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**BRIEF REPORT**

Exome sequencing identifies the first genetic determinants of sirenomyelia in humans

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Abstract

Sirenomelia is a rare severe malformation sequence of unknown cause characterized by fused legs and severe visceral abnormalities. We present a series of nine families including two rare familial aggregations of sirenomelia investigated by a trio-based exome sequencing strategy. This approach identified *CDX2* variants in the two familial aggregations, both fitting an autosomal dominant pattern of inheritance with variable expressivity. *CDX2* is a major regulator of caudal development in vertebrate and mouse heterozygotes are a previously described model of sirenomelia. Remarkably, the p.(Arg237His) variant has already been reported in a patient with persistent cloaca. Analysis of the sporadic cases revealed six additional candidate variants including a de novo frameshift variant in the genetically constrained *NKD1* gene, encoding a known interactor of *CDX2*. We provide the first insights for a genetic contribution in human sirenomelia and highlight the role of Cdx and Wnt signaling pathways in the development of this disorder.

KEYWORDS

caudal dysgenesis, *CDX2*, de novo mutation, exome sequencing, Sirenomelia

Sirenomelia is a severe, immediately recognizable, embryological malformation of the lower body characterized by fused legs and visceral abnormalities (Opitz, Zanni, Reynolds, & Gilbert-Barness, 2002; Orioli et al., 2011). Hindlimb malformation varies between superficial fusion of normally- yet posteriorly-rotated legs, to the most severe forms consisting in an unrecognizable limb with a single bone without feet (Types I–VII, see Figure 1 and Supporting Information; Boer, Morava, Klein, Schepens-Franke, & Oostra, 2017; Kjaer et al., 2003; Stocker & Heifetz, 1987). Visceral malformations are usually incompatible with survival and almost constantly include renal agenesis, external genitalia and gastrointestinal abnormalities, and fetal vascular malformations (Opitz et al., 2002; Orioli et al., 2011). The pathophysiology of this disorder is not understood. Two main hypotheses have been proposed. First, the observation that almost all sirenomelia fetuses exhibit a single umbilical artery (SUA) of abnormal origin has led to a vascular hypothesis, in which the malformations may derive from a nutriment restriction of the lower part of the embryo. Second, the defective blastogenesis hypothesis suggests that sirenomelia might result from a defect occurring earlier, during the final stages of gastrulation, with similarities with the caudal dysgenesis phenotypes (Garrido-Allepuz et al., 2011). Sirenomelia cases have been reported in all ethnic groups with an incidence of 1–3 per 100,000 birth (Castilla & Orioli, 2004; Groisman, Liasovich, Gili, Barbero, & Bidondo, 2016; Källén et al., 1992; Orioli et al., 2011; Seidahmed et al., 2014). No environmental factor has yet been clearly identified, besides the observation that the rate of sirenomelia is much higher in mono or bi-chorionic twin pregnancies usually with one affected and one non-affected fetus (Di Lorenzo, Brandt, & Veilleux, 1991). Some reports found an association with maternal hyperglycemia (Castori et al., 2010; Lynch & Wright, 1997; Ozturk et al., 2014) although the vast majority of cases

occur in absence of any maternal/gestational diabetes (Duesterhoeft, Ernst, Siebert, & Kapur, 2007; Gerard et al., 2012; Orioli et al., 2011; Seidahmed et al., 2014; Thottungal, Charles, Dickinson, & Bower, 2010). Sirenomelia generally occurs sporadically, with very few familial aggregations reported. In contrast to human sirenomelia, several genetic determinants of sirenomelia are known in mice. Both spontaneous mutations and genetically modified animals have been observed to recapitulate the human phenotype. The known genetic bases of sirenomelia in mice include the disruption of *Cyp26a1*, *Cdx2*, or *Por*, which alter the distribution of the retinoic acid (RA) morphogen, the disruption of BMP signaling, either via *Bmp7:Tsg* or *Bmp7:Shh* double mutants, or conditional *Bmp4* knockout in the developing hindlimb field (Garrido-Allepuz, González-Lamuño, & Ros, 2012; Zakin, Reversade, Kuroda, Lyons, & De Robertis, 2005), and hypomorphic alleles in *Wnt3a* (Greco et al., 1996; Wansleebe et al., 2011).

We hypothesized, as for a high proportion of severe developmental disorders (Deciphering Developmental Disorders Study, 2017), that sporadic cases of sirenomelia could result from rare exonic de novo variants. We present the results of a genomic screening based on exome sequencing in an international cohort of (a) seven sporadic cases of classical sirenomelia and (b) two familial aggregations of sirenomelia and related phenotypes.

Following an international recruitment, we first gathered clinical data on seven sirenomelia sporadic cases (Figure 1a; Supporting Information). All seven pregnancies were interrupted for severe fetal malformation between 13 weeks of gestation (WG) and 21 WG+1. All fetuses had fused legs, with two moderate (Type II of Stocker and Heifetz classification; Stocker & Heifetz, 1987), and five severe forms (Type III–IV). We observed no sex predominance, with four males and three females. Two affected fetuses were part of twin pregnancies,

one with a normal dizygotic twin born at term, and the other one with a malformed monozygotic twin, showing prune belly sequence and distended bladder (Figure 1). A SUA was present in six of seven fetuses (85%). Additional malformations were observed in two fetuses: fetus S7 had bilateral radial hypoplasia, and fetus S3 presented with unilateral radial hypoplasia and a thoracic involvement with a ventricular septal defect and an arteria lusoria. No maternal diabetes was reported in the seven pregnancies as part of the systematic screen during pregnancies.

In addition, we included two families exhibiting aggregations of sirenomelia and related malformative phenotypes (Figure 1b). In the previously described family S5 (Gerard et al., 2012), a first female fetus was interrupted in the second trimester of pregnancy presenting with intrauterine growth retardation, total absence of urinary system, agenesis of the uterus and SUA. The lower limbs were normal. A second female fetus from the same parents was interrupted at 12 weeks of gestation after the identification of a phenotype of classical sirenomelia. Then, a male individual was born from the same parents with less severe malformations consisting of unilateral kidney agenesis and imperforate anus. His mother also presented imperforate anus at birth. In the unpublished family S13, a female fetus exhibited sirenomelia. A male sibling had an SUA. Like in family S5, the mother was born with imperforate anus. The mother presented a family history of sirenomelia in one sibling but no autopsy had been performed and no DNA sample was available for genetic testing.

Following exome sequencing in the seven affected fetuses and unaffected parents (see Supporting Information), we identified 14 candidate coding or splice region de novo mutations (DNMs) in four probands, which were subsequently confirmed to be present in the proband and absent in the parents by Sanger sequencing (Table S1). Three affected fetuses did not harbor any DNM within the coding sequence or splicing regions. Overall, no gene was hit twice by a DNM among the seven probands. DNMs were manually classified into two groups regarding the functional predictions and the variant frequency in the general population in the gnomAD database. Eight variants, either with no predicted effect on coding sequence (i.e., intronic and synonymous variants) or nonsynonymous variants observed at a nonnull frequency in gnomAD, were considered as poor candidates. Conversely, six de novo nonsynonymous variants in four fetuses were classified as good candidate variants. These novel candidate variants included three missense variants (*MN1*, *ZFR*, and *DISP1*), one start-lost (*FBXO7*), one frameshift indel variant (*NKD1*) and one multinucleotide variant in *TTC30A*, predicted to result in the substitution of two consecutive amino-acid residues (Table S1). Due to this small number of candidate genes ($n = 6$), standard Gene Ontology terms analysis failed to highlight any statistically overrepresented gene biological function, but manual assessment identified that four of these six genes are developmental genes involved in either morphogenesis of either the skull and face (*MN1*; Pallares et al., 2015), gastrulation (*ZFR*; Meagher & Braun, 2001) or in global morphogenesis regulation (*DISP1*; Etheridge, Crawford, Zhang,

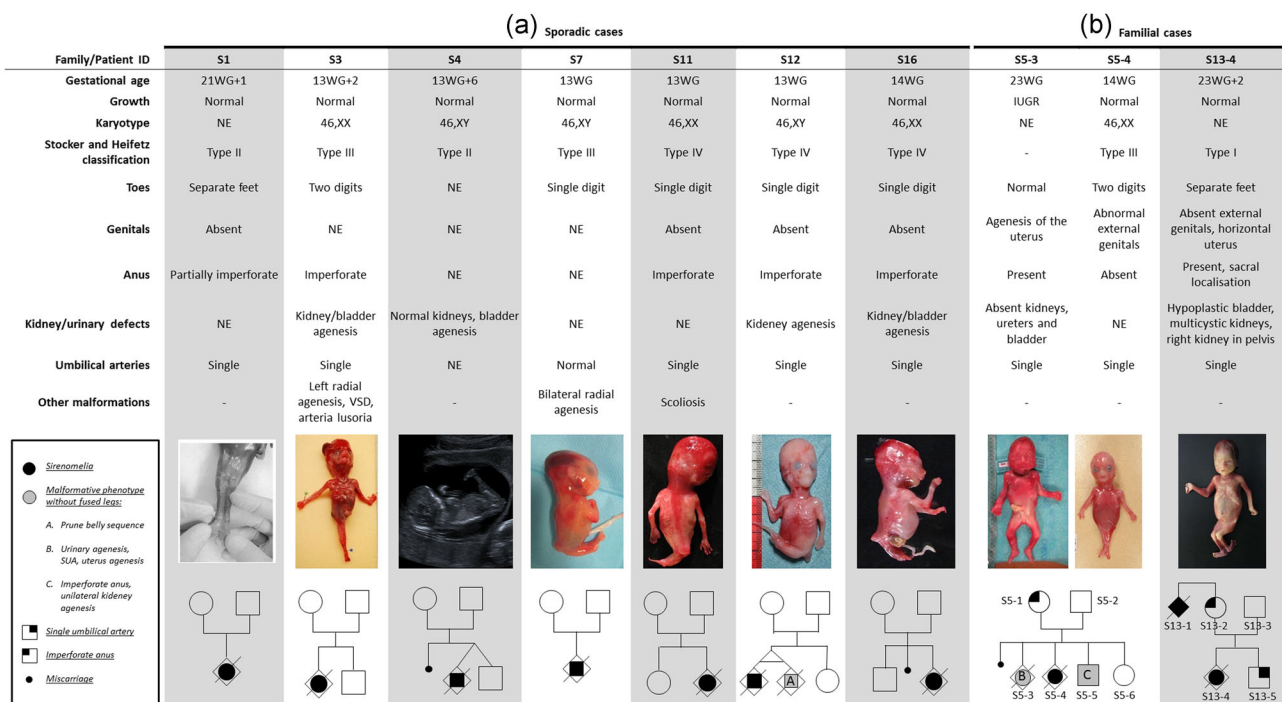


FIGURE 1 Clinical description of all cases. (a) Sporadic cases. (b) Familial cases. Stocker and Heifetz classification: Type I: all bones are present; Type II: fibular fusion; Type III: absence of fibula; Type IV: partial femoral fusion, fibular fusion; Type V: partial femoral fusion, absence of fibula; Type VI: femoral and tibial fusion; Type VII: femoral fusion, absence of tibia. NE, not evaluated; VSD, ventricular septal defect; WG, weeks of gestation

& Roelink, 2010), *NKD1* (Angonin & Van Raay, 2013). PPI analysis did not highlight any direct interaction between the products of these six genes (data not shown). Among these de novo variants, the truncating variant in *NKD1*, NM_033119.4:c.524del, p.(Pro175Glnfs*9), appears as the most promising candidate variant, regarding both variant effect and gene function. Indeed, this variant is absent from the general population bases, it is predicted to disrupt *NKD1*, which is very sensitive to loss of function (LOF) mutations (as confirmed by the 0.08 [90% confidence interval {CI}=0.03–0.27] observed/expected LOF ratio in gnomAD). The *NKD1* protein is a negative regulator of the Wnt signaling pathway (Angonin & Van Raay, 2013), a major determinant of body-axis polarization in vertebrate embryos (Hikasa & Sokol, 2013). Experimental evidence from animal models suggested that canonical Wnt signaling is a good candidate dysregulated pathway in the human phenotypes of caudal dysplasia and caudal dysgenesis (van de Ven et al., 2011). Interestingly, *NKD1* has been implicated in animal models of sirenomelia (Greco et al., 1996; Ishikawa et al., 2004) and in a mouse model of caudal dysgenesis by *Cdx2* conditional knockout (van de Ven et al., 2011), another gene identified in our study (see below). Yet the precise impact of *NKD1* dosage in the patterning of the caudal pole is still unknown.

In addition to de novo variants, we analyzed variants fitting an autosomal recessive model of inheritance in trios. Rare homozygous variants (minor allele frequency <1% in gnomAD popmax) and rare compound heterozygous variants within the coding sequence and canonical splice sites were evaluated. This recessive approach did not lead to the identification of any candidate variant, after excluding variants with either no predicted effect on protein (synonymous variants) or variants observed as homozygous in the gnomAD dataset (Table S2). Among putative copy number variants (CNV) detected by the CANOES read-depth comparison tool (Backenroth et al., 2014; Quenez et al., 2019), none was interpreted as a good candidate based on frequencies in CNV databases and gene content (see Supporting Information).

We then analyzed the two familial aggregations of sirenomelia and associated malformations (Figure 1b). Familial presentation in these two pedigrees argued in favor of an autosomal dominant mode of inheritance with variable expressivity. We applied two strategies to filter rare coding inherited variants of interest: (a) a filtration on a shortlist of candidate genes based on the murine genetic basis of sirenomelia (Table S3), and (b) a filtration on genes simultaneously harboring a rare (MAF < 0.001) coding variant in both families. Strikingly, both approaches highlighted the same gene *CDX2*, as (a) it was the only gene with a rare variant in the candidate gene approach, and (b) it was the best candidate variant (regarding variant frequency and variant function) within the list of nine genes harboring a variant present in affected individuals in both families.

In family S13, the proband (S13–4) harbored a missense *CDX2* variant, NM_001265.5:c.710G>A, p.(Arg237His). The variant was inherited from the mother born with imperforate anus. The mother's affected sibling was not available for testing (S13–1). This variant was absent in gnomAD, and pathogenicity predictors consistently

indicated a deleterious effect (Polyphen-2 score: 1.000 [probably damaging]; SIFT score: 0.00 [deleterious], Mutation Taster probability score: 1 [disease causing]; CADD score: 33). Remarkably, this same missense variant was recently identified as the probable cause of a closely related phenotype of persistent cloaca (PC; Hsu et al., 2018).

In family S5, the *CDX2* variant was predicted to result in the loss of the natural stop codon: NM_001265.5:c.940T>C, p.(Ter314ArgextTer13). This variant was absent from gnomAD, as well as any other stop-loss variants affecting this transcript. It was present in both affected fetuses (S5–3 and S5–4 in Figure 1b), and was inherited from the mother born with imperforate anus. Targeted Sanger sequencing showed that it was also carried by the other sibling presenting with imperforate anus and unilateral kidney agenesis (S5–5; Figure 1b and Table 1). To assess the potential effect of this variant predicted to increase protein length by 13 aberrant residues, we used the I-TASSER resource (Zheng, Zhang, Bell, & Zhang, 2019) to predict the protein structure in the wild-type and mutant contexts. This analysis suggested possible disorganization of the 3D conformation in the mutant protein, therefore losing its predicted ability to bind DNA and putatively modifying critical functions of this transcription factor (Figure S1). The screening by Sanger sequencing of six additional cases of sirenomelia did not identify any other rare *CDX2* variant. In addition, we assessed the presence of another variant in the coding sequence of *CDX2*, whatever the frequency or the coding effect, in fetuses S5 and S13, and found no additional variant. Then, we went back to the exome sequencing data of all seven trios and did not identify any inherited candidate variant in the gene list used for the analysis of familial cases.

CDX2 is a central component of the caudal type homeobox transcription factors, also comprising *CDX1* and *CDX4*. It is notably expressed in the caudal part of the elongating embryo, in the presomitic mesoderm where it is hypothesized to act as an integrator of caudalizing information (Savory et al., 2009). Several mice models have been used to evaluate the functions of *CDX2*. Homozygous knockout animals do not survive due to a lack of embryo implantation, and heterozygous mice present a variable association of tail abnormalities, growth restriction, vertebral and rib abnormalities, and polyps of the gastrointestinal tract (Chawengsaksophak, James, Hammond, Köntgen, & Beck, 1997). To bypass the early lethality caused by the absence of *Cdx2*, more complex mice models have been developed, based on tetraploid fusion (Chawengsaksophak, de Graaff, Rossant, Deschamps, & Beck, 2004) and conditional inducible knockouts (Savory et al., 2009). These *Cdx2*-null embryos presented axial truncation posterior to the forelimbs. Interestingly, heterozygous mutants exposed to RA presented the phenotype of sirenomelia while wild-type littermates showed an absence of tail (Savory et al., 2009). These results suggest that sirenomelia can be triggered by the combination of altered RA signaling in the predisposing genetic context of a heterozygous *Cdx2* variant.

In a recent study, de novo variants in *CDX2* have been identified in two individuals with sporadic isolated PC, a rare condition caused by the absence of development of the urorectal septum during early fetal

TABLE 1 Review of the phenotype associated with pathogenic CDX2 variants

Family	-	-	S13	S13	S5	S5	S5	S5	S5
Individual	VL21	VL6	S13-2	S13-4	S5-1	S5-3	S5-4	S5-5	S5-5
Reference	Hsu et al. 2017	Hsu et al. 2017	This study	This study	This study	This study	This study	This study	This study
Variant type	Missense	Stop-gain	Missense	Missense	Stop-loss	Stop-loss	Stop-loss	Stop-loss	Stop-loss
Variant (NM_001265.5)	c.710G>A	c.396C>A	c.710G>A	c.710G>A	c.940T>C	c.940T>C	c.940T>C	c.940T>C	c.940T>C
Consequence	p.(Arg237His)	p.(Cys132 ^a)	p.(Arg237His)	p.(Arg237His)	p.(Ter314Ar-gextTer13)	p.(Ter314Ar-gextTer13)	p.(Ter314Ar-gextTer13)	p.(Ter314Ar-gextTer13)	p.(Ter314Ar-gextTer13)
Segregation	de novo	de novo	familial	familial	familial	familial	familial	familial	familial
Sex	F	F	F	F	F	F	F	F	M
Phenotype synopsis	Persistent cloaca	Persistent cloaca	Imperforate anus	Sirenomelia	Imperforate anus	Visceral malformations	Sirenomelia ^a	Visceral malformations	Visceral malformations
Genito-urinary	PC	PC	N	N	Multicystic kidneys	N	Right kidney in pelvis	Bilateral renal agenesis	Unilateral renal agenesis
								Vesical agenesis	
								Uterus agenesis	
								Absent external genitals	
Digestive	PC	PC	Imperforate anus	Anus in sacral localisation	Imperforate anus	N	Absent anus	Imperforate anus	Imperforate anus
Vasculary	NA	NA	NA	SUA	NA	SUA	SUA	NA	NA
Lower limbs	N	N	N	Lower limbs fusion	N	N	Lower limbs fusion	N	N

Note: Patients from ref (Hsu et al., 2018) and this study are reviewed.

Abbreviations: N, normal; NA, not available; PC, persistent cloaca; SUA, single umbilical artery.

^aExternal examination only.

development, leading to the persistence of a common channel between rectum, vagina, and urethra (Hsu et al., 2018, p. 2). The well-established role of *CDX2* in the morphogenesis of the caudal region of the embryo and the insights from animal models, as well as the very low probability of identifying two de novo variants in the same gene in a small cohort were strong arguments in favor of the association of *CDX2* to PC. However, the molecular mechanism remained unclear, as both variants were found to result in apparently opposite effects. The first variant had a stop-gain consequence and was predicted to result in a loss of *CDX2* function. In contrast, when overexpressed in cell lines, the second variant, leading to the p.(Arg273His) missense change, showed an upregulation of the messenger RNA of *CDX2*'s direct target gene *CYP26A1*, in favor of a possible gain of function. Interestingly, we observed the exact same missense variant in the affected cases of family S13, thus extending the phenotype associated with this variation. The other *CDX2* variant that we identified, in family S5, was predicted to result in a loss of the natural stop codon. In silico analysis was in favor of disorganization of the 3D protein conformation, which could hypothetically lead to a loss of *CDX2* function, but further functional evidence will be needed to validate a putative deleterious effect. According to the data from gnomAD v2.1, *CDX2* appears moderately constrained in the general population with an observed/expected LOF ratio of 0.19 (CI: 0.07–0.59), but the small size of the gene, and, therefore, the small amount of observed and expected variants, precludes definite conclusions. No deletions or duplications are present in the Database of Genomic Variants and in the gnomAD Structural Variant database (Collins et al., 2019).

In total, a *CDX2* variant has been observed in eight individuals with caudal abnormalities (clinical and genetic descriptions summarized in Table 1), ranging from isolated imperforate anus to the severe complete phenotype of classical sirenomelia. Individual S13–5 from family S13 presented with an isolated SUA, but the DNA sample was unavailable, preventing the association of this mild phenotype to the clinical spectrum of *CDX2* pathogenic variants. The observation of the high intrafamilial variation caused by pathogenic *CDX2* variants is clinically important and should result in the incorporation of *CDX2* in gene panels for caudal malformation, given the risk for more severely affected offspring when a pathogenic variant in *CDX2* is identified. However, given the variable clinical expression, its use of genetic counseling may be difficult.

In summary, we identified *CDX2* as the first likely causal sirenomelia gene in humans and several novel candidate genes with DNMs. *CDX2* variants appeared to be associated with a wide spectrum of caudal abnormalities, including sirenomelia in its most severe form. This phenotypic variability is reminiscent of the inconsistency of the occurrence of sirenomelia in most mice models, where genotype and environment are controlled, arguing for a stochastic expression of the disease in individuals with a genetic predisposition. Alternatively, phenotypic variability in humans could result from epigenetic/environmental factors or even other genetic variants modulating the phenotype. Among trios, the observation of a de novo truncating variant in *NKD1*, which is a biologically and genetically

plausible gene, makes us propose *NKD1* haploinsufficiency as a novel genetic determinant in humans as well. The validation of *NKD1* null variants as a risk factor for sirenomelia would require replication in other affected individuals. Similarly, variants in *MN1*, *ZFR*, *DISP1*, *FBXO7*, and *TTC30A* will need further investigations regarding their functional effect, and a recurrence with other affected cases to be considered as putatively contributing to the development of sirenomelia. In addition, other mechanisms including noncoding variations should be assessed to better understand the genetic determinism of sirenomelia.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

Variants of interest have been submitted to the Clinvar database (<https://www.ncbi.nlm.nih.gov/clinvar/>): SUB6960321, SUB6960338. Variant data can be made available upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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