Research Article

Clinical and molecular characterization of a Brazilian cohort of campomelic dysplasia patients, and identification of seven new SOX9 mutations

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Abstract

Campomelic dysplasia (CD) is an autosomal, dominantly inherited, skeletal abnormality belonging to the subgroup of bent bone dysplasias. In addition to bowed lower limbs, CD typically includes the following: disproportionate short stature, flat face, micrognathia, cleft palate, bell-shaped thorax, and club feet. Up to three quarters of 46,XY individuals may be sex-reversed. Radiological signs include scapular and pubic hypoplasia, narrow iliac wings, spaced ischia, and bowed femora and tibiae. Lethal CD is usually due to heterozygous mutations in SOX9, a major regulator of chondrocytic development. We present a detailed clinical and molecular characterization of nine Brazilian CD patients. Infants were either stillborn (n = 2) or died shortly after birth and presented similar phenotypes. Sex-reversal was observed in one of three chromosomally male patients. Sequencing of SOX9 revealed new heterozygous mutations in seven individuals. Six patients had mutations that resulted in premature transcriptional termination, while one infant had a single-nucleotide substitution at the conserved splice-site acceptor of intron 1. No clear genotype-phenotype correlations were observed. This study highlights the diversity of SOX9 mutations leading to lethal CD, and expands the group of known genetic alterations associated with this skeletal dysplasia.

Keywords: campomelic dysplasia, skeletal dysplasia, osteochondrodysplasias, SOX9, prenatal diagnosis.

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Introduction

Campomelic dysplasia (CD) is an autosomal dominant condition classified among the bent bone skeletal dysplasias (Warman *et al.*, 2011). Typical manifestations of CD include shortening and bowing of the long bones, hypoplasia of the scapula, absence of ossification of the dorsal vertebral pedicles, and an abnormal pelvic bone pattern, with narrow iliac wings, spaced ischia, and hypoplastic/absent pubis bones. These findings warrant the diagnosis of CD in both prenatal ultrasonographic evaluation and postnatal radiological examination.

Clinically, CD is characterized by disproportionally short stature, short and bowed limbs, pretibial skin dimples, club feet, hip dislocation, thoracic constriction, cleft palate, micro- and/or retrognathia, and midface hypoplasia (Maroteaux *et al.*, 1971; Spranger *et al.*, 2002). Additionally, up to three quarters of karyotypically male

(46,XY) patients are sex-reversed, with phenotypically normal external female genitalia (Massardier *et al.*, 2008). Most infants with typical CD die shortly after birth due to pulmonary hypoplasia. However, patients diagnosed with a CD variant known as acampomelic campomelic dysplasia (ACD) usually survive the neonatal period and may reach adult life without major disabilities, but give birth to affected children (Gordon *et al.*, 2009; Lecointre *et al.*, 2009).

CD has been the subject of intense investigation for over twenty years, since its original genetic linkage to human chromosome 17 (Tommerup *et al.*, 1993) and the subsequent characterization of SOX9 as the locus involved (Foster *et al.*, 1994; Wagner *et al.*, 1994). The vast majority of CD cases (as well as some ACD cases) are attributed to haploinsufficiency, due to *de novo* mutations in the coding region of SOX9, a master regulator of chondrogenesis and SRY-mediated testicular development (Foster *et al.*, 1994; Wagner *et al.*, 1994; Gordon *et al.*, 2009). Conversely, a greater proportion of ACD patients is characterized by genomic imbalances in the vicinity of SOX9, a gene desert

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region that encompasses approximately 2 Mb in chromosome 19q24.3-25 (Gordon *et al.*, 2009).

In this paper, we present nine Brazilian CD patients who were diagnosed either prenatally or shortly after birth. Major clinical and radiologic findings are detailed for all cases. Sequencing of SOX9 revealed mutations in seven of them, all of which had not been previously described. This work is a report on one of the largest cohorts of CD patients, and it highlights the mutational diversity of SOX9 mutations.

Material, Subjects and Methods

From April 2012 to November 2013, clinical data from patients suspected of having CD were either retrospectively (for patient 1 and patients 3 to 7) or prospectively (patients 2, 8, and 9) referred to our Institution from different Brazilian medical genetics services, and here they were evaluated by two clinical geneticists (MTVS or DPC). Photographs, radiographs, and medical records were used to characterize the findings of each patient, both clinically and radiologically. Standard growth curves were utilized to determine percentiles for birth length (BL), birth weight (BW), and occipitofrontal circumference (OFC).

DNA was extracted from peripheral blood and used to amplify all exons and exon-intron boundaries of SOX9 by polymerase chain reaction (PCR), in nine patients. For patient 9 only, DNA from both parents was also obtained. Amplification products were purified by treatment with an exonuclease I / shrimp alkaline phosphatase protocol and subjected to conventional Sanger sequencing using an ABI 3130*xl* Genetic Analyzer (Applied Biosystems). CodonCode Aligner software, version 4.2.1 demo (CodonCode Corporation), was used to align sequences and analyze electropherograms. SOX9 reference sequences ENSG00000125398.5 (genomic) and ENSP00000245479.2 (protein) from ENSEMBL were used as wild type references. Nucleotide numbering of mutations followed the base positions of SOX9 cDNA reference sequence CCDS_11689.1 from NCBI.

When the karyotype was not available, the genotypic sex of patients was inferred from the amplification of X and Y chromosome-specific sequences in *zinc finger protein, X-linked* (*ZFX*), and *sex determining region Y* (*SRY*) genes, respectively. Primer sequences and PCR conditions are available upon request. The Spliceman web server (Lim and Fairbrother, 2012) was used to estimate the pathogenicity of the splice-site mutation identified in patient 3.

Ethical approval for this study was obtained from both the Institutional Review Board of the Hospital de Clínicas de Porto Alegre, and the Brazilian National Committee for Ethics in Research (project number 07044212.3.0000.5327).

Results

The main clinical data from the nine CD patients are summarized in Table 1. Examples of typical clinical and radiological findings are also illustrated in Figure 1. All infants displayed the typical CD phenotype, and they were either stillborn or died shortly after birth. Sex reversal was found in one of the 46,XY patients (patient 7). The mean gestational age (GA) at birth, considering live births only, was 36.4 ± 2.5 weeks. BW was within the normal range in the majority of patients (mean = $2,672.8 \pm 699.0$ g), while BL was below the 10th percentile in all patients whose measurement data were available (mean = 40.7 ± 3.1 cm). Most infants had macrocephaly, with OFCs above the 90th percentile for the GA (mean = 36.1 ± 1.8 cm). No live born infants survived the neonatal period. Shortened and bowed limbs were found in all patients — additional dysmorphic features are detailed in Table S1. Of note, craniofacial anomalies (micro/retrognathia, flat face, flat nasal bridge) were highly prevalent in this cohort. Post-natal radiographies were obtained for all patients — a detailed assessment of skeletal abnormalities is presented in Table S2. Bowing of the femora and tibia and malformation of the pelvic bones were consistently found.

DNA was available for molecular analysis for all patients, and putative pathogenic heterozygous SOX9 mutations were found in seven individuals, which equates to a mutational detection rate of 78% (see Table 1). Mutations shown in Figure 2 for patients 1, 2, 5, 6, 8, and 9 predicted the insertion of a premature termination codon, either due to a stop codon mutation (patients 2, 5, and 6), or a frame shift mutation (patients 1, 8, and 9).

An A-to-G substitution was identified in the conserved 3' splice-site of intron 1 of patient 3. *In silico* analysis using the Spliceman web server, which ranks putative donor and acceptor splice-site mutations based on exon/intron sequence conservation among different species, predicted a pathogenic consequence for this mutation, with a probability of 67%. Representative sequencing results are shown in ± for each patient with an identified mutation. Because of the absence of DNA from parents in most cases, molecular confirmation of a *de novo* mutation was only possible for patient 9, although parents of all patients were phenotypically normal.

Discussion

In this study, we sought to characterize a large cohort of CD patients in Brazil at the clinical, radiological and molecular levels. Our study recapitulated several observations previously reported, and this illustrated a striking degree of homogeneity among CD patients, considering clinical and radiological findings. We were able to identify different heterozygous mutations in the coding region of SOX9, in seven of the nine patients. However, not unlike others (Wagner *et al.*, 1994; Kwok *et al.*, 1995; Meyer *et al.*,

Table 1 - Clinical characteristics of the investigated CD patients.

| Patient	Phenotypic sex	Karyotype	SRY status	Sex reversal	GA	BW (p[a])	BL (p[a])	OFC (p[a])	Survival time	SOX9 Mutation
1	Female	NS	SRY -	No	32	1260 (p10)	NS	NS	1 day	p.Gly263fs277* (PTC)
2	Male	NS	SRY +	No	23[b]	632	29	22.5	NA	p.Lys151Ter (PTC)
3	Female	46,XX	NA	No	NS	2740 (p50)	41 (<p10)	33 (p50)	NS	IVS1-2A > G (splice site)
4	Female	NS	SRY -	No	40[b]	2740 (p10-25)	43 (<p10)	NS	NA	None
5	Male	NS	SRY +	No	37	2770 (p50)	44 (p10)	35 (p90)	Neonatal[c]	p.Ser41Ter (PTC)
6	Female	46,XX	NA	No	37	2830 (p50)	37 (<p10)	37 (>p90)	11 days	p.Gln232Ter (PTC)
7	Female	46,XY	NA	Yes	NS	3150 (p75)	42 (<p10)	38 (>p90)	7 days	None
8	Female	46,XX	NA	No	38	3470 (p75-90)	43.5 (<p10)	37 (>p90)	Neonatal[c]	p.His131fs248* (PTC)
9	Female	NS	SRY -	No	38	2490 (p10-25)	37 (<p10)	36.5 (> p90)	82 days	p.Phe204fs218* (PTC)

[a]Percentile distribution according to gestational age at birth; [b]stillbirth; [c]death within first 3 months, not detailed; BL: birth length (cm); BW: birth weight (g); GA: gestational age (weeks); NA: not applicable; NS: not specified; OFC: birth occipitofrontal circumference; PTC: premature termination codon. Patients were referred from medical institutions from Rio Grande do Sul (1-2), São Paulo (3-8), and Pernambuco (9) Federative States. Retrospective cases (n = 6) corresponded to patients evaluated from years 2000 to 2011, while two patients (2, 8 and 9) were diagnosed prospectively.

1997), a subset of the screened patients (2 out of 9) did not have any nucleotide variation in the coding region of SOX9. Of course, a heterozygous deletion within SOX9 or imbalances of regulatory elements in its genomic vicinity cannot be ruled out. These hypotheses shall be further investigated for patients 4 and 7.

Although SOX9 is a small gene, spanning little more than 5 kb and three exons, a plethora of studies have characterized regulatory DNA elements located both upstream and downstream from the gene that regulates SOX9 expression, both temporally and tissue-specifically (Bagheri-Fam *et al.*, 2006). For instance, cumulative evidence has suggested that a 78 kb genomic region upstream from SOX9 plays a role in testicular expression, given that deletion of this putative regulatory element has been observed in some 46,XY sex-reversed individuals (Pop *et al.*, 2004). Likewise, isolated Pierre-Robin (Fukami *et al.*, 2012), brachydactyly-anonychia (Kurth *et al.*, 2009), and congenital heart defects (Sanchez-Castro *et al.*, 2013) have already been linked to pathological copy number variations of putative regulators of SOX9 expression in this chromosomal region.

Different studies have tried to establish genotype-phenotype correlations in CD/ACD, but these have mostly remained elusive (Wagner *et al.*, 1994; Meyer *et al.*, 1997; Ninomiya *et al.*, 2000; Pop *et al.*, 2005). Mutations in SOX9 also display a great degree of variable expressivity (Cameron *et al.*, 1996). Moreover, it has been demonstrated that patients with the same SOX9 mutation may develop CD or ACD, have different degrees of sexual development disorders, and even stark differences in survival (Wagner *et al.*, 1994; Meyer *et al.*, 1997; McDowall *et al.*, 1999; Friedrich *et al.*, 2000; Moog *et al.*, 2001; Wada *et al.*, 2009).

Since changes in SOX9 associated with CD are mainly private mutations, the alterations identified here expand the spectrum of SOX9 pathogenic variations to about seventy (Table S3) (Thong *et al.*, 2000; Giordano *et al.*, 2001; Preiss *et al.*, 2001; Sock *et al.*, 2003; Michel-Calemard *et al.*, 2004; Hsiao *et al.*, 2006; Shotelersuk *et al.*, 2006; Beaulieu *et al.*, 2009; Gentilin *et al.*, 2010; Okamoto *et al.*, 2010; Staffler *et al.*, 2010; Kim *et al.*, 2011; Stoeva *et al.*, 2011; Chen *et al.*, 2012; Gopakumar *et al.*, 2013; Matsushita *et al.*, 2013; Tonni *et al.*, 2013). Premature termination codons (PTCs) are the most prevalent mutational class in CD, accounting for approximately 45% of all the alterations identified. Consistently, six of the nine patients (67%) in our series had a PTC mutation. In these cases, non-sense mediated mRNA decay (NMD) could potentially be employed to prevent translation of truncated peptides (Chang *et al.*, 2007). Even if expression from the mutated alleles escaped NMD, these would generate SOX9 proteins lacking all, or at least some, of the essential domains for proper protein activity (Figure S1).
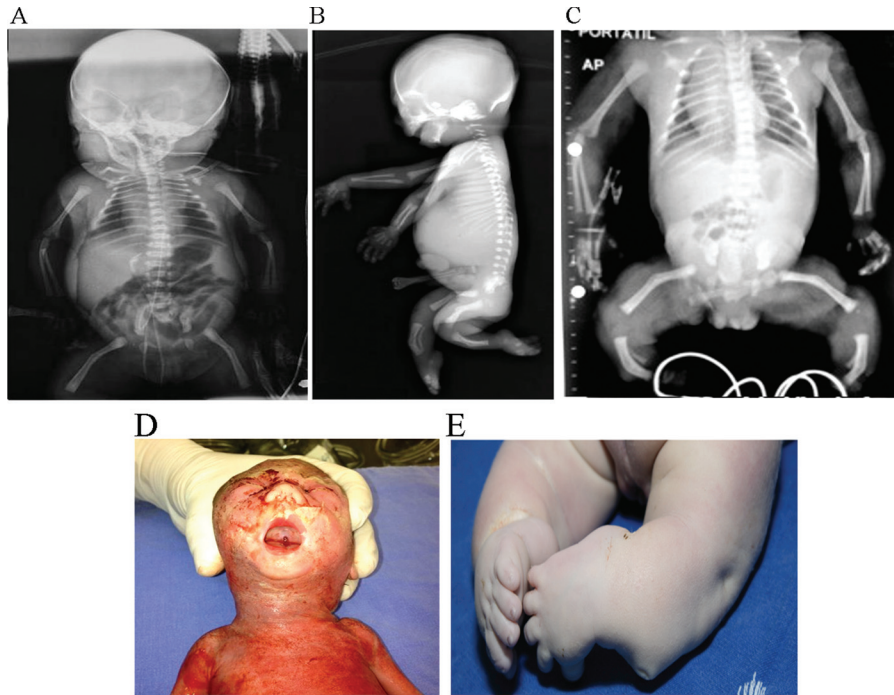
Figure 1 - Typical campomelic dysplasia findings observed in the patients included in this study. A: Antero-posterior (AP) radiography of patient 7, showing short long bones with bowed femora and tibiae, a short thorax with eleven pairs of ribs, and hypoplastic pubic bones, although no SOX9 mutation was identified. B: Lateral radiography of patient 2. Bowing of the femora and tibiae, as well as thoracic constriction, are identifiable. C: AP radiography of patient 9. In addition to the skeletal abnormalities already described in patients 1 and 2, hypoplastic scapulae are present. D: Clinical picture of patient 2 at necropsy. A small, flat face can be observed, as well as micro- and retrognathia, and cleft palate. E: Clinical picture of patient 1 at necropsy, illustrating the club feet and the pre- and post-tibial skin dimples characteristic of CD.
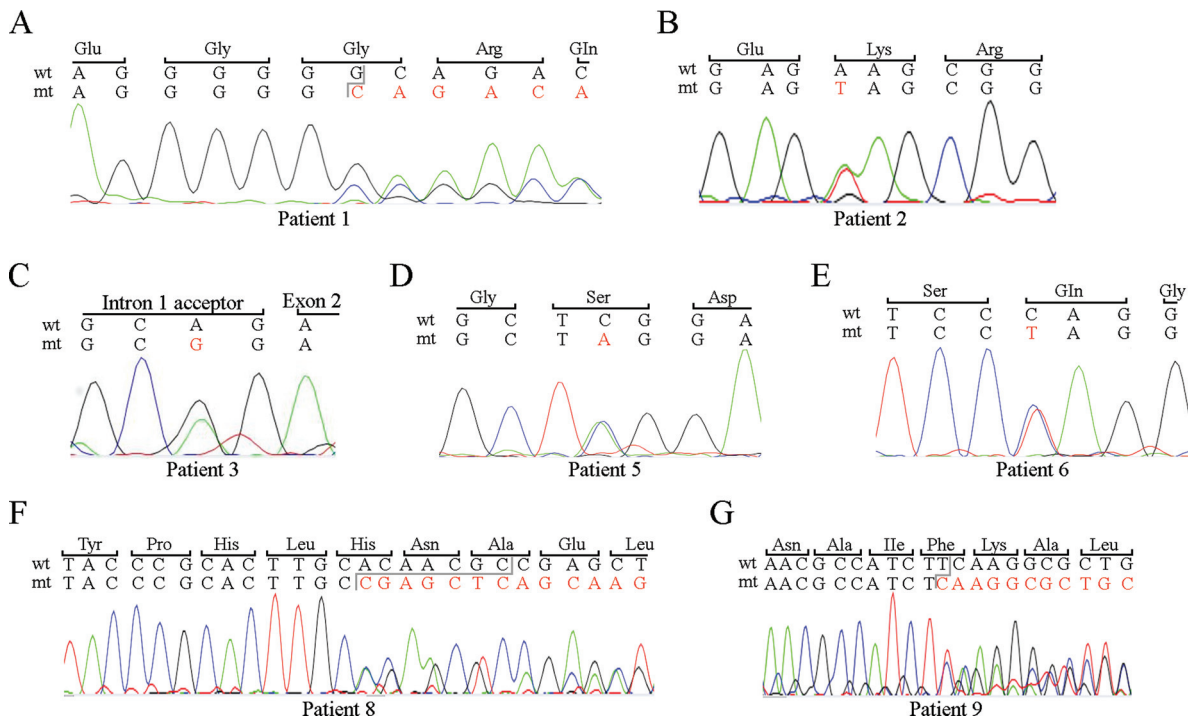
Figure 2 - SOX9 mutations identified in six CD patients included in the study. For each image, a fragment of the wild type (wt) allele with the corresponding codified amino acids is shown (except for C, where the junction between the first exon and intron is depicted). Altered nucleotides in the mutant (mt) allele are depicted in red. A: frame shift mutation of patient 1 due to a 1-bp deletion. B-E: Single-nucleotide change observed in patients 2, 3, 5, and 6, respectively. F: frame shift mutation in patient 8 due to a 1-bp deletion. G: frame shift mutation in patient 9 due to a 7-bp deletion.

Since truncated SOX9 polypeptides have been shown to retain some residual activity (Cameron *et al.*, 1996), different authors have associated larger protein truncations with greater life expectancy of CD patients (Meyer *et al.*, 1997; Pop *et al.*, 2005). In this cohort, patient 1 would, theoretically, retain the largest portion of SOX9, with approximately 54% of the wild-type peptide. However, this observation did not translate into less severity, because we did not identify any correlation between the identified mutations and survival of patients.

Splice-site mutations in SOX9 account for approximately only 4% of CD mutations reported to date (see Table S3). Only one patient in this series had a splice-site mutation. This gives rise to an A-to-G transition at nucleotide position -2 from the acceptor splice-site of intron 1 (IVS1-2A > G), which was predicted *in silico* to affect splicing with a probability of 67%. Kwok *et al.* (1995) identified an A-to-C transition at the same nucleotide position -2 in a female infant with typical CD findings and 46,XY karyotype (Kwok *et al.*, 1995). Our patient had a normal 46,XX karyotype.

Interestingly, while many studies report mutations in the coding sequence of SOX9 in 46,XY sex-reversed CD individuals, we did not identify any nucleotide alteration in a patient with sex-reversal. CD patients were observed to encode mutations of any of the mutational classes already described (see Table S3). Some researchers argue that sex reversal may be a phenotype with incomplete penetrance (Meyer *et al.*, 1997), but this proposition needs further support. Further investigations of the molecular basis of CD and related disorders are likely to contribute to a better understanding of the physiological roles of SOX9, which is a key transcription factor in the early embryonic development of several tissues.

Acknowledgments

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Internet Resources

Spliceman Web Server, http://fairbrother.biomed.brown.edu/spliceman/index.cgi (April 28, 2014).

Supplementary Material

The following online material is available for this article:

Table S1 - Detailed clinical evaluation of CD patients included in the study.

Table S2 - Detailed radiological evaluation of CD patients included in the study.

Table S3 - Compilation of all reported *SOX9* mutations associated to CD and ACD.

Figure S1 - Putative SOX9 proteins resultant from the premature stop codon mutations identified in six CD patients.

This material is available as part of the online article from http://www.scielo.br/gmb.

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