a phenotypically unaffected mother suggests that it is either a benign change or a predispositional variant with incomplete penetrance but unlikely to be the sole pathogenic factor. The other CNE variants identified in 3 other patients (2 with PRS-Plus; 1 with family history of cleft) were reported with higher frequency and did not occur within any known transcription factor binding sites, thus making their role in pathogenesis less likely.

CNE sequencing in our cohort of PRS-Plus children with musculoskeletal anomalies and family history of PRS/cleft did not identify any likely causal variants that might impact upon *SOX9* expression, suggesting that these are unlikely in this cohort. The significance of the variant overlying the GATA1 transcription factor binding site is unclear, although functional studies beyond the scope of this manuscript may provide clarity. Chromo-

some microarray has been used to detect large chromosomal deletions 5′ of *SOX9* in individuals with PRS [Fukami et al., 2012; Amarillo et al., 2013; Smyk et al., 2015; Castori et al., 2016]. Although chromosome microarray is the genetic investigation of choice in all children with PRS seen after 2010 in our centre, probe coverage is typically low in the agenic 5′ region of *SOX9*, so it is possible that small copy number variants in this region have been missed in our cohort. Furthermore, our sequencing analysis is unable to detect small copy number variations at our locus of interest. Customized targeted analysis with MLPA of noncoding agenic regions [White et al., 2011] may be more likely to detect such molecular lesions as a cause for PRS-Plus.

Conclusion

A broad spectrum of phenotypes exists for children with nonsyndromic PRS. Many of these children have anomalies outside of the craniofacial system (PRS-Plus), with musculoskeletal and ocular anomalies being the most common. The PRS-Plus group is fascinating, and it remains unclear as to what genetic factors exist that differentiate it from isolated PRS. This, and all previous work examining the clinical features of PRS, underscores the broad phenotypic spectrum ranging from simple micrognathia to complex syndromes.

The phenotypic spectrum in children with PRS as well as the growing number of syndromes with PRS as a component feature suggests that review by a clinical geneticist is desirable. Our analysis of the CNEs of *SOX9* in a selected group of children with musculoskeletal anomalies and/or a family history of cleft or PRS did not reveal any significant mutations in this genomic region, suggesting that these are likely to be rare at least in this subset of PRS. A more agnostic whole genome approach is likely to be of higher yield than a targeted analysis. The breadth of the phenotypic spectrum observed in the PRS-Plus group is difficult to reconcile with a single embryological aetiology. Genetic heterogeneity in PRS has been proposed previously and is a broadly accepted concept. The recent explosion in genetic diagnoses following exome sequencing has highlighted the requirement for detailed phenotyping in gene discovery. However, isolated PRS is by definition a highly constrained phenotype, making dissection of the isolated PRS group to clarify genetic heterogeneity problematic. In contrast, the breadth of the PRS-Plus phenotypes provides an opportunity to define subcategories of PRS based on detailed phenotyping. Once defined, these subgroups will be amenable to contemporary exome-based gene identification strategies, which in turn will provide candidates for examination in isolated PRS cohorts. Further studies are required to fully elucidate the genetic mechanisms underlying the nonsyndromic PRS phenotype, but systematic subphenotyping may provide the most efficient path to this end.

Statement of Ethics

Approval for this study was granted by The Royal Children's Hospital Human Research Ethics Committee (approval numbers 32268A and 31190B and C).

Disclosure Statement

The authors have no conflicts of interest to declare. The authors have not received any grant, honorarium or other form of payment to produce the manuscript.

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